Ph.D. disszertáció

MULTIFUNKCIONÁLIS FEHÉRJÉK A SEJTMETABOLIZMUSBAN

A gliceraldehid-3-foszfát dehidrogenáz, mint adenin-uracil - gazdag szekvenciát felismerő RNS-kötő fehérje vizsgálata

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TARTALOMJEGYZÉK

ELŐSZÓ

Izgalmas dolog *ma* sejtbiológusnak lenni. Izgalmas, mivel a sejtbiológia paradigmaváltás küszöbén áll, és mert valószínűleg ez még az én életemben bekövetkezik.

Mire alapozódik ez a feltételezés? 1. A sejtfiziológia (sejtbiológia) korai teóriái átfogó koncepció alapján közelítették meg és magyarázták a kísérleti tényeket. A sejtfiziológia fragmentálódásával - membrán fiziológia, biokémia, molekuláris biológia (genetika), stb. - az egyes résztudományok hihetetlen mennyiségű információt szolgáltattak, és egy idő után már a sejtről való átfogó koncepció alkalmazása nélkül is képesek voltak funkcionálni, a mind finomabb és finomabb molekuláris részleteket feltárni. Azt hiszem azonban, hogy elérkezett a sejtbiológia egy olyan stádiumba, amikor ezek a mind finomabb részletek (amelyek mennyisége hihetetlen mértékben növekszik) már nem segítik az alapkérdések lényeges megértését, hanem egyre inkább azt sejtetik, hogy valahogy más módon kell az információkat összetennünk. Hiányzik egy koherens és átfogó szemlélet, mely rávilágít a részeredmények összefüggéseire, és eloszlatja a látszólagos ellentmondásokat. Tehát a divergencia által lehetővé váló specializálódás és a részletek felismerése egy magasabb szinten újból megteremtette a konvergencia igényét. 2. A sejtbiológiában *ma* uralkodó molekuláris biológia klasszikus érája lezáródóban van. A genetikus információ továbbadásának, a sejt lineáris memóriájának feltárása 50 éve indult és hamarosan befejeződik. A watsoni predikció szerint a XXI. század első évtizedében a humán genom telejesen ismeretessé válik. Ezután már nincs több új szereplő, a meglévők között kell a molekuláris interakciókat és azok szabályozását megérteni. 3. Az új paradigma fontos feledata lesz, hogy az élő sejtben a molekuláris asszociációk dinamikus átrendeződését (mely részben egy program alapján (autonóm), másrészt a homeosztázist veszélyeztető befolyás hatására jön létre) ill. annak térbeli és

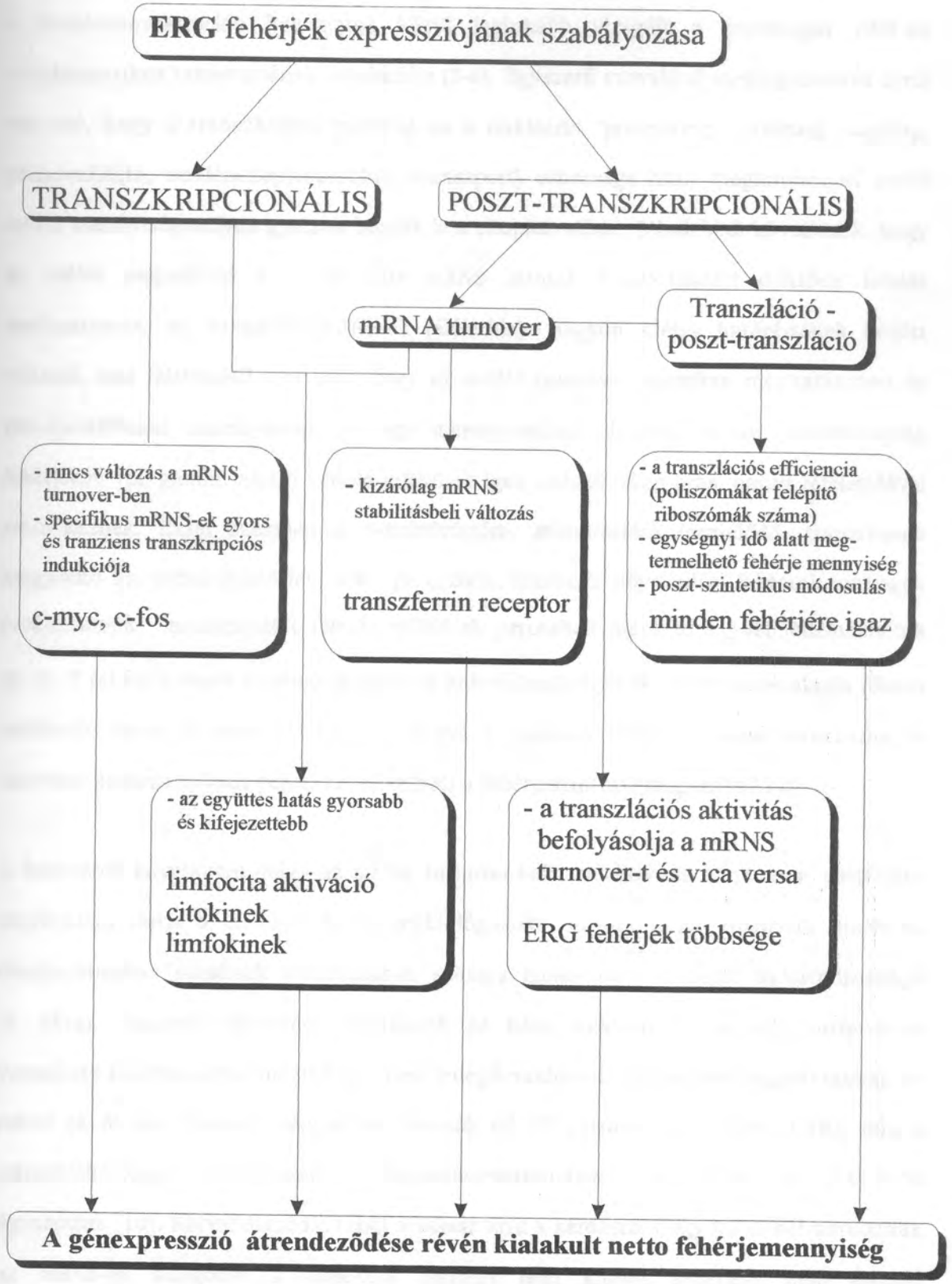
időbeli szabályozását molekuláris szinten megfejtse és megmagyarázza, továbbá új kérdések újfajta megfogalmazásával új horizontot nyisson, és kijelölje az élővé szerveződés megismerésének elérhető határait.

A nem-nukleinsav alapú információátvitel, a térmemória globális megértése, és egyszerű modellben való leírása tehát még várat magára. Létezését meglévő kísérleti eredmények ill. elméleti feltételezések előrevetítik. A szemlélet-váltást jelző mérföldkövek már megnevezhetők: a finomabb fehérjestruktúrák felismerése (a citoszkeleton felfedezése, a microtrabecular lattice), a metabolite chanelling-koncepció megszületése, a struktúrált víz, az ionok, kis molekulák és makromolekulák mozgásszabadságának intracelluláris korlátozottságának és kompartmentalizáltságának felismerése, stb. Úgy gondolom, hogy a multifunkcionális fehérjék létezése ill. jobban mondva a fehérjék multifunkcionalitásának egyre növekvő számú bizonyítéka fontos részét képezi e szemléletváltásnak. Ami a frusztrációt okozza ezzel kapcsolatban az az, hogy sokszor nem nyilvánvaló egyazon fehérje különböző funkciói közötti összefüggés. Ennek feloldására szolgálhat a következő goldolatmenet. Ismeretes, hogy a fehérjék különböző doméneket tartalmaznak, amelyek mint mobilis modulok többféle polipeptidláncba is inkorporálhatók. Az ugráló gének felfedezése, a mobilis genetikai elemek felismerése, a fehérjék harmadlagos és negyedleges szerkezetének fontossága, a protein folding mind rávilágítanak arra, hogy az egyes funkciók betöltéséhez szükséges térbeli konfiguráció elérhető a lineárisan és diszkontinuusan elhelyezkedő struktúr-elemek térbeli újrarendeződésével. Ez azt is jelentheti, hogy a dinamikusan létrejövő fehérje-komplexek egyes tagjai külön-külön hozzájárulhatnak a polipeptidláncukban rejlő információjukkal, és az egyedi fehérje hozzájárulása az együttesen kialakított struktúra információ tartalmához attól is függ, hogy mely fehérjékkel és más molekulákkal, hol és mikor kerül "egy csapatba".

BEVEZETÉS

A DNS, mint örökítő anyag azonosítását, és a gének felismerését követően intenzív kutatás indult meg a génexpresszió szabályozása terén, melynek fókuszában hosszú ideig kizárólag a transzkripciós reguláció állt. A 80-as évektől kezdődően azonban az e területen végzett kísérletek mindinkább rávilágítanak a génexpresszió poszt-transzkripciós szabályozásának jelentőségére is (1). Számos gén esetében kimutatott mind a konstitutív, mind az indukált génexpresszió kapcsán, hogy egy adott időpontban jelenlévő fehérje mennyisége ill. annak változásai nem magyarázhatók csupán a transzkripciós aktivitással, ill. annak változásaival. A különböző sejtfehérjék kifejeződésében és annak módosulásában a transzkripciós és a poszt-transzkripciós mechanizmusok hozzájárulása eltérő mértékű. Bizonyos gének esetében az előbbi, míg mások esetében az utóbbi a döntő szabályozási lépcső, általánosságban beszélve azonban mindkettő együttesen.

A poszt-transzkripciós szabályozáson belül is több mechanizmus határozza meg a génexpresszió mértékét, amelyek egymással természetesen összefüggenek (1. ábra). Jól példázza ezt az mRNS-ek transzlációjában bekövetkező változások mRNS stabilitást befolyásoló hatása. Az történik ugyanis, hogy fehérjeszintézist gátló szerekkel kezelve a sejteket a rövid életű mRNS-ek turnover-e lelassul, stabilabb lesz a molekula (2). Több lehetséges magyarázata lehet a jelenségnek. Némelyek nagyon gyors turnover-ű proteinek fontosságát hangsúlyozzák, mások inkább a riboszómákon-poliszómákon jelenlévő nukleázok szerepét feltételezik (3). E többlépcsős szabályozás egy igen erőteljes eltolódást okozhat a génexpresszió mértékében, hiszen példának okáért egy ötszörös emelkedés a transzkripciós aktivitásban párosulva egy négyszeresére megnyúlt mRNS féléletidővel ($t_{1/2}$) és egy háromszor hatékonyabb transzlációval, valamint felére csökkent protein degradációval, 5x4x3x2=120-szor több génproduktumot, vagyis fehérjét jelent.



1. ábra A génexpresszió szabályozásának szintjei

A poszt-transzkripciós folyamatok közül leginkább vizsgált a messenger RNS-ek citoplazmatikus turnover-ének regulációja (3-8). Egyszerű szavakkal megfogalmazva arról van szó, hogy a transzkripció mértéke és a nukleáris "processing" (splicing, capping, poliadeniláció, nukleocitoplazmatikus transzport) sebessége által meghatározott nettó mRNS mennyiség milyen gyorsan bomlik le a citoplazmában. Mindebből következik, hogy az mRNS degradáció a steady-state mRNS szintek kialakításáért döntően felelős mechanizmus. Az eukarióta mRNS-ek féléletideje nagyon széles határértékek között változik, ami feltétlenül arra utal, hogy az mRNS turnover szigorúan meghatározott és gén-specifikusan szabályozott. A nagy mennyiségben jelenlévő és ún. housekeeping fehérjéket (pl. globin, aktin) kódoló mRNS-ek igen hosszú, több órás, napos féléletidővel rendelkeznek. Ezzel szemben a sejtaktivációra, stimulációra termelődő, tranziensen megjelenő ún. indukálható fehérjék - pl. citokin, limfokin, növekedési faktorok, onkogén produktumok - szintéziséért felelős mRNS-ek percekben mérhető $t_{1/2}$-vel jellemezhetők (3, 9). E fehérjék rapid és rövid ideig tartó indukálhatóságának molekuláris alapja főként mRNS-ük gyors turnover-ében rejlik, mivel a gyorsan felfutó, magas transzkripciós aktivitás abbamaradását percekkel követheti a fehérjeszintézis megszakadása.

A fentiekből következik, hogy az mRNS turnover-beli szelektivitás alapjainak megértése izgalomban tartja a molekuláris és sejtbiológusokat egyaránt. Az eukarióta mRNS-ek citoplazmatikus sorsának alakulásában számos molekuláris szereplő kulcsfontosságú (2. ábra). Alapvető kérdésnek kínálkozik az RNS szekvenciákban rejlő turnover-re vonatkozó információkat megfejteni. Ilyen jellegű nukleotid szekvenciák leggyakrabban az mRNS-ek 3' nem átíródó régiójában lelhetők fel (3' untranslated region, UTR), míg a transzlálhatóságot befolyásoló szekvencia-determinánsok leginkább az 5'UTR-ba ágyazottak (10). Körvonalazódik tehát a válasz arra a kérdésre, hogy mi célból hordoznak az mRNS-ek átlagosan a molekula mintegy felét kitevő, fehérjévé nem átíródó szekvenciákat, amelyek sok esetben szigorúbban konzerváltak a különböző fajokat összehasonlítva, mint maguk a kódoló szekvenciák (11). A válasz: a reguláció.

A. RNS primer szekvencia

-protektív elemek
5' cap-strutúra
3' végi poli(A) farok

-destabilizáló elemek
ARE az ERG mRNS-ek 3'UTR-ban

-stabilizáló elemek
IRE a TfR mRNS 3'UTR-ban

B. RNS szekunder struktúra - térbeli szerkezet

-stem-loop

-pseudoknot

-RNS duplex (dsRNS) - antisense reguláció

-5' és 3' végek interakciója - transzlálhatóság regulációja

C. RNS-protein interakciók (mRNP komplexek)

-szekvencia-specifikus felismerés

-szekunder struktúra-specifikus felismerés

-nem szekvencia-specifikus kapcsolat

D. mRNS lokalizáció

-citoszkeletonnal való kapcsolat
aktin/lokalizációs szekvenciák a 3'UTR-ban

-kompartmentalizáció
poliszómális szubfrakciók
anterior-poszterior megoszlási különbség (Drosophila)

E. Ribonukleázok

-exonukleázok
3'→5' irányban emésztők
5'→3' irányban emésztők

-endonukleázok
szekvencia-specifikus hasítás

2. ábra Az mRNS féléletidőt meghatározó citoplazmatikus faktorok

A jelenlegi elképzelés szerint az eukarióta mRNS stabil molekula, amennyiben instabilitást determináló szekvenciát nem hordoz. A stabilitást számos struktúra biztosítja. A legáltalánosabb ezek közül a 3' végi poli(A) farok, mely kb. 250 adenint tartalmaz, és kevés kivételtől eltekintve (hiszton mRNS-ek) minden eukarióta mRNS-nek része. Némely adat arra utal, hogy 3'→5' exonukleázok ellen véd, mások a transzlációban betöltött szerepével magyarázzák mRNS stabilitásra való általános hatását (12-15). Az 5' végi cap struktúra - a transzlációban játszott szerepe mellett - szintén fontos az mRNS molekula integritásának megőrzésében, valószínűleg a 5'→3' irányban dolgozó exonukleázok ellenében (1).

Destabilizációs szignálként különböző mRNS target szekvenciák szerepelhetnek. Az ez ideig egyik legjobban karakterizált instabilitás-elem a rövid életű, indukálható fehérjéket, az ún. early response gene-eket (ERG) kódoló mRNS-ek 3' nem transzlálódó régiójában jellegzetesen előforduló AUUUA pentamerek AU- (adenin-uracil) gazdag RNS szekvenciába ágyazottan (3, 16) (3. ábra). Az ERG mRNS-ek jellegzetesen instabilak. Közel 100 molekulát sorolnak ebbe csoportba, köztük a proto-onkogéneket (c-myc, c-fos, c-jun), limfokineket (interleukinek, interferonok) és citokineket (GM-CSF, TNF, stb.) kódoló messenger RNS-eket (3). Shaw es Kamen kísérletei igazolták, hogy az adenin-uracil gazdag régiók (AU-rich elements, ARE) jelenléte okozza, de lagalábbis nagy mértékben hozzájárul e molekulák instabilitásához. Kísérleteikben a GM-CSF mRNS 3'UTR régiójából származó ARE szekvenciákat a globin mRNS 3'UTR-ba helyezték át, mely az egyébként nagyon stabil molekula gyors degradációját okozta a hibrid mRNS-t kódoló plazmid-DNS transzfekcióját követően (17). Legújabb kísérletek egy nonamér jelenlétének fontosságát igazolják, vagyis a pontosított motívum: UUAUUUA(U/A)(U/A) (18, 19). Azóta sem alakult ki azonban egy egységes kép arról, hogy a destabilizálódás pontosan hogyan következik be. Úgy tűnik, hogy az AU-gazdag szekvenciák nem szolgálnak endonukleázok felismerési helyéül. A c-myc és c-fos mRNS-ek esetében igazolt, hogy az ARE jelenléte a poli(A) farok gyors degradációját okozza, mely valószínűleg az instabil mRNS-ek lebomlásának első története (20, 21).

hu GM-CSF	UAAUAUUUAUAUAUUUAUAUUUUUAAAAUAUUUAUUUAUUUAUUUAUUUAA
hu G-CSF	UAUUUAUCUCUAUUUAAUAUUUAUGUCUAUUUA
hu IFN-α	UAUUUAUUUAUUUAA
hu IFN-β	UUUUGAAAUUUUUAUUAAAUUAUGAGUUAUUUUUAUUUAUUUAAAUUUUA UUUUGGAAAA
hu IFN-γ	UAUUUAUUAAUAUUUAACAUUAUUUAUAU
hu TNF-α	AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA
hu IL-1	UUAUUUUUUAAUUAUUAUUUAUAUAUGUAUUUAUAAAUAUAUUUAAGAUA AUUAUAAUAU
hu IL-2	UAUUUAUUUAAAUAUUUAAAUUUUAUAUUUAUU
hu IL-12	UAUUUAAAUAUUUAAGUAAUUUAUGUAUUUAUUAGUAUAUUACUGUUAUUUAA
hu c-myc	UAAUUUUUUUUAUUUAAGUACAUUUUGCUUUUUAAAGUUGAUUUUUUUCUAU UGUUUUUA
hu c-fos	GUUUUUAAUUUAUUUAUUAAGAUGGAUUCUCAGAUAUUUAUAU UUUUAUUUUAUUUUUUU
hu c-myb	AUUUUUUAAAAAAAAUAAAUGAUUUAUUUGUAUUUUA

3. ábra ERG fehérjék 3' nem átíródó régiójának AU-gazdag szekvenciái

Az e motívumokat felismerő szekvencia-specifikus RNS-kötő fehérjéknek (AU-rich binding proteins, AUBP) nagy szerepet tulajdonítanak AU-gazdag mRNS-ek által kódolt fehérjék expressziójának szabályozásában (22-32). Irodalmi adatok azt sejtetik, hogy az AU-gazdag RNS-szekvenciáknak, és az ezeket kötő fehérjéknek az RNS-metabolizmus számos pontján fontos regulatórikus szerep tulajdonítható. Leginkább vizsgált ezek közül az AU-gazdag mRNS-ek stabilitásának (24, 26, 27, 32, II. Közlemény), transzlációjának (33-36) és nukleocitoplazmatikus transzportjának kontrollja (30, 37).

Általánosságban is igaz, hogy az mRNS stabilitás regulációjában az RNS-kötő fehérjék kulcsszerepet játszanak, melyek szekvencia-specifikusan, vagy nem szekvencia-specifikusan kapcsolódnak RNS partnerükhöz. Kiemelkedő fontosságú szerepüket alátámasztja az a tény, hogy az eukariota mRNS-ek a citoplazmában csakis fehérjék által kísérve fordulhatnak elő. Jóllehet még nem sokak által használt, de egyre gyakrabban felbukkanó, és általunk is preferált az RNS chaperon kifejezés, mely plasztikusan leírja a fehérjéknek azt az odaadó guardedam-ságát, kíséretét, melyben az RNS molekulának részesülnie kell a citoplazmában, hogy a nukleázoknak áldozatul ne essék, és a megfelelő időben és a megfelelő helyen vehessen részt a sejt metabolizmusában.

Az utóbbi évek nagy felfedezése, hogy az mRNS-ek kapcsolódnak a citoszkeletonhoz (38-46). Az aktin mRNS például a 3'UTR-jában lévő lokalizációs szignálok révén a saját maga által kódolt aktinhoz kötődik (47). Letapadó sejttenyészetekben *in situ* hibridizációval jól látható az aktin mRNS "stress fiber" -ekre lokalizálódása, tehát az mRNS a sejtnek abban a régiójában tartózkodik, ahol a fehérjévé átíródásra lokálisan szükség van (48). Ebből adódóan a citoplazma különböző részein lévő riboszómák, poliszómák más-más mRNS-ek transzlációját intézik (49). A transzportfehérjék szintézise membrán-asszociált riboszómákon történik, amely szintén arra utal, hogy a sejt egy hihetetlen mértékben organizált és szabályozott rendszer, hiszen az exportra kerülő fehérjéknek meg kell járniuk a membrán-gazdag vezikuláris "fehérjefeldolgozót". A hiszton mRNS-ek például a citoszkeleton-asszociált poliszómákon íródnak át. Amennyiben az e helyre lokalizáló szignált a hiszton mRNS molekula 3' UTR-ból eltávolítják, a messenger egy másik poliszómális szubpopulációba kerül, és teljesen felborul a hiszton mRNS-stabilitás sejtciklus-függő szabályozása (50, 51). Az ERG mRNS-ek közül leginkább vizsgált a c-myc mRNS lokalizáció, mely szintén a 3' UTR-ban lévő jellegzetes RNS szekvenciák függvénye (52). Jól ismert példa az mRNS lokalizációra vonatkozólag, hogy a Drosophila normális egyedfejlődéshez elengedhetetlen az mRNS aszimmetrikus megoszlása a sejtben. Az egyes fehérjék anterior-poszterior megoszlási különbségéért az általuk kódolt mRNS-ek eltérő lokalizációja felelős (53, 54). A hely-specifikus lehorgonyzásban szintén az egyes mRNS-ek 3'UTR-jában lévő lokalizációs szekvenciák szerepelnek (53, 54). Amennyiben ezeket a specifikus RNS motívumokat eltávolítják, úgy az mRNS egyenlő eloszlást mutat a citoplazmában. Ha pedig "beragasztják" egy másik, egyébként nem polarizálódó mRNS 3'UTR-ba, úgy az a lokalizációs szekvenciának megfelelően helyezkedik el vagy az anterior vagy a poszterior póluson.

Számos kísérleti modellrendszerben igazolt, hogy a szignálok és stimulusok széles skálája képes változásokat indukálni specifikus mRNS-ek degradációjának mértékében, eképpen

igazítva az egyes géntermékek termelését a sejt aktuális igényéhez, a stimulusnak megfelelő válaszhoz. A jelenség különösen nyilvánvaló a fentiekben emlegetett, sejtaktivációra megjelenő, indukálható fehérjék expressziójának szabályozásában (3). Miután nyilvánvalóvá vált, hogy sejtaktivációra fiziológiásan, és malignus transzformáció kapcsán patológiásan az ERG mRNS-ek stabilitásában drámai változások következnek be, nagy figyelem fordult e fehérjék poszt-transzkripcionális regulációja felé (3, 55-62).

Többszöri utalásom és a célzott szakirodalmi áttekintésből már sejthető, hogy e molekulákkal kerültem munkám során közelebbi tudományos kapcsolatba. Kezdeti kísérleteinkben a nyugvó T limfociták stimulációját (G_0-G_1 tranzíció) követően aktiválódó poszt-transzkripciós mechanizmusokat vizsgáltuk. Számos - saját és más laboratóriumból származó - kísérleti adat támogatta azt az elképzelésünket, hogy a T sejt aktivációban kiemelkedően fontos szerepet játszó regulátor fehérjék - limfokinek, citokinek, növekedési faktorok - expressziója a megfelelő gének transzkripciójának indukálásán túl jelentős mértékben poszt-transzkripciósan szabályozott (55-62). Konkrétan, a mitogénnel aktivált T limfocita még akkor is folytatja a limfokinek, citokinek megnövekedett termelését, amikor a transzkripciós indukció már lecsengőben van. mRNS $t_{1/2}$ mérésekkel igazolást nyert a megnyúlt féléletidő szerepe. Miután a 3'UTR-ban lévő jellegzetes ARE szekvenciák instabilitás elemként való szerepe már kiderült (17), és az első ARE-specifikus fehérjéket is leírták (22-27), kézenfekvőnek tűnt a mi modellünkben is az AUBP-k szerepét megvizsgálni. Az hamar világossá vált, hogy a nyugvó - tehát limfokin, citokin mRNS-eket nem, vagy nagyon kis mennyiségben tartalmazó - limfocitákban jóval kisebb AUBP aktivitás detektálható *in vitro*, mint az aktivált sejtekben, melyekben e specifikus mRNS populáció jelentősen felszaporodik. Azonban az is sejthető volt, hogy a sejtextrakció során kritikus interakciókat és információt veszítünk, amely az *in vitro* RNS-kötés során nem rekonstruálható. Ez stimulálta azt az elképzelésünket, hogy a mi rendszerünkben detektálható AUBP-eket tisztán, nagyobb mennyiségben kinyerjük, biokémiailag

detektálható AUBP-eket tisztán, nagyobb mennyiségben kinyerjük, biokémiailag jellemezzük, azonosítsuk (esetleg már ismert RNS-kötő fehérjékkel), és specifikus antitesteket nyerjünk funkcionális ill. *in vivo* vizsgálatokhoz. Ennek érdekében humán limfocitákból ill. emberi lépből AUBP-eket tisztítottunk. Kísérleteink során egy 36-kDa molekulatömegű fehérjéhez jutottunk, mely *in vitro* RNS-kötési vizsgálatokban uracil- ill. adenin-uracil gazdag RNS szekvenciákat ismert fel. Aminosavszekvencia-analízissel megállapítottuk, hogy fehérjénk az eddig glikolítikus enzimként ismert gliceraldehid-3-foszfát dehidrogenázzal (GAPDH) azonos. E meglepő azonosságot további immunológiai és biokémiai vizsálatokkal igazoltuk.

Első meglepetésünket követte az a felismerés, hogy az irodalomi adatok két vonalon is megtámogatják eredményeinket. Először is, újabb kísérleti eredmények egész sora utal arra, hogy már eddig ismert, a sejt metabolizmusában kulcsszerepet játszó enzimek is rendelkezhetnek RNS-kötő sajátsággal (63). A legismertebb és legmeggyőzőbb, *in vivo* is bizonyított, metabolikus enzim/RNS-kötő fehérje analógia az akonitáz/IRE-BP (Iron-Response Element Binding Protein) példája. Az IRE-BP az intracelluláris Fe^{++} -szinttől és celluláris lokalizációjától függően RNS-kötő fehérje avagy citrát-köri enzimként funkcionál (64, 65). E fehérje specifikus RNS szekvenciát és térbeli struktúrát ismer fel, melyet iron responsive element-nek (IRE) kereszteltek. Jellemzően a vasanyagcserében fontos szerepet játszó proteinek messenger RNS molekuláiban lelhető fel. A ferritin és az ALA-szintáz esetében az 5'UTR-ban, a transzferrin receptor (TfR) mRNS 3'UTR-ban. E specifikus szekvencia-elemeket ismeri fel az IRE binding protein (IRE-BP), méghozzá intracelluláris vas koncentráció függő módon. Amennyiben az aktuális Fe^{++} mennyiség meghaladja a sejt pillanatnyi igényét, az IRE-BP Fe^{++}-kötő kapacitása révén érzékeli, és ezzel megszűnik RNS-kötő fehérjének lenni. Intracelluláris vashiány állapotában az IRE-BP újra képes lesz IRE kötésre. Ahogy a fentiekben már említettem, az 5'UTR leginkább a

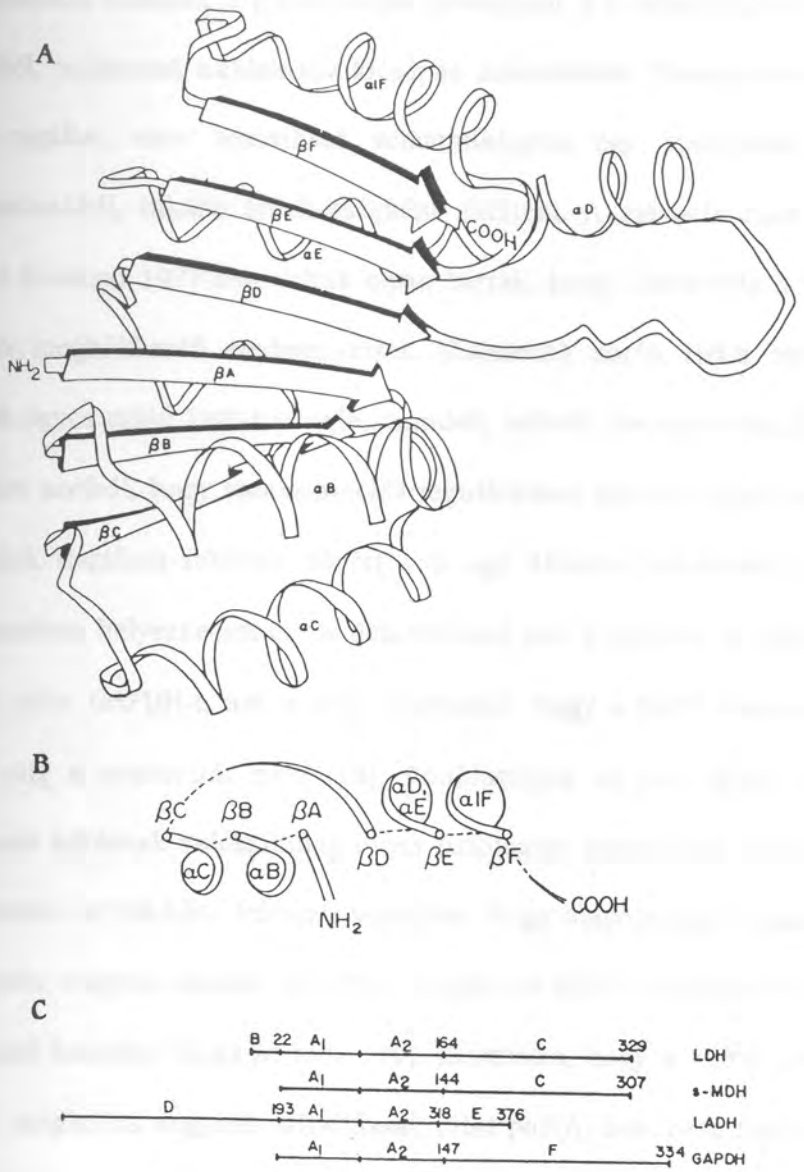
transzláció, míg a 3'UTR az mRNS stabilitás regulációjában kulcsszereplő. Ennek megfelelően alacsony intracelluláris [Fe^{++}] esetén az IRE-BP rákötődik a ferritin 5'UTR-ban lévő IRE-re és megakadályozza annak transzlációját, vagyis leállítja a vashiány miatt feleslegessé váló tároló funkciójú ferritin termelést. A transzferrin receptor mRNS 3'UTR-ban lévő IRE-vel való interakció viszont a molekula stabilitását növeli, elősegítve ezzel a nagyobb mértékű intracelluláris vas felvételhez szükséges több receptorfehérje molekula jelenlétét. Olyannyira specifikusan és mondhatni mechanisztikusan működik ez a rendszer, hogy a specifikus IRE szekvenciát más mRNS - amelynek egyébként semmi köze a vasanyagcseréhez - 5'UTR-jába inkorporálva, a hibrid RNS transzlációja is [Fe^{++}]-függő módon felfüggeszthető mind *in vitro*, mind *in vivo* (65). Vas-kelátor (dezferroxiamin) hozzáadásával a transzláció újra lehetségessé válik, természetesen csak olyan sejtrendszerben, amelyben az IRE-BP jelen van. Az RNS-kötés és az akonitáz aktivitás egyazon fehérjére kimutatható, de nem egy időben. Amennyiben a fehérje RNS-t köt, úgy enzimatikusan inaktív, és vica versa. A mechanizmust igazán "élővé" a kompartmentalizáció teszi, vagyis a fehérje intracelluláris lokalizációja határozza meg, hogy mely szereposztásban lép fel a sejtmetabolizmus színpadán. A mitokondriumban citrát-köri enzimként a citrát-izocitrát átalakulást katalizálja, a citoplazmában pedig a másik, RNS-kötő életét éli.

Az akonitázon kívül számos más enzimről mutattak ki RNS-kötő képességet. Legjellemzőbben NAD^+ ill. NADH kötésére képes dehidrogenázok okoztak meglepetést RNS-kötő tulajdonságukkal (63). Lightowlers és laboratóriuma kimutatta, hogy a glutamát dehidrogenáz a citokróm C mRNS-t köti a mitokondriumban (66), méghozzá szekvencia- és szekunder szerkezet (tetraloop) specifikus módon (67). A NAD^+-függő izocitrát dehidrogenázról azt találták, hogy a mitokondriális mRNS-ek jellegzetesen AU-gazdag 5' nem transzlálódó régióját ismeri fel (68). Egyéb nem dehidrogenáz, de

(di)nukleotid-kötő régiót tartalmazó enzimek is felbukkantak az RNS-kötő fehérjék között. A dihidrofolát reduktáz (69), a timidilát szintáz (70), a kataláz (71), a treonin szintáz (72) saját mRNS-üket kötik, rámutatva egy autoregulációs mechanizmusra, mellyel önmaguk expresszióját szabályozzák. Különösen érdekes az utóbbi enzim esete, mely egy molekuláris mimikri által válik saját mRNS-ének felismerőjévé. Ugyanis az RNS molekula 5'UTR-ja képes a treonin tRNS-hez nagyban hasonlatos térszerkezetet felvenni és megtéveszteni a treonin szintázt, amely a treonin tRNS szintjének csökkenésekor "ráfanyalodik" saját mRNS-ére. Ennek következménye pedig a transzláció leállítása. Teleológikus regulációs értelme pedig az lenne, hogy treonin tRNS hiányában nincs szükség treonin tRNS szintázra sem. A szeril-tRNS szintázról szintén kimutattak mRNS-kötő képességet (73).

A fentiekben kivonatosan felsorakoztatott enzimek mind rendelkeznek egy jellegzetes struktúrával, az ún. (di)nukleotid- vagy NAD^+-kötő régióval, amely koenzim vagy nukleotid kötéséért felelős. A 70-es évek közepén Rtg krisztallográfiás vizsgálatok mutattak arra rá, hogy a NAD^+-függő dehidrogenázok és más NAD^+-koenzimet használó enzimek is ezt a térben hasonlatos szerkezetet veszik fel, melyet leírójukról Rossmann hajlatnak (fold) is neveznek (74). Ez a struktúra tulajdonképpen két mononukleotid-kötő részből tevődik össze, egy nagyon hidrofób adenozin-kötő "zsebből" és egy inkább ionos és hidrofób interakciókat is biztosító nikotinamid felismerő részből. A treonil- és a szeril-tRNS szintáz, ahogy az I. osztályba sorolt összes tRNS-szintáz, szintén rendelkezik Rossmann hajlattal (75). A 4. ábrán látható sematikus rajz a 6 β-redőből és 4 α hélixből álló Rossmann hajlat térbeli ábrázolását mutatja, amely számos NAD^+-függő dehidrogenáz Rtg krisztallográfiás elemzésén alapul (4. ábra A, B). A GAPDH-ban ez a régió a molekula N-terminálisán van, de mint egy molekuláris építőelem "mozgatható", más dehidrogenázokban a polipeptidlánc közepén, vagy C-terminálisán is elhelyezkedhet

(4. ábra C). A NAD^+-dehidrogenázok koenzim-kötő régióinak térbeli hasonlósága annál is inkább rejtélyesnek tűnik, mivel az aminosavszekvencia homológia igen alacsony szintű. Wierenga munkássága hívta fel a figyelmet a kritikus, hidrofób aminosav-pozíciók szerepére a jellegzetes βαβ hajlat kialakításában (76, 77) .



4. ábra A NAD^+-függő dehidrogenázok koenzim-kötő régiójának (Rossmann hajlat) sematikus ábrázolása (A) Térbeli konfiguráció felülnézetben, a β-redők által alkotott síkra merőleges függőleges (perpendukuláris) irányból. (B) Térbeli konfiguráció oldalnézetből, a szemlélőhöz legközelebb eső N-terminális végtől a legtávolabb eső C-terminális felé a polipeptidláncon végignézve. (C) A különböző dehidrogenázok NAD^+-kötő régióinak intramolekuláris elhelyezkedése lineáris ábrázolásban. LDH: laktát dehidrogenáz, LADH: liver (máj) alkohol dehidrogenáz, MDH: malát dehidrogenáz (Az ábrák Rossmann és mtsai összefoglaló közleményéből származnak (74).)

Visszatérve a GAPDH-nak, mint AU-gazdag RNS-kötő fehérjével való azonosításunk kérdéskörére, ismertetem azokat az irodalomban fellelhető eredményeket, melyek szintén kapcsolatba hozzák e fehérjét a nukleinsav anyagcserével. Az első utalásom áttételes ugyan, de már a molekula "felfedezése" idejére datálódik. Otto Wartburg és mtsa 1939-ben tisztította elsőként a gliceraldehid-3-foszfátot 1,3-difoszfogliceráttá alakító enzimet élesztőből, méghozzá nukleinsav kicsapás módszerével. Természetesen ez az adat csak távoli sugallat, nem tekinthető semmmiképpen egy specifikus módszernek a mi szempontunkból, inkább érdekességként említem. A második adat már annál inkább. Perucho és mtsai 1977-ben voltak olyan bátrak, hogy leközöljék a biokémia klasszikus érájában meghökkentő eredményeiket. Kísérleteik során HeLa sejtek magfrakciójából izoláltak egyes szálú DNS-t (single stranded, ssDNS), de natív, kettős szálút nem - kötő fehérjéket acélból, hogy transzkripciós regulációban szereplő proteineket azonosítsanak. Az általuk tisztított fehérjék között volt egy 36-kDa molekulatömegű, amely főleg a citoplazmában helyezkedett el, de detektálható volt a magban is. Miután azonosították e fehérjét, mint GAPDH-t, azt is megállapították, hogy a NAD^+ koenzim gátolja a ssDNS-kötést, míg a szubsztrát nem (78). Konklúziójuk az volt, hogy az általuk detektált nukleinsav kötésnek valószínűleg nincs fiziológiás jelentősége. Karpel és laboratóriuma élesztősejtek ssDNS-kötő fehérjéit vizsgálta. Nagy alapossággal végzett kísérleteit 1981-ben közölte, melyben élesztő GAPDH-t vizsgált, és újfent megállapította, hogy egyes szálú nukleinsav kötésére képes *in vitro* (79). Kimutatta, hogy a ssDNS mellett poli(U)-hoz is kötődik, méghozzá nagyobb affinitással, mint poli(A)-hoz. Nem frakcionált emlős RNS is szerepelt a GAPDH által felismert nukleinsavak között (79). Biokémiai vizsgálatoknak alávetve azt találták, hogy e fehérje nukleinsavhoz kötődése megváltoztatja a T_m (melting temperature) értéket, vagyis a molekula kicsavarodását (unwinding) okozza, és ez alapján helikáz aktivitással ruházták fel. A rövid, elágazó poli(AU) RNS hélixekre való hatása volt a legkifejezettebb. Azt is ismertették, hogy a GAPDH számos izoformja közül csak a

legbázikusabb kötődött a poli(U)-oszlophoz, és mutatott helikáz aktivitást (79). Eredményeik specificitását azzal támogatták, hogy a GAPDH-n kívül más dehidrogenázt nem tudtak azonosítani. 1985-ben egy neurofiziológiai munkacsoport jelentkezett a GAPDH magbéli szereplésére vonatkozó újabb adatokkal (80). Neuronok transzkripciós regulátor fehérjéit vizsgálták, s közöttük ismét azonosították a GAPDH-t. Azt állapították meg, hogy a teljes GAPDH populációnak csak mintegy 5%-a az, amely ssDNS-kötő képességgel rendelkezik. Az ezt követő években számos más glikolítikus enzimet is kimutattak biokémiai ill. immunhisztológiai módszerekkel a sejtmagban (81). 1991-ben Michael Sirover és mtsai lepték meg a tudományos világot azzal az eredményükkel, hogy a 37-kDa-os bázis excíziós DNS-repair enzimként jellemzett uracil DNS glikoziláz (UDG), amelyet HeLa sejtek magjából izoláltak nem más, mint a GAPDH (82). Gyári, tisztított GAPDH-ról is kimutatták, hogy hordoz UDG aktivitást *in vitro*, tehát képes dUTP-t eltávolítani szintetikus DNS-ből (82).

A GAPDH RNS metabolizmussal való kapcsolata a '80-as évek közepén vetődött fel. Ryazanov a nyúl retikulocita lizátumot (rabbit reticulocyte lysate, RRL) vizsgálva állapította meg, hogy eme *in vitro* transzlációs rendszer három RNS-kötő fehérjét tartalmaz nagy mennyiségben. A két, már addigra azonosított elongációs faktor (EF-1a, EF-2) mellett a harmadik, 36-kDa-os riboszómális RNS oszlophoz kötődő fehérjét GAPDH-nak identifikálta (83). Két-dimenziós elektroforézis vizsgálatokkal azt is igazolta, hogy az RRL-ban található GAPDH-nak számos izoformja van, és ő is megerősítette, hogy csak a legbázikusabb rendelkezik RNS kötéssel. Modellrendszeréből adódóan azt feltételezte, hogy a GAPDH-nak egy "laza", nem szekvencia-specifikus kapcsolata van ribonukleinsavakkal, és helikáz aktivitása révén a transzlációban volna szerepe. A GAPDH fehérjeszintézis-beli szerepét egy másik munkacsoport is felvetette, miután modellrendszerükben az enzim jelentős mértékben fokozta az immunglobulin termelést, egy Actinomycin D-től független, de cikloheximid-érzékeny mechanizmus révén (84, 85).

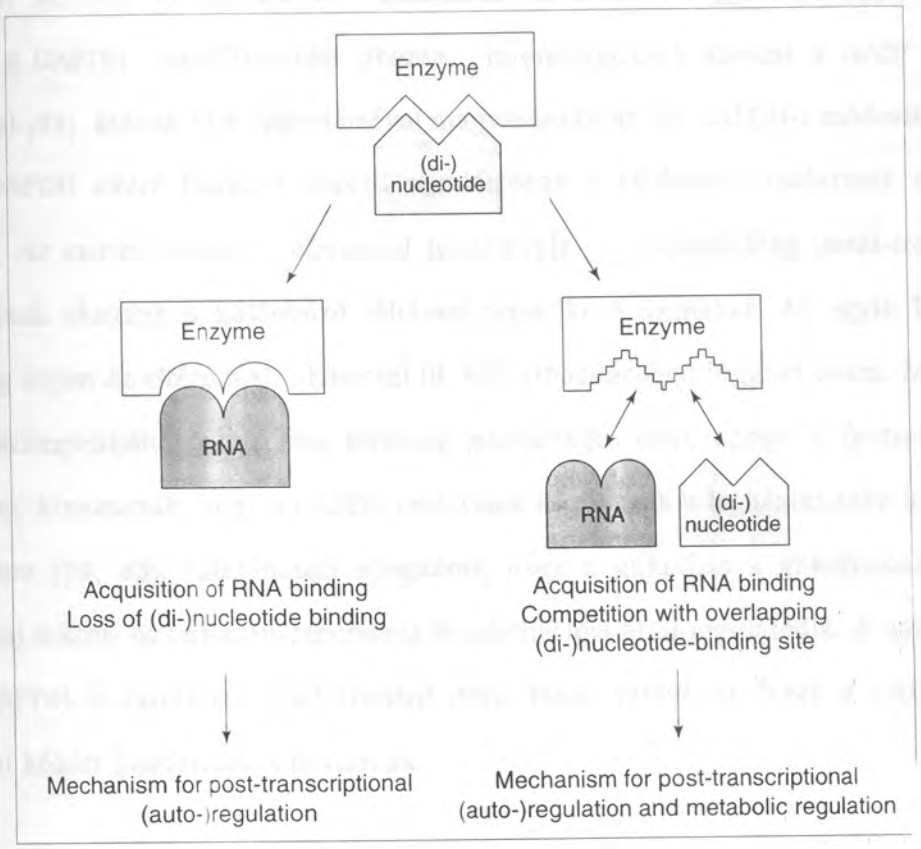
Eddig a pontig senki sem állapított meg szekvencia-specifikus nukleinsav felismerést. 1993-ban azonban Michael Green és laboratóriuma meglepő adatokat szolgáltatott a Science hasábjain (86). tRNS exportot vizsgáltak HeLa sejtekben, és olyan magfehérjék után kutattak, amelyek képesek megkülönböztetni transzport-deficiens mutáns tRNS molekulákat normálisan transzportált vad-típúsuaktól *in vitro* RNS-kötésükkel. Egy 36-kDa molekulatömegű fehérje "akadt a horogra", amely - a felvezetés után már nem meglepő módon - GAPDH-nak bizonyult. A GAPDH által kísérleteikben felismert különböző tRNS molekulákra általános konszenzus-szekvenciát nem sikerült ténylegesen kimutatniuk, ennek ellenére feltételezték, hogy egy részben primer, részben szekunder RNS struktúra-felismerésen alapuló specifikus interakciót találtak, amely koncentráció-függő módon gátolható volt a NAD^+ koenzimmel (86).

Az e dolgozatban ismertetendő eredményeink megjelenését követően más laboratóriumok is jelentkeztek és közöltek igen érdekes további adatokat a GAPDH RNS-kötésével kapcsolatban. McGowan és Pekala a glukóz transzporter (GLUT1) mRNS AU-gazdag 3' nem transzlálódó régiójának különböző dehidrogenázok általi kötését vizsgálta. A dehidrogenázok szerepe a GLUT1 mRNS poszt-transzkripciós szabályozásában többek között a mi vizsgálataink alapján merült fel (87). Kísérleteikben igazolták a GAPDH AU-specificitását, a glutamát dehidrogenáz és izocitrát dehidrogenáz nem ismerte fel RNS-próbájukat, az LDH és a glukóz-6-foszfát dehidrogenáz pedig nem mutatott AU-specificitást. Sioud és Jaspersen azt közölte, hogy humán T limfocitákban a GAPDH kötődik a TNFα-ribozim-hez NAD^+-dal és ATP-vel gátolható módon. Az interakció hatására a ribozim RNS-hasító aktivitása jelentősen megnőtt, amit a GAPDH RNS unfolding-ot okozó hatásával hoztak összefüggésbe (88). Schultz és mtsai a hepatitis A virus 5'UTR - kötő fehérjék után kutatva találkoztak a GAPDH RNS-kötő képességével (89), és megerősítették a poli(U)-hoz való affinitást, valamint az RNS-kicsavarodást okozó hatást.

A NAD^+-kötő régió felmerülése különösen érdekesnek tűnt számunkra, mivel 1993-ra, mikor a kérdéssel foglalkozni kezdtünk már nyilvánvalóvá vált, hogy az RNS-kötő fehérjék jellegzetes RNS-kötő doménokat (RNA-binding domain, RBD) tartalmaznak, és egy-egy nagy családba tartozó fehérjék egy jellegzetes RBD révén ismerik fel ill. kötik meg RNS partnerüket (90). Példának említem a legnépesebb és legjobban jellemzett heteronukleáris RNS-kötő fehérje-családot, melynek tagjai egy ~80 aminosavból álló RBD-t hordoznak, 2-4x ismételve egy polipeptidlácon belül. Delécios mutáns ill. fúziós hibridfehérjékkel kimutatták, hogy ez a régió, mint egy modul áthelyezhető más, nem RNS-kötő fehérjébe, és azt RNS-kötővé teheti, ill. eltávolítva a hnRNP RNS-kötése megszüntethető (91). A család minden tagjára igaz, hogy az RNS-kötés tényleges helye az RBD-ben van, de a szekvencia-specificitáshoz a családtagok egy részének fehérjemolekuláik egyéb részei is szükségesek. NMR spektroszkópiás tanulmányok arra utalnak, hogy a β redők, ill. a bázikus aminosavak területére lokalizálható a fehérjék érintkezése az RNS-sel (92).

A GAPDH esetében az RNS-kötő domén kérdés jelentőségét az a tény is hangsúlyozza, hogy számos NAD^+-függő dehidrogenázról kimutattak RNS-kötő képességet (ahogy a fentiekben ezt már említettem), tehát a GAPDH/AU-gazdag RNS interakció vizsgálata, valamint e kapcsolat NAD^+-függésének mechanizmusa ezen enzimek RNS-kötését is megmagyarázhatja. Elképzelésünk az volt, hogy a GAPDH RNS-kötő régiója részben, vagy teljesen azonos lehet a (di)nukleotid-kötő doménnal. Ebből következik, hogy a glikolítikus funkció és az RNS-kötő képesség egymást egy időben kizáró módon, reciprok szabályozás révén valósulna meg. Ezt a mechanizmust feltételezve a Rossmann hajlatban specifikusan kötődő molekulák kompetitíve gátolják a GAPDH/AU-gazdag RNS interakciót. Ezen hipotézisünket (szinte keletkezésének pillanatában) Hentze is megfogalmazta a

metabolikus enzimek (különösképpen a NAD^+-függő dehidrogenázok) RNS-kötéséről, ill. annak molekuláris alapjáról írt összefoglaló közleményében (63) (5. ábra).



5. ábra Az átfedő RNS- és nukleotid-kötő domének által megvalósuló autoreguláció és metabolikus reguláció hipotetikus modellje

Mivel a GAPDH egy tömegesen előforduló citoplazmatikus glikolítikus enzimként vált ismeretessé, nyilvánvalóan kell egy magyarázattal szolgálni, hogy miképpen vehet részt szelektív mRNS-ek metabolizmusában. A klasszikus glikolítikus funkciót betöltő enzim négy 36-kDa-os monomerből áll össze. A maximális aktivitást a tetramer forma mutatja, de a dimer is rendelkezik glikolítikus aktivitással (93). A glikolítikus és az RNS-kötő aktivitás közötti reguláció egyik lehetséges módja az oligomerizáció foka lehet. Ezt támogatja a Sirover laboratóriumából származó adat, mely arra utal, hogy csak a

monomer rendelkezik UDG aktivitással. (82). Az is ismeretes, hogy a monomerek funkcionálisan különbözőek, pl. NAD^+-kötő affinitásuk eltérő (93). Különösen az emberi GAPDH esetében olyan erős is lehet a koenzim rögzülése a Rossmann hajlatban, hogy csak aktív szénnel távolítható el. McDonald és Moss vizsgálatai alapján nitrogén monoxid a GAPDH modifikációját okozza, interpretációjuk szerint a NAD^+ kovalens fixálásával (94). Mások ADP-ribozilációval magyarázzák az NO GAPDH-t módosító hatását (95). A GAPDH eltérő funkciói közötti regulációban a különböző izoformok szerepe is felmerül. Az enzim számos izoformmal rendelkezik, és valószínűleg poszt-transzlációs módosulások okozzák a különböző töltéssel rendelkező formákat. Az egyik lehetséges különbség éppen az eltérő NAD^+-kötéssel ill. ADP-ribozilációval függhet össze. Mindennek a téma szempontjából azért van különös jelentősége, mert ahogy a fentiekben már említettem, kimutatták, hogy a GAPDH izoformok közül csak a legbázikusabb képes RNS-felismerésre (79, 83). Általánosan elfogadott, hogy a glikolízis a glikolítikus enzimek növekedési faktor- és onkogén-dependens foszforilációja által regulálódik. A nyúl izomból izolált GAPDH is tartalmaz foszfotirozint (96). Tehát valószínű, hogy a GAPDH eltérő izoformjai között foszforiláltak is vannak.

Fontosnak tartom még a Bevezetőben ismertetni azokat az irodalmi adatokat is, amelyek a GAPDH-t megmutatják más, igen sokoldalú "arcáról". Már az enzim biokémiai jellemzése során felismerték, hogy számos nem-dehidrogenáz aktivitással is rendelkezik, például aciltranszferáz és észteráz aktivitással bír (97). Az észteráz reakciót NAD^+-mentes állapotában katalizálja a GAPDH, és főként aril-észter kötéseket bont. Az aciltranszferáz aktivitással hozhatók kapcsolatba Kawamoto és Caswell eredményei, melyek arra utalnak, hogy a GAPDH-nak szerepe van egyes izomfehérjék foszforilációjában, sőt autofoszforilációra is képes lenne (98).

Számos fehérjével érték már tetten a GAPDH-t, s ezen interakciók alapján különböző funkciókat rendeltek hozzá. A GAPDH/aktin "viszony" az egyik legismertebb, és a

legkevésbbé megütközést keltő, mivel számos más glikolítikus és egyéb metabolikus enzimről ismeretes, hogy kapcsolatban van az aktin-citoszkeletonnal. Meghatározták az aktin GAPDH-val kapcsolatba lépő régióját, és a GAPDH aktin-kötő helyét is feltérképezték (99, 100). Az aktinhoz kapcsolt GAPDH más glikolítikus enzimekkel egy "csapatban" effektívebb szubsztrát átalakításra képes, mint a nem aktin-asszociált populáció. Ez a kísérletes eredmény részét képezi a "metabolite channeling" koncepciónak, amely értelmében az egy reakciósorba tartozó enzimek egy térbeli és funkcionális komplexet képeznek, az egyik enzim által előállított produktum egy másiknak szubsztrátjaként szerepel (101). Tehát a metabolitok nem "kószálnak" céltalanul a citoplazmában, arra várva, hogy valamely enzimmel összetalálkozzanak, hanem egy térben és időben dinamikusan szabályozott, direkt módon kerülnek molekuláról molekulára, fehérjéről fehérjére. Érdekes módon többen kimutatták, hogy a metabolikus enzimek komplex képződésében RNS is szerepel (102-104). Mazurek és mtsai vizsgálatai szerint a GAPDH, a foszfoglicerát kináz, a piruvát kináz és az enoláz egy olyan komplexet képeznek, mely RNáz kezelésre szétesik (104).

Valószínüleg a GAPDH aktinnal való kapcsolata az oka annak is, hogy az eritrociták membránszkeletonjában tömeges enzim (105). Úgy tűnik keresi organizált, struktúrát kialakító fehérjék társaságát. Bár kissé ellentmondásos az irodalom e téren, de tetten érni vélték többen is a citoszkeleton mikrotubuláris elemeivel (106-108). Az egyik modell szerint ATP-függő módon a mikrotubulusok kötegekbe rendeződését (bundling) okozza (106).

Számos egyéb sejtbiológiai funkció kapcsán megmutatkozott már a GAPDH multifunkcionális jellege. Egy munkacsoport hősokk fehérjékre emlékeztető tulajdonságait írta le (109). Legújabban az apoptózissal foglalkozók csodálkozhattak rá a GAPDH felbukkanására (110-112).

Bizonyára többekben felmerül, ahogy bennem is, hogy vajon tényleg egy ilyen sokoldalú, a sejtmetabolizmus számos útvonalán felbukkanó fehérje-e a GAPDH, avagy valamiképpen *in vitro* furcsán viselkedik, mutatva számos aktivitást, amihez talán nem rendelhető fiziológiás szerep. Ennek a kérdésnek eredtünk nyomába az RNS-kötő képessége kapcsán, bízván abban, hogy előkísérleteink és az irodalmi ismeretek alapján inkább az előbbi tűnik igaznak.

A molekuláris biológia és biokémia ezen új eredményei jól illeszkednek laboratóriumunk sejtbiológiai koncepciójába, amely az életfolyamatok térbeli és időbeli koordináltságát, valamint az élő sejten belüli kompartmentalizáció fontosságát hangsúlyozza. A kompartmentalizáció - azáltal, hogy számos eltérő, de egyben dinamikusan szabályozott mikrokörnyezetet teremt és tart fenn - megteremti az alapját multifunkcionális fehérjék működésének. Tehát megmagyarázza és integrálja azt az egyre növekvő számú kísérletes megfigyelést, amely egy fehérjéhez (enzimhez) több funkciót rendel.

KÉRDÉSFELVETÉS

1. A T limfociták stimulációját követően számos limfokin, citokin gén aktiválódik, ám génexpressziójuk nem magyarázható csupán a transzkripció mértékével. Az irodalmi ismeretek és általunk is előkísérletekben megfigyeltek alapján, poszt-transzkripciós mechanizmusok is szerepelnek a nyugvó T sejtből aktivált limfocitává válás során. E poszt-transzkripciós szabályozáson belül **szekvencia-specifikus RNS-kötő fehérjék vizsgálatát** terveztük. Olyan fehérjékre voltunk elsősorban kíváncsiak, melyek mennyisége ill. aktivitása változik a limfociták aktivációjára, és felismerik azt a jellegzetes RNS-szekvenciát (AUUUA ill. AU-gazdag) *in vitro*, amely minden citokin, limfokin mRNS 3'UTR-ban jelen van, tehát az előzőleg vizsgált limfokin **mRNS stabilitás-beli változásokkal kapcsolatba hozhatók**.

2. E fehérjék kimutatását követően a **biokémiai jellemzés, az RNS-kötési sajátságok vizsgálata** vált kísérleti célkitűzésünkké, valamint annak megállapítása, hogy az általunk detektált molekulák más, már **ismert fehérjékkel azonosíthatók-e**. A jobb karakterizálás érdekében eme **AUBP fehérjék tisztítását** is tervbe vettük.

3. Miután az egyik AUBP a **gliceraldehid-3-foszfát dehidrogenáz enzimmel mutatott azonosságot**, erős biokémiai és immunológiai bizonyítékokat kerestünk a biztos identifikációhoz. A molekuláris biológiai jellemzés során azt szándékoztuk vizsgálni, hogy mi lehet a **molekuláris alapja az RNS-felismerésnek**, mi a kapcsolata az RNS-kötő aktivitásnak a jól ismert glikolítikus funkcióval, és hogyan történik a **két funkció szabályozása**. Kiemelt figyelmet szenteltünk az **RNS-kötő hely meghatározásának**. Többoldalú megközelítéssel arra törekszünk, hogy az RNS-kötő szubpopulációt meghatározzuk. A várható nehézségek ellenére ill. mellett a direkten vagy indirekten *in vivo* adatokat szolgáltató kísérleti stratégiák kidolgozását tartottuk és tartjuk a legfontosabb célnak.

MEGKÖZELÍTÉS ÉS MÓDSZEREK

Anyagok, reagensek

A monoklonális anti-humán UDG/GAPDH antitest (40.10.09) Michael Sirover laboratóriumából (Temple University School of Medicine, Philadelphia, PA, USA) származik. A nyúl izom GAPDH (G 2267), NADH, CAPS, DRB és poli(U) Sepharose a Sigma-tól (St. Louis, MO, USA), a NAD^+, az ADH (ló máj), az LDH (nyúl izom), az MDH (sertés szív, mitokondriális) választottunk. a proteáz inhibitorok, és a nem-jelölt nukleotidok a Boehringer-Mannheim cégtől (Indianapolis, IN, USA), míg az [α-^{32}P]UTP (~3000 Ci/mmole), Heparin Sepharose CL-6B, Protein A Sepharose, pGEX vectorok a Pharmacia-tól (Uppsala, Sweden) való.

Sejttenyésztés

Önkéntes donoroktól leukoferezis technikával leukocita-dús vért nyertünk, melyből Ficoll-Hypaque sűrűség gradiens centrifugálással mononukleáris sejteket (limfociták, monociták) izoláltunk. A sejteket $4x10^6$ sejt/ml koncentrációban 8% hővel inaktivált (56°C, 1 h) FBS-sel (Flow Laboratories, Inc.) és 50 μg/ml gentamycin szulfáttal (U.S. Biochemical Corp.) komplettált RPMI 1640 médiumban (KC Biologicals, St. Louis, MO) tenyésztettük 37 °C-on 5 % CO_2 koncentráció mellett. A sejteket 1 μg/ml koncentrációjú PHA-val az egyes kísérletekben jelzett ideig aktiváltuk.

Sejtextrakció

Az aktivált és a különböző módon kezelt sejteket PBS-sel vagy szérum-mentes médiummal 4 °C-on kétszer megmostuk, majd lízis pufferben (10 mM Pipes, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM szaharóz, 1 mM PMSF, 1 μg/ml leupeptin és pepstatin A) a sejtpelletet finoman felszuszpendáltuk. Ezt követően Triton X-100 detergenst adtunk a

sejtekhez 1 % végső koncentrációban, 3 percig jégen extraháltunk, amit 3 perces 900g centrifugálás követett 4 °C-on. Némi módosítással Cerveca és mtsai leírását követtük (38). A protein koncentrációt Micro BCA Protein Assay Reagent kittel (Pierce) határoztuk meg.

RNS-kötő fehérjék izolálása

Az AUBP-k izolálását, tisztítását egy ikerpár hemolítikus anémia miatt eltávolított lépeiből kezdtük meg. A szövetek homogenizálását, és a magfrakció eltávolítását követően szekvenciális ammónium szulfát precipitációt (30, 60 and 80 %) végeztünk. Dializálás után először Heparin Sepharose, majd poli(U) affinitás kromatográfiával tisztítottuk az RNS-kötő fehérjéket. Az AUBP aktivitás nyomonkövetésére a következőkben részletezett módon előállított, radioaktívan jelzett AU-gazdag RNS próbát (IFN-γ 3'UTR) használtunk. (A módszer részletes ismertetése a közleményekben.)

In vitro RNS-szintézis, RNS próbák

RNS-kötő vizsgálataink során magas specifickus aktivitású (~10^8 cpm/μg) RNS próbákat használtunk, melyeket linearizált transzkripciós plazmid DNS-ről (pT7/T3α19) szintetizáltunk 50 μCi [α-^{32}P]UTP, 20 μM UTP, valamint 4 mM ATP, GTP, és CTP jelenlétében, bakteriális T7 és T3 RNS polimeráz segítségével. A limfokin, citokin és proto-onkogén mRNS-ek - AU-gazdag RNS szekvenciákat hordozó - 3' UTR szakaszait tartalmazó rekombináns transzkripciós vektorokat a közleményekben részletezett módon állítottuk elő. A szekvencia-specificitást célzó kísérleteinkhez a "hideg" próbákat 4 mM ATP, GTP, CTP, és UTP jelenlétében szintetizáltuk.

In vitro RNS-kötés (label transfer)

A sejtlizátumok és a fehérjetisztítás során nyert frakciók fehérjéit, valamint a tisztított GAPDH preparátumokat 4-8x10^4 cpm [α-^{32}P]UTP-jelölt AU-gazdag RNS próbával

inkubáltuk 12 mM HEPES pH 7.9, 15 mM KCl, 0.2 μM DTT, 0.2 μg/ml élesztő tRNS, és 10% glicerol jelenlétében, 10 percig, 30°C-on. Ezután a képződött RNS/fehérje komplexeket 254 nm hullámhosszúságú monokromatikus UV-fénnyel kovalensen fixáltuk (Stratalinker 1800, Stratagene, 5 min., 3000 μW/cm^2), hogy az SDS-PAGE analízis során is együtt maradjanak. A nem kötött, tehát fehérjék által nem védett RNS molekulákat kiterjedt RNáz kezeléssel (7.5 U RNáz T1 és 15 μg RNáz A, 30 min., 37 °C) elemésztettük, így a specifikus RNS-kötés detektálását nem zavarták. A mintákat gélelektroforézist követően autoradiográfiával analizáltuk.

Fehérje elektroforézis

A klasszikus Laemmli féle egy dimenziós SDS-poliakrilamid gélelektroforézis technikát használtuk (113), általában 12.5%-os akrilamid koncentrációval. Ettől a metodikától abban az esetben tértünk el, mikor 10-kDa-nál kisebb fehérjéket, GAPDH peptid fragmentumokat vizsgáltunk V8 proteáz emésztést követően. Ez utóbbi esetben 17 %-os Tris-Tricine géleket használtunk (114). Molekulasúly markerként ^{14}C-jelölt fehérjekeveréket használtunk (GIBCO BRL, Gaithersburg, MD). A proteineket Coomassie Brillant Blue festéssel, vagy ezüstözéssel (Silver Stain Kit, Bio-Rad) tettük láthatóvá. A fehérjedetektálást követően a géleket szárítottuk, és Rtg filmen exponáltuk (általában 8-16 h). RNS-kötésre vonatkozó összehasonlító vizsgálatainkkor az autoradiográfián megjelenő aktivitást minden esetben összevetettük az adott fehérjék festődési intenzitásával, tehát mennyiségével.

RNS izolálás, mRNS féléletidő meghatározás - Northern blotting

Az mRNS féléletidőt, ill. a különböző kezelésekkel indukált mRNS stabilitás-beli változásokat az RNS-szintézis gátlását (Actinomycin D, DRB) követő specifikus RNS-szint csökkenésének detektálásával mérjük. Mivel a limfociták rendkívül gazdagok RNáz-

okban, ezért az RNS izolálás során a sejt megnyitása pillanatában igen hatékonyan és gyorsan kell e számunkra "kártékony" enzimeket bénítani. E célt szolgálja az erős fehérjedenaturánst alkalmazó, guanidium isotiocianát módszer (115). Az OD_{260} alapján azonos RNS mennyiséget tartalmazó mintákat 1%-os agaróz-formaldehid gél-elektroforézissel méret szerint elválasztottuk, és nylon membránra transzferáltuk, amelyen a limfokin mRNS-eket random priming módszerrel előállított, [α-^{32}P]UTP-jelölt gén-specifikus DNS próbákkal azonosítottunk. A Northern blot hibridizáció további részleteit a (III) Közlemény Materials and Methods része tartalmazza.

Western blotting

SDS-PAGE analízist követően a fehérjéket nitrocellulóz membránra transzferáltuk 48 mM Tris, 39 mM glycine puffer pH 9.1 vagy 10 mM CAPS pH 11.0 puffer oldatokban, 15% methanollal komplettálva. A Ponceau S festéssel vizualizált membránokat TBS-T (0.075% Tween-20) pufferben mostuk, a nem specifikus háttérjelölődést 1% BSA-val blokkoltuk. Primer antitestként monoklonális anti-humán GAPDH-t használtunk, a specifikus antigén-antitest reakciót kemilumineszcenciás módszerrel (ECL Chemiluminescence Kit for Western blotting, Amersham, Arlington Hts., IL) hívtuk elő.

Northwestern blotting

A fehérjéket a Western blotting-hoz hasonlóan nitrocellulóz membránra transzferáltuk, majd immunológiai detektálás helyett az oldatban történő *in vitro* RNS-kötésnél már ismertetett radioaktív RNS-próbákkal inkubáltuk az ott részletezett hibridizációs oldatban. A 15 perces, szobahőn történő hibridizációt követően a membránokat 3x10 percig mostuk próbát nem tartalmazó hibridizációs oldatban, Ponceau S-sel fehérjére festettük, majd Rtg filmen exponáltuk.

Peptid térképezés specifikus endoproteázzal

Tisztított GAPDH-t (Sigma, nyúl vázizom) *Staphylococcus* aureus V8 proteázzal emésztettünk, mely az általunk használt körülmények között (50 mM NH_4HCO_3, pH 7.8) csak a glutaminsav aminosavaknál hasítja a peptidláncot. Az aminosavszekvencia ismeretében a várható peptidek molekulatömege kiszámítható és a szakaszok többnyire biztonsággal azonosíthatók. A fragmentálást követően ill. azt megelőzően radioaktív RNS-t kötöttünk a fehérjére, annak megállapítására, hogy a GAPDH mely részére lokalizálható az RNS felismerés. A peptideket 17%-os Tris-Tricine géleken szeparáltuk, és ezüst-nitráttal festettük, valamint autoradiográfiával azonosítottuk az RNS-kötő fragmentet.

Aminosavszekvencia-analízis

A 36-kDa-os AUBP-t tartalmazó poli(U) affinitás kromatográfiával nyert frakciót, ill. a V8 proteázzal emésztett GAPDH-t tartalmazó mintát SDS-PAGE-t követően PVDF (polivinylidene difluoride) (Problott, Applied Biosystems, Foster City, CA, USA) membránra blottoltuk. CBB festést követően (0.1% CBB, 1% ecetsav, 40% methanol) a megfelelő mólsúlyú fehérjéket kivágtuk, és a membráncsíkokat mikroszekvencia-analízisre bocsájtottuk.

Immunprecipitáció

Az emberi lépből tisztított GAPDH-t ^{32}P-jelölt IFN-γ 3'UTR RNS próbával inkubáltunk, UV-keresztkötöttük, RNázzal emésztettük, annak megfelelően, ahogy azt a fentiekben az *in vitro* RNS-kötésnél ismertettem. Ezt követően a reakcióhoz protein A Sepharose gyöngyökhöz kötött anti-GAPDH antitestet adtunk immunprecipitációs pufferben (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 1 μM Pefabloc, 1 μg/ml leupeptin és pepstatin A). Az RNS/GAPDH komplexet a gyöngyök centrifugálásával

távolítottuk el. Kontrollként a monoklonális anti-GAPDH előállításakor használt hibridóma partner tenyésztőmédiumából tisztított antitestet használtuk. A immundepletált és immunprecipitált frakciókat egyaránt analizáltuk SDS-PAGE-t követő autoradiográfiával.

Poliszóma izolálás

A poliszómális frakciókat Brewer és Ross szerint preparáltuk (116). A limfocita sejtszuszpenzióból hipotóniás foszfát pufferrel (10mM, 15mM KCl) lizátumot készítettünk, melyből szaharóz grádiensben végzett ultracentrifugálással (130.000g, 16h, 4°C) nyertük a riboszómákat, poliszómákat tartalmazó pelletet. A mintákból az $OD_{260/280}$ alapján számított azonos anyagmennyiséget analizáltunk GAPDH tartalomra Western blot analízissel.

Fúziós fehérjék szintézise

Expressziós vektorként a pGEX vektorcsalád (Pharmacia) tagjait használtuk, melyben fúziós partnerként a gluthation-S-transferáz (GST) gén szolgál. Ehhez in-frame "ragasztottuk" a humán GAPDH cDNS-t, ill. egyes szakaszait. A Michael Sirovert-től kapott pChug1.20 (GAPDH cDNS-t tartalmazó pUC8) plazmidból EcoRI emésztéssel kinyertük a GAPDH inzert-tet, amit a pGEX 5x-1 plazmid polilinker régiójának EcoRI helyére klónoztunk be. Miután igazoltuk, hogy az így nyert rekombináns plazmidról szintetizálódik a teljes hosszúságú, 36-kDa molekulatömegű fehérje (a GST-vel együtt ~63-kDa), ezen plazmid NcoI emésztésével eltávolítottuk a molekula N-terminális 1/3-át kódoló génszakaszt. Ezt azért tehettük, mert a GAPDH inzertben lévő első és utolsó Nco I hasítási hely összeillesztése nem okoz "frame-shift"-et, és a plazmid maga nem tartalmaz Nco I hasítási helyet (lásd 7. ábra). Íly módon nyertünk egy ~50-kDa-os hibrid fehérjét, amely a 26-kDa-os GST mellett tartalmazza a a GAPDH C-terminális 226 aminosavból álló

részét. A feltételezett RNS-kötő hely pontosabb feltérképezéséhez 3 további, rövidebb DNS-szakaszt PCR amplifikációval nyertünk. Az N-terminális 141, 81 és 43 aminosavból álló szakaszokat egy közös 5' primer-rel (5'-GGGAATTCCATGGGGAAGGTGAAGGTCGGA-3') és három, egyenként 423, 243 ill. 129 bp hosszúságú DNS szakaszokat eredményező 3' primer-rel (5'-CCGTCGACTTAGTCATACTTCTCATGGTTCA-3', 5'-CCGTCGACTTAATCTCGCTCCT GGAAGATGG-3' ill. 5'-CCGTCGACTTACATGTAGTTGAGGTCAATGA-3') állítottuk elő. Mivel a primer-eket úgy terveztük, hogy az 5' végükön egy-egy restrikciós enzim (EcoR I és Sal I) hasítási helyet (aláhúzott nukleotidszekvenciák) is hordozzanak, direkcionális klónozással a két génszakaszt az EcoR I/Sal I emésztéssel megnyitott pGEX 4T-3 vektorba ligáltuk. Eredményül egy ~40-, ~35- és egy ~30-kDa molekulatömegű fúziós fehérjét kaptunk. A hibridfehérjéket szonikálással szolubilizáltuk, majd kihasználva a GST specifikus szubsztrát-kötését, Gluthatione Sepharose affinitás kromatográfiával nagy fokban tisztítottuk. Amennyiben a fúziós fehérjék zárványtestbe kerültek, úgy ureával vagy elektroforézis mintapufferben vittük oldatba.

EREDMÉNYEK ÉS MEGBESZÉLÉS

A. Közlemények, kézirat

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Association of Heterogeneous Nuclear Ribonucleoprotein A1 and C Proteins with Reiterated AUUUA Sequences*

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Post-transcriptional regulatory mechanisms have been shown to play a major role in gene expression in eukaryotic cells. The presence of a reiterated pentamer (AUUUA) in the 3′-untranslated region (UTR) of mRNAs encoding lymphokines, cytokines, transcription factors, and proto-oncogenes has been shown to be associated with rapid turnover and translation attenuation. Cytoplasmic proteins (70, 50, 43, 36, and 25 kDa) capable of specifically binding to RNAs containing these AU-rich sequences were identified in human peripheral blood T lymphocytes. Levels of the 36-kDa protein were markedly increased following transcriptional, but not translational inhibition, a feature recently reported for hnRNP A1, a protein of comparable mass. Antibodies directed against heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 and C immunoprecipitated 36- and 43-kDa proteins that had bound the AUUUA-rich region contained in the 3′-UTR of granulocyte-macrophage colony-stimulating factor mRNA. Recombinant hnRNP A1 was shown to preferentially bind to RNAs containing AUUUA sequences in a specific manner, and displayed comparable patterns to the 36-kDa AU-specific binding proteins following partial proteolysis. These data identify for the first time hnRNP A1 and C as cytoplasmic proteins in human lymphocytes that are capable of specifically associating with reiterated AUUUA sequences present in the 3′-UTR of labile mRNAs. As such, they may play a role as *trans*-acting factors in the modulation of cytoplasmic mRNA turnover and translation, in addition to their previously characterized roles as pre-mRNA binding proteins involved in nuclear mRNA processing.

In eukaryotic cells, the regulation of mRNA turnover is considerably more varied and complex than in prokaryotes (reviewed in Ref. 1). This heterogeneity in mRNA stability presumably permits eukaryotic cells nontranscriptional means to modulate the expression of genetic information. Various environmental stimuli (heat shock, viral infection, growth stimulation) can result in substantial changes in the turnover and translation rates of specific mRNAs (1). The role of *trans*-acting proteins in mediating post-transcriptional gene expression through binding to common *cis*-acting mRNA sequences has been compellingly demonstrated in the regulation of cellular iron metabolism. In this system, coordinate reciprocal changes in translation and mRNA stability are mediated by a single protein (iron-response binding protein) capable of binding to a specific iron-response element located in the 5′ and 3′ ends of the ferritin and transferrin receptor mRNAs, respectively (2–4).

A variety of proto-oncogene, transcription factor, and lymphokine genes encode mRNAs distinguished in their cytoplasmic lability (half-life < 30 min). Many of these unstable mRNAs contain reiterations of a specific sequence (AUUUA) in their 3′-untranslated region (3′-UTR)[1] (5, 6). Reiterations of this sequence are capable of conferring instability on a previously stable mRNA in heterologous gene constructs (6). Other studies have implicated these sequences in regulating translation of mRNA (7, 8). The characterization of AU-rich motifs as *cis*-acting sequences which regulate mRNA stability has prompted investigation of the *trans*-acting factors that recognize and bind these sequences. Using differing cell lines and preparations (nuclear *versus* cytoplasmic) a variety of AU-specific binding proteins (AUBPs) have been described (9–13). Malter and co-workers (9, 10) have described a cytoplasmic AUUUA-specific mRNA-binding protein of 36 kDa (denoted AUBF) in T lymphocyte cell lines and peripheral blood mononuclear cells. Using human T lymphocytes, Bohjanen (11, 12) has described three AUBPs in human T lymphocytes, AU-A, a cytoplasmic/predominantly nuclear 34-kDa protein, as well as AU-B (30 kDa) and AU-C (43 kDa) which are cytoplasmic in location and induced with activation. Vakalopoulou (13) described a 32-kDa AUBP present in nuclear and cytoplasmic extracts of HeLa cells and demonstrated a correlation between binding of this protein and reduced accumulation of both nuclear and cytoplasmic mRNA. This group also identified a 38-kDa nuclear AUBP as hnRNP C, but concluded that the 32-kDa protein was not an hnRNP A protein (13).

We now present evidence that human peripheral blood lymphocytes contain several cytoplasmic proteins (70, 50, 43, 36, and 25 kDa) capable of specifically binding to RNAs containing these AU-rich sequences. Levels of the 36-kDa protein were markedly increased following transcriptional,

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[1] The abbreviations used are: UTR, untranslated region; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; AUBP, AU-specific binding proteins; DRB, 5,6-dichlororibofuranosyl benzimidazole; PHA, phytohemagglutinin; hnRNP, heterogeneous nuclear ribonucleoprotein.

but not translational inhibition, a feature recently reported for hnRNP A1, a protein of comparable mass. Immunoprecipitation with specific antibodies demonstrated that the 36- and 43-kDa proteins share antigenic reactivity with hnRNP A1 and C. Recombinant hnRNP A1 demonstrated similar AU binding activity as the putative 36-kDa protein. To our knowledge, these data represent the first identification that some cytoplasmic AUBPs are members of the family of hnRNPs, and combined with the previous studies suggest the role of hnRNP A1 in the regulation of mRNA turnover and translation. Finally, these data implicate hnRNPs in cytoplasmic regulation of RNA metabolism in addition to their well recognized roles in pre-mRNA processing and splicing.

EXPERIMENTAL PROCEDURES

Materials—Actinomycin D, cycloheximide, 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), and trypsin were purchased from Sigma and freshly made up prior to use, except for actinomycin D, which was prepared and stored as a stock solution (5 mg/ml, 4 °C). Monoclonal antibodies SP20, 4F4 (anti-hnRNP C), and 9H10 (anti-hnRNP A1) were generously provided by Dr. Gideon Dreyfuss as were recombinant human hnRNP A1 and C (14–18). [α-^{32}P]UTP (3000 Ci/mmol) was purchased from Amersham, while unlabeled nucleotides were obtained from Boehringer Mannheim.

Cell Culture and Lysate Preparation—Human peripheral blood mononuclear cells obtained from volunteer donors by leukapheresis were isolated by Ficoll-Hypaque discontinuous gradient centrifugation and cultured at 2×10^6 cells/ml in RPMI 1640 medium (KC Biologicals, St. Louis, MO) supplemented with 8% heat-inactivated (56 °C, 1 h) neonatal bovine serum (Sigma) and 50 μg/ml gentamicin sulfate (U. S. Biochemical Corp.) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were stimulated with that concentration of PHA (1 μg/ml, Wellcome Reagent Ltd., Beckenham, United Kingdom) found to cause maximal stimulation.

Cytoplasmic lysates were prepared by washing the cells twice in ice-cold phosphate-buffered saline, followed by gentle resuspension of the cell pellet in 1% Triton X-100 lysis buffer containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and incubation for 3 min on ice followed by a 3-min centrifugation at $500 \times g$ (19). Incubation of the cell pellet in lysis buffer from 1 to 10 min prior to centrifugation did not alter the profile of AUBP detected by this method, nor did the inclusion of leupeptin (1 μg/ml) in the lysis buffer. Cell clumping was not observed. The use of this lysis buffer and method was chosen as it has been shown to leave the cytoskeleton intact with minimal polyribosome disruption (19). This extraction technique was slightly modified as the lysis buffer lacked 1 mM $CaCl_2$ and included simultaneous exposure of the cell pellet to the detergent and the other components of the lysis buffer. In multiple experiments, each of these modifications was shown not to affect the levels of the 25-, 36-, and 43-kDa AUBP in cytoplasmic lysates relative to the original method (19), nor were levels of AUBP altered in comparison to lysates prepared with Nonidet P-40-containing lysis buffers (9). These data are consistent with observations in the original report that the presence of calcium in the lysis buffer was shown not to be essential (19).

RNA Probes and AUBP Assay—The Δ2R1 probe, which contains a sequence found in the 3′-UTR of GM-CSF mRNA (9), was prepared by T7 RNA polymerase transcription of *Eco*RI-linearized pT7/T3α19 plasmid with 4 AUUUA sequences in the *Bam*HI site of the multiple cloning site. Δ2H3 was prepared by T3 RNA polymerase transcription of *Hin*DIII-linearized plasmid described above. The 3′-IL-2 RNA probe was prepared by transcription of *Eco*RI-linearized pT7/T3α19 plasmid with the 270-base pair *Stu*I-*Eco*RI fragment of human IL-2 (20) inserted into the *Bam*HI site of the multiple cloning site with transcription by the T7 RNA polymerase. The antisense 3′-IL-2 probe (3′-IL-2AS) was prepared by transcription of *Hin*dIII-linearized plasmid described for 3′-IL-2 with T3 RNA polymerase. 3′ c-*myc* was prepared by SP6 RNA polymerase transcription of *Ssp*I-linearized pRK5 plasmid with the 400-base pair *Nsi*I-*Afl*II human c-*myc* fragment (21) inserted into the *Sma*I site of the multiple cloning site. The transforming growth factor-β2 fragment was prepared by SP6 RNA polymerase transcription of a *Hpa*I-linearized pSP72 plasmid with the 595-base pair *Pst*I-*Hin*dIII fragment corresponding to nucleotides 253–853 of the original sequence (22).

α-^{32}P-Labeled mRNAs with specific activity of $>10^6$ cpm/μg RNA were prepared by *in vitro* transcription in the presence of 50 μCi of [^{32}P]UTP (3000 Ci/mmol) from Amersham, 0.0125 mM UTP, 2.5 mM ATP, GTP, and CTP from Boehringer Mannheim. The AUUUA sequences in each RNA are *underlined* in Fig. 1. RNA probes (8×10^4 cpm; ~3–14 fmol) were incubated with 10–20 μg of protein from lysates in 12 mM Hepes, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol for 10 min at 30 °C. In previous experiments, we have determined that maximal RNA-protein binding interaction is achieved in less than 5 min at this temperature. UV cross-linking is performed at 4 °C using a Stratagene UV Stratalinker 1800 (5 min, 3000 microwatts/cm^2) followed by RNase digestion (5 units of RNase T1 and 10 μg of RNase A for 30 min at 37 °C). The sample was then analyzed under denaturing conditions by 12% SDS-PAGE, followed by autoradiography.

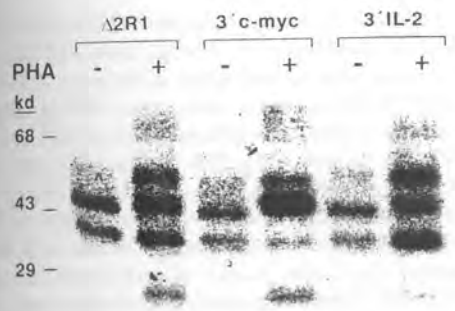
Immunoprecipitation of AUUUA-binding Proteins with Monoclonal Antibodies to HnRNP C and A1 Proteins—Human lymphocyte cytoplasmic lysates were incubated with α-^{32}P-Δ2R1 RNA, UV cross-linked, and digested with RNase as described above. The RNA-protein complexes were added to 1:500 dilution of SP2O (negative control antibody), 4F4 (anti-hnRNP C), or 9H10 (anti-hnRNP A1) ascites in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 1 mM PMSF and incubated 2 h at 4 °C. 5 μg of goat anti-mouse F(ab′)$_2$ and 25 μl of protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc.) were added and incubated with gentle mixing for 1 h at 4 °C. Beads were pelleted by brief centrifugation, washed 5 times in phosphate-buffered saline, boiled in SDS sample buffer, and analyzed by 12% SDS-PAGE. Using this method, 9H10 antibody was capable of completely immunoprecipitating 1 μg of recombinant hnRNP A1, with no evidence of cross-reactivity with SP20 and 4F4 antibodies.

Analysis of AUUUA-binding Proteins by Partial Proteolysis—Cytoplasmic lysates (5 μg) prepared from 20-h PHA (1 μg/ml) + 2-h actinomycin D (5 μg/ml) treatment or recombinant hnRNP A1 (0.5 μg) or hnRNP C (0.1 μg) were incubated with ^{32}P-Δ2R1 (8×10^4 cpm), UV cross-linked, and digested with RNase as described above. RNA-protein complexes were incubated with trypsin (ranging from 0.25 to 2.5 ng, 2000:1, w/w) in 50 mM Tris-HCl, pH 8.0, 325 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A for 0, 5, 15, and 60 min at room temperature. Trypsin digestion was stopped by adding to SDS-PAGE sample buffer and boiling, followed by analysis under denaturing conditions by 15% SDS-PAGE.

RESULTS

Cytoplasmic Proteins with AUUUA Binding Activity (AUBP) from Resting and PHA-activated Peripheral Blood Lymphocytes—Cytoplasmic lysates were assayed for binding to *in vitro* transcribed, radiolabeled AU-rich sequences present in the 3′-UTR of lymphokine (IL-2, GM-CSF) or c-*myc* mRNAs. The Δ2R1 probe contains a sequence found in the 3′-UTR of GM-CSF mRNA (9) (Fig. 1). After coincubation of AUUUA-containing RNA with cytoplasmic lysates, ultraviolet cross-linking and RNase digestion were performed, the samples were then analyzed by SDS-PAGE. Three major AU-rich RNA-protein complexes (36, 43, and 50 kDa) were detected in cytoplasmic lysates from resting and activated cells (Fig. 1). The intensity of each of these bands was increased with activation. Activation also induced the appearance of two additional AUBPs (70 and 25 kDa), with the level of the 25-kDa protein being quite variable, and each usually much less relative to the other proteins. The increases in AUBP upon activation were not readily apparent before 8–12 h of lectin activation. Similar patterns of AU-specific binding proteins were observed with purified (>90% CD3+) T cell preparations (data not shown).

In previous studies, we found that the interaction between the AUUUA-containing RNA probes and the 30–40-kDa AUBP is specifically with the reiterated AUUUA sequence (5). Unlabeled 3′ GM-CSF mRNA was able to block binding of the 36-, 43-, 50-, and 70-kDa proteins to radiolabeled 3′ GM-CSF mRNA probe, thus demonstrating the AUUUA



Δ2R1 5′ T7- GGAUCCAUUUAUUUAUUUAUUUAAGCUUGG-3′

Δ2H3 5′ T3- CCAAGCUUAAAUAAAUAAAUAAAUGGAUCC-3′

5′IL-2 5′ T7- AGGCCUUCUAUUUAUUUAAUAUUUAAAUUUUA
UAUUUAUU-$(N)_{120}$-CACUUAUUUAUCCCAAAUAU
UUAUUA-$(N)_{40}$-AAAACUAUUUAAUAAAUUUGAUA
AAUAU-$(A)_{12}$-C-$(A)_{11}$-3′

3′IL-2AS 5′ T3- $(U)_{11}$-G-$(U)_{12}$-AUAUUUATCAAAUUUAUUAAAUAGUUU
UACUAACCAAUCUACAUAGAUACUAUAUUUAACAUU-
CAACA-$(N)_{80}$-UAUUUAUAGUUUUUUGAUUAAGA-$(N)_{40}$
AAAUAUAAAAUUUUAAAUAUUUAAAUAAAUAGAAGGCCU

3′c-myc 5′ SP6- UGAUCAAAUGCUUCCUCACAACCUUGGCUGAAGUC
UUGAGACUGAAAGAAUUUUAAGCCAUAAUGUAAACU
GCCUCAAAUUGGAACUUUGGGCAUAAAAGAACUUU
UUUAUGCUUACCAUCUUUUUUUUUCUUUAACAGAUU
UGUAUUUUAAGAAUUGUUUUUAAAAAAUUUUAAGAU
UUACACAAUGUUUCUCUGUAAAU-3′

TGF-β2 5′ SP6-$(N)_{574}$-GAACCUGGGAUUUAAAAUA

FIG. 1. **AUUUA-binding proteins detected in human resting and PHA-activated PBMC.** *Upper panel*, cytoplasmic lysates (20 μg of protein) from human lymphocytes cultured 24 h ± PHA were incubated with 8×10^4 cpm of indicated ^{32}P-RNA probes, UV cross-linked, and RNase digested before analyzing under reducing conditions by SDS-PAGE. *Lower panel*, sequence of RNA probes with AUUUA sequences *underlined* are shown.

specificity of the RNA-protein interaction (Fig. 2, *upper panel*). The cross-reactivity and specificity of the AUBP for reiterated AUUUA sequences was investigated (Fig. 2, *middle panel*). Radiolabeled IL-2 3′-UTR binding to AUBP was competed by the unlabeled 3′-UTR of GM-CSF, c-*myc*, and itself, while the antisense of the 3′-UTR of GM-CSF did not compete at all. To further insure that the observed AUBP binding did not require the presence of additional sequences, the antisense RNA of the 3′-UTR of IL-2 mRNA (IL-2AS which contains 5 AUUUA multimers) was examined. Comparable profiles of AUBPs were observed with IL-2AS relative to those of sense IL-2, GM-CSF, and c-*myc* UTRs (Fig. 2, *lower panel*). The binding of AUBP to the antisense of the 3′-UTR of IL-2 mRNA was competed equally well by both unlabeled 3′-UTR of GM-CSF and the antisense IL-2, further indicating the specificity of the observed binding. The size of the 36-, 43-, and 70-kDa AUBPs are similar to those that have been differentially observed in separate reports (9–13). As found previously (9), pretreatment of cytoplasmic lysates with proteinase K abolished all AUBP activity, confirming the polypeptide nature of their binding activity (data not shown).

Modulation of Cytoplasmic AUBP by Transcriptional but Not Translational Inhibition—Previous work has shown that after prolonged transcriptional inhibition, the stability of AUUUA-containing c-*fos* mRNA was selectively increased over time (23). The effect of transcriptional inhibition upon the activity of AUBPs was therefore examined (Fig. 3). Treatment of PHA-activated lymphocytes with actinomycin D or DRB increased the level of the 36-kDa AU-specific binding protein after 30–60 min. Cycloheximide treatment alone, or

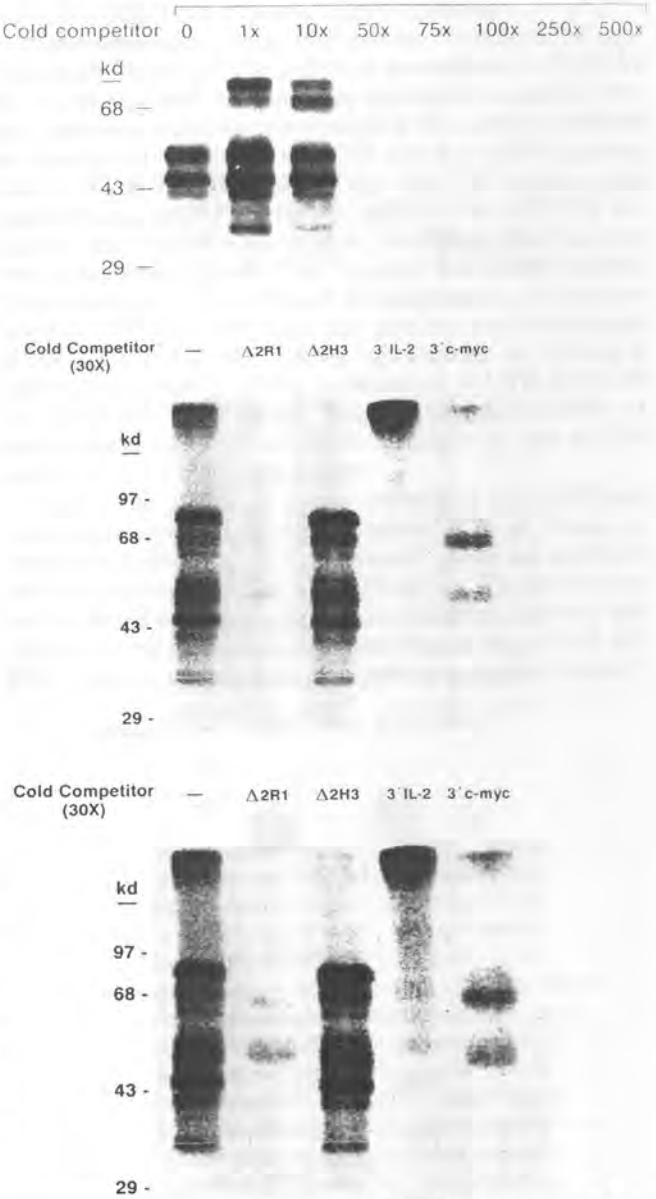


FIG. 2. **Specific competition of AUUUA binding activity.** *Upper panel*, ^{32}P-Δ2R1 was transcribed and incubated with 20 μg of activated (8 h PHA) human lymphocyte cytoplasmic lysate in the presence of 0–500 × molar excess of cold Δ2R1 and analyzed as described above. ^{32}P-3′-IL-2 (*middle panel*) and 3′-IL-2AS (*lower panel*) were incubated with lymphocyte cytoplasmic lysates (20 μg of protein, 8 h PHA) in the absence or presence of a 30 × molar excess of the specified unlabeled RNA probes, UV cross-linked, RNase digested, and analyzed by 12% SDS-PAGE.

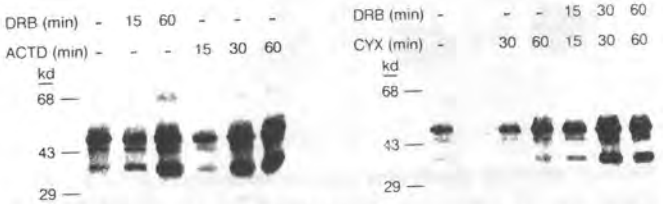


FIG. 3. **Transcriptional inhibition increases AUUUA-binding proteins.** Cytoplasmic lysates were prepared from 20-h PHA-activated human lymphocytes before and after addition of 100 μM DRB, 5 μg/ml actinomycin D (*Act. D*), or 20 μg/ml cycloheximide (*CYX*) for the indicated times and assayed for binding to ^{32}P-Δ2R1 by 12% SDS-PAGE.

in concert with DRB treatment failed to modulate cytoplasmic levels of AUBPs, even up to 3 h of treatment (data not shown). Smaller, but substantial increases in the binding activity of the other AUBPs were also observed with transcriptional inhibition. Earlier work by Dreyfuss demonstrated a 38-kDa protein that associated with cytoplasmic poly(A)$^+$ mRNA following transcriptional, but not translational, inhibition (14). This protein was eventually identified as the hnRNP A1, and has recently been shown to shuttle between the nucleus and cytoplasm in HeLa cells, with increased cytoplasmic accumulation following transcriptional inhibition (16).

Identification of the Cytoplasmic 36- and 43-kDa AUBPs as HnRNPs—The selective modulation of the 36-kDa AU-specific binding protein by transcriptional inhibitors as well as the size similarity of the 36- and 43-kDa AUBP with the hnRNP A1 and hnRNP C proteins (molecular mass 41 and 43 kDa) (24, 25), suggested that some or all of these proteins were hnRNPs. This issue was addressed using monoclonal antibodies directed against the hnRNPs A1 and C proteins (14–16). Cytoplasmic lysates from activated lymphocytes were incubated with radiolabeled GM-CSF 3′-UTR mRNA, UV cross-linked, and immunoprecipitated (Fig. 4). Antibody directed against the anti-hnRNP A1 (9H10) precipitated a single radiolabeled RNA-protein complex of 36 kDa from each lysate. In contrast, antibody (4F4) which recognizes the hnRNP C1 and C2 proteins (41 and 43 kDa by SDS-PAGE) precipitated two bands (43 and 36 kDa). The relative ratio of the 43-kDa RNA-protein complex to that of the 36-kDa RNA-protein complex appeared to vary in immunoprecipitation experiments with the 4F4 antibody. In addition, 4F4 occasionally weakly precipitated a 70-kDa protein (experiment 2). These latter results are not unexpected, as previous studies have demonstrated that this anti-hnRNP C antibody (4F4) immunoprecipitates multiple hnRNPs (including hnRNP A1) in the absence of ionic detergents (15). Nevertheless, the detection of these specific immunoprecipitated protein-RNA complexes indicates their direct physical contact with the radiolabeled AUUUA-containing RNA probe (26), and establishes both of these proteins as having the appropriate size and immunologic cross-reactivity with the hnRNP A1 and C proteins. Given the apparent cross-reactivity of the 4F4 antibody with hnRNP A1, we have also demonstrated that under these conditions, 9H10, but not the 4F4 or SP20 ascites, can immunoprecipitate recombinant hnRNP A1·radiolabeled 3′-UTR GM-CSF RNA complex (data not shown).

These observations suggested that the 36-kDa AUBP represented hnRNP A1. The ability of recombinant hnRNP A1 to specifically bind to sequences containing reiterated AU-rich sequences was therefore examined. Recombinant hnRNP A1 specifically bound the GM-CSF and IL-2 mRNA probes, while binding the antisense of the GM-CSF probe or the transforming growth factor-β2 mRNA (one AUUUA sequence) was markedly diminished, parallelling that observed with cytoplasmic lysates (Fig. 5, *upper* and *middle panels*). This specificity was confirmed by demonstrating that unlabeled 3′-UTR GM-CSF, IL-2, or c-*myc*, but not the antisense of the 3′-UTR GM-CSF mRNA, could inhibit the binding of recombinant hnRNP A1 to radiolabeled 3′-UTR GM-CSF mRNA (Fig. 5, *lower panel*). Thus, recombinant hnRNP A1 demonstrates identical sequence specificity as the 36-kDa protein in our lymphocyte lysates.

Finally, the effects of partial proteolysis on AUBP and recombinant hnRNP A1 were compared (Fig. 6). Either recombinant hnRNP A1 or a cytoplasmic lysate was incubated with radiolabeled 3′-UTR GM-CSF mRNA, UV cross-linked, and subjected to partial proteolysis with trypsin. With 5 min of trypsin treatment, a marked reduction in the hnRNP A1·RNA complex is observed, disappearing completely after 15

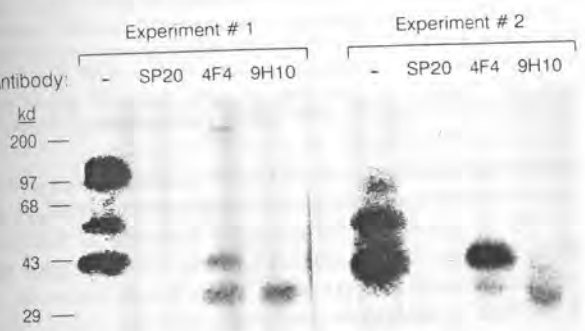


FIG. 4. **Immunoprecipitation of AUUUA-binding proteins with monoclonal antibodies for hnRNP C and A1 proteins.** Human lymphocyte cytoplasmic lysates (PHA-activated for 8 h) were incubated with ^{32}P-Δ2R1, UV cross-linked, and digested with RNase. The RNA-protein complexes were immunoprecipitated with protein A-Sepharose beads following incubation with SP20 (negative control antibody), 4F4 (anti-hnRNP C), or 9H10 (anti-hnRNP A1) ascites, boiled in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography for 7 days. Untreated lysates are shown as shorter exposures, with the 36-kDa AUBP poorly visualized.

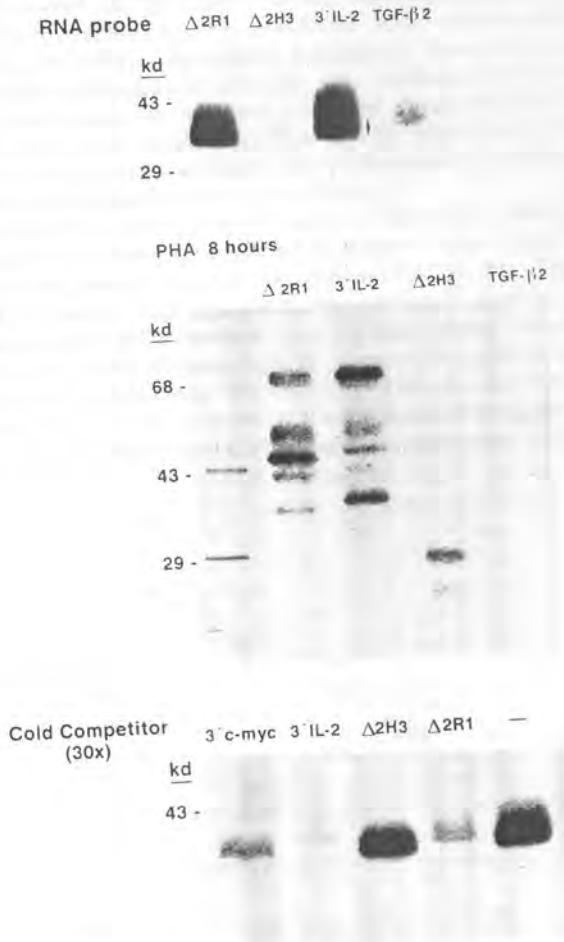


FIG. 5. **Comparison of binding specificity between hnRNP A1 and AUBP.** Recombinant hnRNP A1 protein (0.1 μg) (*upper panel*) or cytoplasmic lymphocyte lysate (20 μg) (*middle panel*) was incubated with the indicated ^{32}P-RNA, UV cross-linked, digested with RNase, and analyzed by 12% SDS-PAGE. *Lower panel*, recombinant hnRNP A1 (0.1 μg) was incubated with radiolabeled Δ2R1 in the absence or presence of a 30 × molar excess of the specified unlabeled RNA, and analyzed as described in the legend to Fig. 1.

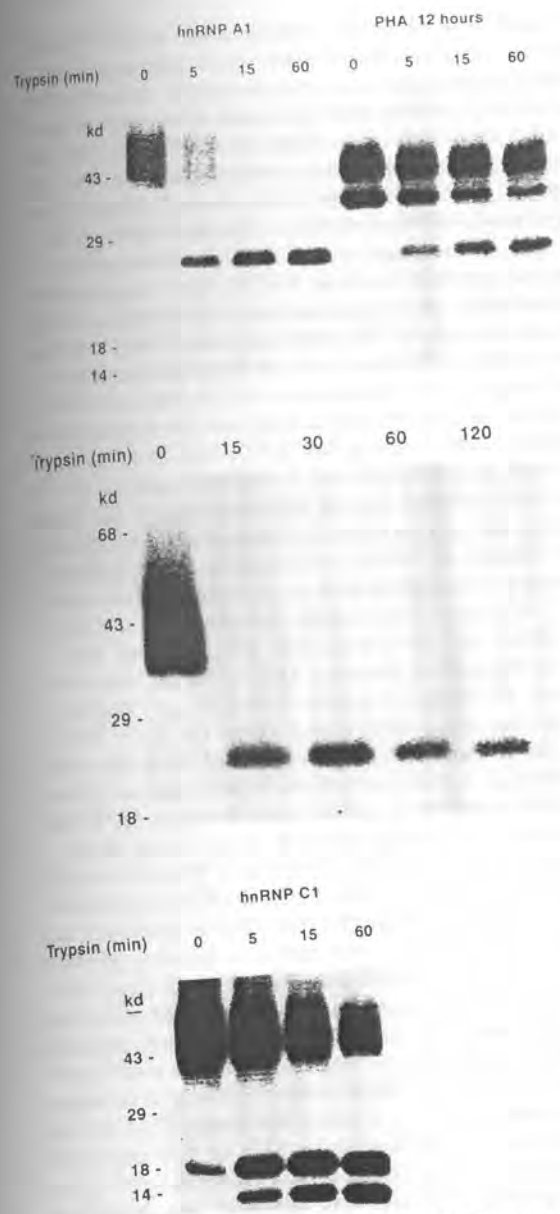


FIG. 6. **Trypsin digestion of AUUUA-binding proteins and recombinant hnRNP A1.** Cytoplasmic lysate (5 μg) from 20-h PHA-activated (with actinomycin D (*Act. D*) treatment for the last 2 h-treated) human lymphocytes or recombinant hnRNP A1 or C protein (0.5 μg) was incubated with ^{32}P-Δ2R1, UV cross-linked, and digested with RNase. RNA-protein complexes were incubated with trypsin for the indicated times and analyzed by 15 (*upper* and *lower panel*) or 12% (*middle panel*) SDS-PAGE.

min of trypsin treatment. With the disappearance of the larger RNA·hnRNP A1 complex, a corresponding increase in a 25-kDa AUBP is observed, probably corresponding to UP-1, a single-stranded DNA-binding protein which has been shown to be generated from hnRNP A1 by trypsin digestion (27–29). In a 15% SDS-polyacrylamide gel, recombinant hnRNP A1 appeared to run as a 43-kDa RNA·protein complex. Utilizing a 12% SDS-polyacrylamide gel, the recombinant hnRNP A1 yielded a molecular mass of 36 kDa as previously demonstrated (Fig. 6, *middle*), thus indicating that the apparent mass of recombinant hnRNP A1 size was influenced by the increasing the percentage of polyacrylamide from 12 to 15%.

A similar sensitivity to trypsin was observed with the cytoplasmic lysates, with progressive disappearance of the 36-kDa AUBP (34% of control after 60 min) and a corresponding appearance of the 25-kDa AUBP (Fig. 7). When proteolytic digestion was performed following RNA binding, a significant dimunition in the 43-kDa AUBP was not observed (77% of control after 60 min), allowing us to conclude that, under these conditions, the 25-kDa AUBP appeared to derive primarily from the 36-kDa AUBP, which we have identified as hnRNP A1. Recombinant hnRNP C was considerably more resistant to trypsin digestion than recombinant hnRNP A1 (Fig. 6, *bottom*), and therefore approximates what was observed for the 43-kDa AUBP in the cytoplasmic lysates. Trypsin digestion of recombinant hnRNP C·RNA complex yields 18- and 14-kDa AUBPs. These studies provide further evidence that the 25-kDa AUBP observed with partial proteolysis of the cytoplasmic lysates is generated from the 36-kDa AUBP that we have identified as hnRNP A1, and not the 43-kDa AUBP protein we have identified as hnRNP C.

DISCUSSION

These data indicate that two of the AUBPs detected in these experiments are members of the family of hnRNPs. In particular, the identification of the cytoplasmic 36-kDa protein as hnRNP A1 would seem strongly supported by our findings: (i) specific immunoprecipitation; (ii) modulation of cytoplasmic levels with transcriptional, but not translational inhibitors; (iii) comparable patterns of AU-specific RNA binding between the 36-kDa AUBP and recombinant hnRNP A1; (iv) comparable sensitivity and AUBP patterns generated by partial proteolysis. By similar, but slightly less stringent criteria (size and immunoreactivity), the cytoplasmic 43-kDa AUBP would appear to represent a member of the hnRNP C protein family, but another immunologically related hnRNP cannot be conclusively excluded due to the lack of absolute specificity of the anti-hnRNP C antibody.

The hnRNP proteins are a family of abundant nuclear proteins that has been generally implicated in mRNA metabolism at the level of pre-mRNA splicing (25, 30, 31). The hnRNP A1 and C proteins have been shown to bind to a polypyrimidine stretch bordered by AG at the 3′ end of introns (30, 31), and antibodies to hnRNP C have been shown to inhibit splicing reactions *in vitro* (25). With the discovery that hnRNP A1 shuttles between the nucleus and cytoplasm (16), the possibility exists that hnRNP A1 may exert addi-

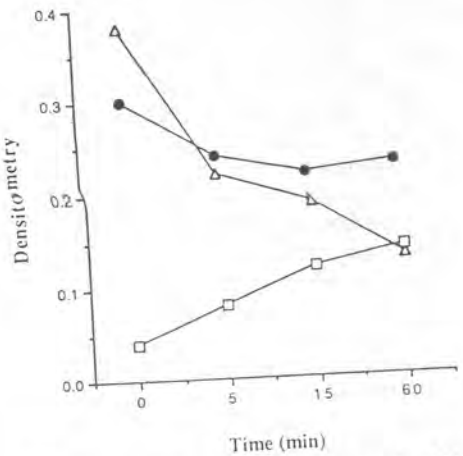


FIG. 7. **Quantitative analysis of changes in AUBP activity in response to partial proteolysis.** Densitometric scanning was performed of the cytoplasmic lysate autoradiograph shown in Fig. 6, *upper panel*. Data is graphed as densitometric units of absorbance relative to duration of trypsinization prior to analysis by SDS-PAGE. After 60 min of proteolysis, the 43- (●) and 36 (△)-kDa AUBPs had declined by 23 and 66%, respectively, while the 25-kDa (□) AUBP had increased by 350%, relative to their levels prior to trypsinization.

tional influences upon mRNA metabolism beyond that of nuclear processing. Since intron-containing pre-mRNAs are spliced prior to nuclear export, and cytoplasmic hnRNP A1 has been shown to be associated with mRNA (16), the findings reported here raise the possibility that the hnRNP A1 protein we have described may favor association with RNA polymerase II transcripts that contain reiterated AUUUA sequences.

The possible etiology of hnRNPs as AUBPs was first raised by Vakalopoulou *et al.* (13), who reported 32- and 43-kDa nuclear proteins with AUBP activity. Through immunoprecipitation, this 43-kDa nuclear protein was identified as being immunologically related to an hnRNP C protein, thus supplying the first identification of an hnRNP protein's ability to bind to reiterated AUUUA sequences. Our studies thus serve as confimation of this original description, but differ significantly as the 43-kDa AUBP was found in the cytoplasm, and not the nucleus. Because of the above finding, these authors considered, and rejected, the notion that the 32-kDa AUBP found in the nucleus and, to a lesser extent, the cytoplasm of HeLa cells, might represent an hnRNP A protein. This conclusion was based in part, on their inability to immunoprecipitate this protein with the relevant antibody (13).

Their findings raise the issue that the identity of the cytoplasmic 32-kDa AUBP reported by this group might not be hnRNP A1, although another possibility may be that hnRNP A1 is not easily precipitated in certain cell types or subcellular sites. Indeed, we have found that under conditions that would completely immunopreciptate 1 μg of recombinant hnRNP A1, only a fraction of the cytoplasmic 36-kDa AUBP is immunoprecipitable. A similar inability to immunodeplete with anti-hnRNP C antibody was observed (data not shown). A second possibility is that hnRNP A1 proteins in the nucleus and cytoplasm of these two different cell types may represent differing isoforms or alternately spliced forms (hnRNP A2) of the same hnRNP A1 gene family (17, 33, 34). However, antibodies to hnRNP A1 have been shown to detect these alternate forms (33, 34), making this possibility unlikely. Third, these two proteins may be completely unrelated, as is suggested by the report that partial proteolysis of the 34-kDa nuclear AUBP from marmoset T cells was quite distinct from that of purified hnRNP A1 (35). It is unclear if these experiments involved assay of hnRNP A1 binding to AUUUA multimers, and whether any AUUUA binding activity of hnRNP A1 was observed.

In any case, the evidence we have presented (modulation by transcriptional inhibitors, immunologic cross-reactivity, comparable binding profiles with recombinant hnRNP A1 protein) would seem to establish that the 36-kDa cytoplasmic protein is a member of the hnRNP A1 family of proteins. Moreover, hnRNP A1 has been shown to have a trypsin-sensitive site which, when cleaved, generates a 25-kDa protein, UP-1 (27, 28), initially described in thymic extracts for its ability to bind single-stranded DNA (29). Given identification of the cytoplasmic 36-kDa AUBP as hnRNP A1, as well as the partial proteolysis experiments, the occasionally observed 25-kDa AUBP would seem likely to represent UP-1, which may occur *in vivo* (28). Finally, hnRNP A1 has been demonstrated in the cytoplasm of activated lymphocytes and is modulated by transcriptional inhibitors, thus confirming the cytoplasmic location observed in these experiments.[2] Unresolved in these studies is the identity of the other cytoplasmic AUBPs that we have detected. Our findings suggest that the AUBPs might be either members of, or related to, the family of hnRNPs, all of which contain RNA-binding domains (36, 37). The reactivity of the 4F4 antibody with the 43- and 70-kDa AUBP would suggest that the latter protein might also be a member of the hnRNP family.

The finding that hnRNP C protein is a cytoplasmic AUBP differs from previous work (13), where hnRNP C was identified as a nuclear AUBP in HeLa cells. These studies raise the possibility that the hnRNP C detected in our experiments may represent hnRNP C leakage from the nucleus. Alternatively, the different subcellular localization of these proteins may result as a consequence of differing cell types and growth conditions (resting peripheral blood lymphocytes as opposed to an actively replicating HeLa cell line). Several lines of evidence support the hypothesis that nuclear leakage of hnRNP C does not occur under the conditions utilized in these experiments. First, Western blotting of cytoplasmic lysates did not demonstrate the 70-kDa U1 small nuclear (sn) RNP (data not shown), indicating that leakage of this nuclear protein had not occurred. Thus, nuclear contamination of our cytoplasmic lysates was therefore not evident by this index. Second, cytoplasmic preparations of human peripheral blood monocytes are devoid of AUBP activity relative to resting lymphocytes (data not shown), indicating that nuclear leakage of hnRNP C from a cell closely related to the lymphocyte is not observed. Moreover, it indicates that the lymphocyte appears distinct from the monocyte in terms of the presence of cytoplasmic hnRNP, making our findings with those of Vakalopoulou *et al.* (13) less surprising. Third, previous studies have indicated that hnRNP are tightly associated with nuclear hnRNA (38), such that hnRNP leakage from the nucleus would not be expected to easily occur. In support of this view has been our finding that the profile of cytoplasmic AUBP was unchanged over 1–10 min of detergent exposure (data not shown). Moreover, using different methods of cytoplasmic lysate preparation, other groups have also observed a cytoplasmic 43-kDa AUBP (10, 11). Despite these considerations, it is clear that definitive localization of hnRNP C as a cytoplasmic protein is beyond the scope of this study. These studies identify the 43-kDa AUBP that we have found in cytoplasmic preparations of peripheral blood lymphocytes as the hnRNP C protein, which thereby raises interesting issues relative to both the cytoplasmic localization and the biologic role of hnRNP C in lymphocytes.

Given the well characterized role of the AUUUA sequences in the regulation of cytoplasmic mRNA turnover and translation (5–8), this characterization of two hnRNPs as AUBPs leads to the consideration of a novel biologic role for the hnRNP protein family. An analysis of the functional relevance of their binding to lymphokine mRNA is beyond the scope of this article and promises to be difficult, given other studies that have sought a direct consistent correlation between AUBP binding *in vitro* and mRNA turnover. In a previous study (13), an AUUUA motif flanked by polyuridines efficiently bound a 32-kDa AUBP, but mRNA lability (both nuclear and cytoplasmic) required at least three AUUUA sequences. This finding would suggest that binding of an AUBP to the reiterated AU sequences facilitates RNA turnover, as would the report that 37- and 40-kDa c-*myc*-specific AUBPs exhibited intrinsic nuclease activity (39). Although we are unaware of any reports of nuclease activity being exhibited by hnRNPs, we have found that increases in cytoplasmic AUBP do not occur prior to 8–12 h of PHA activation, a time when lymphokine mRNA levels begin to decline despite continued high rates of gene transcription (40).

These data would be most consistent with the hypothesis that cytoplasmic levels of the 36-kDa AUBP/hnRNP A1 directly correspond to increased mRNA turnover. This theory is in general agreement with the observation that co-stimu-

[2] E. Nagy and W. F. C. Rigby, manuscript in preparation.

lation of T cells with a phorbol ester and anti-CD3 antibody induces the disappearance of a 30-kDa AUBP, while increasing lymphokine mRNA stability (11). In contrast, the increase in cytoplasmic hnRNP A1 that occurred with inhibition of RNA polymerase II would suggest an mRNA stabilizing activity given the report that transcriptional inhibition reduces the turnover of mRNA containing reiterated AUUUA sequences (23). However, this effect of transcriptional inhibition requires prolonged (>2 h) treatment with DRB or actinomycin D, in contrast to the more rapid (30 min) modulation of hnRNP A1, suggesting that another mechanism might mediate this effect. It is clear that AUUUA-dependent post-transcriptional regulation of gene expression will involve multiple mechanisms, as we found that cycloheximide treatment had no effect of cytoplasmic AUBP levels although it has been reported to increase the stability of lymphokine mRNA (41, 42). The complexity of these pathways is further supported by the observation that the turnover of cytokine mRNA can be uncoupled from that of proto-oncogenes such as c-*fos* and c-*myc* (43–45).

In conclusion, these studies would suggest that hnRNP A1 and C proteins present in human T lymphocytes are capable of specifically binding to the AU-rich sequences contained in the 3′-UTR of lymphokine and c-*myc* mRNA. As these *cis*-acting sequences have been shown to be functionally important determinants in the post-transcriptional regulation of gene expression, the association of these proteins with the reiterated AUUUA sequences may play an important role in mRNA turnover and translation. With the identification of these proteins, future studies can now be more carefully directed at defining their functional role(s) in the post-transcriptional regulation of lymphokine gene expression.

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Enhanced Stability of Interleukin-2 mRNA in MLA 144 Cells

POSSIBLE ROLE OF CYTOPLASMIC AU-RICH SEQUENCE-BINDING PROTEINS*

(Received for publication, August 6, 1993, and in revised form, October 13, 1993)

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The MLA 144 gibbon T cell line is infected with a type C retrovirus and constitutively expresses interleukin-2 (IL-2) and granulocyte macrophage colony-stimulating factor (GM-CSF). IL-2 mRNA levels are 10-fold more abundant than GM-CSF in these cells. Comparable transcriptional rates for these lymphokines suggested the involvement of post-transcriptional mechanisms in selective IL-2 mRNA accumulation. IL-2 mRNA is exceptionally stable in MLA cells with a $t_{1/2}$ of more than 8 h. The presence of reiterated AUUUA sequences in the 3′-untranslated region (UTR) has been shown to confer mRNA lability. The provirally altered MLA IL-2 allele encodes an mRNA in which three AUUUA motifs have been deleted. Six major cytoplasmic proteins bound *in vitro* transcribed RNA probes containing sequences from the 3′-UTR of normal human IL-2 (3′-IL-2), GM-CSF (Δ2R1), and the virally altered MLA IL-2 (3′-IL-2 PV) mRNA. Increased binding of these proteins to 3′-IL-2 PV was observed relative to 3′-IL-2 or Δ2R1. Northwestern blotting demonstrated similar differential ability of a 36- and 43-kDa protein to bind, as well as showed that these proteins colocalized by immunoblotting as hnRNP A1 and C, respectively. These findings suggest a direct correlation between differential binding of cytoplasmic proteins to AU-rich 3′-UTRs *in vitro* and lymphokine mRNA stability *in vivo*.

Post-transcriptional processes provide an important level of regulation in eukaryotic gene expression in response to various external stimuli (1, 2). Biologically active molecules (cytokines, proto-oncogenes, lymphokines) important in cell growth and differentiation are frequently encoded by mRNA that exhibit rapid cytoplasmic turnover (half-life = $t_{1/2}$ < 30 min) (3–5). Reiterated AUUUA pentamers (ARE) found within the 3′-untranslated region (UTR)[1] of these intrinsically labile mRNAs have been shown to confer mRNA lability using heterologous gene constructs (6, 7). These ARE appear to function as *cis*-regulatory elements capable of modulating both the turnover and translation of specific mRNAs (6–8). Cytoplasmic proteins that specifically bind to ARE of the 3′-UTR of lymphokines (9), cytokines (10), and proto-oncogenes (11, 12) have been identified as potential *trans*-acting factors which transduce this activity. Nevertheless, direct functional correlations between specific cytoplasmic protein binding to an ARE and mRNA turnover are lacking. This correlation may be of importance in understanding the disordered cell growth and differentiation that characterize neoplastic transformation. Considerable evidence exists to suggest that ARE-dependent mRNA turnover is important in tumorigenesis (13–15). Additionally, enhanced proto-oncogene or growth factor mRNA stability is observed frequently in neoplastic cells (16, 17). Thus, evolution and/or maintenance of the transformed state might possibly be mediated through enhanced expression of active growth factors or proto-oncogenes. In this paper, we describe a cell line in which abnormal growth factor mRNA stability is observed and provide a direct correlation between AUBP-ARE interactions *in vitro* with cytokine mRNA stability *in vivo*.

Constitutive production of biologically active lymphokines, such as interleukin 2 (IL-2) and granulocyte macrophage colony-stimulating factor (GM-CSF), is a characteristic feature of the cell line MLA 144 (18). These cells were derived from lymphoid tumor tissue and are infected with a type C retrovirus, gibbon ape leukemia virus, which has been associated with several hematopoietic neoplasms (19). Constitutive IL-2 gene transcription occurs in the presence of two retroviral insertions within one allele of the MLA IL-2 gene (20). A viral 5′ long terminal repeat (LTR) is found in the 5′-flanking region in the antisense orientation, about 1200 bases upstream from the coding region. Transcriptional analysis demonstrated that this gibbon ape leukemia virus sequence has weak enhancer activity (21). Additionally, a proviral insertion with an internal 3.25-kb deletion is present within the 4th exon of the IL-2 gene. This insertion results in a 1.1-kb mRNA (≈200 bases longer than the native IL-2 message) in which the coding region and the first 100 bases of the 3′-UTR are that of IL-2, and the remaining 495 bases are that of the LTR of the provirus. This 3′ proviral integration event deletes three of the seven AUUUA pentamers of the IL-2 mRNA, but preserves the AU-rich region containing the remaining four ARE, 43 bases 3′ to the coding region (20). At the site of proviral insertion, there is also a deletion of four bases from the native IL-2 gene.

We observed that IL-2 mRNA is more abundant than GM-CSF despite comparable rates of transcription in MLA 144 cells. Message stability studies revealed IL-2 mRNA to be exceptionally stable with a half-life ($t_{1/2}$) of more than 8 h. In contrast, GM-CSF mRNA turnover was rapid ($t_{1/2}$ < 30 min), indicating that a generalized derangement of ARE-dependent mRNA turnover was not operant. Six major cytoplasmic proteins (36, 41, 50, 70, 85, and 90 kDa) from MLA 144 cells were

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[1] The abbreviations used are: UTR, untranslated region; IL-2, interleukin-2; GM-CSF, granulocyte macrophage colony-stimulating factor; LTR, long terminal repeat; ActD, actinomycin D; DRB, 5,6-dichloro-1-β-ribouranosylbenzimidazole; PHA, phytohemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid; cpm, counts/minute; PAGE, polyacrylamide gel electrophoresis; hnRNP, heteronuclear ribonucleoprotein.

observed to bind to the ARE of provirally modified IL-2 mRNA to a greater degree than to those found in native IL-2. Similar data were observed with cytoplasmic lysates of activated human T lymphocytes. Characterization of the proviral sequences necessary for altered AUBP binding were performed. Enhanced binding of the provirally modified IL-2 3′ UTR was apparent by Northwestern blotting, which permitted colocalization of the 36- and 43-kDa AUBPs with hnRNP A1 and C, respectively. Recombinant human hnRNP A1 and C1 proteins demonstrated comparable patterns of RNA probe binding. These data suggest a relationship between AUBP and hnRNP binding *in vitro* and ARE-dependent mRNA turnover *in vivo*. Moreover, these findings indicate that the context in which ARE exist appears to be of importance in determining AUBP binding *in vitro*.

MATERIALS AND METHODS

Reagents—Actinomycin D (ActD) and 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB) were purchased from Sigma. ActD was kept at 4 °C as a 5 mg/ml stock solution whereas DRB was made up freshly before use. Phytohemagglutinin (PHA) was obtained from Wellcome Reagent Ltd., Beckenham, United Kingdom. [^{32}P]UTP (3000 Ci/mmol) was purchased from Amersham Corp., and unlabeled nucleotides were obtained from Boehringer Mannheim. Recombinant human hnRNP A1 and C1 as well as antibodies against these proteins were generously provided by Dr. Gideon Dreyfuss, whereas recombinant human La protein was a generous gift of Dr. William St. Clair.

Cell Culture—MLA 144 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 medium (JHR Biosciences, Lenexa, KS) supplemented with 8% heat-inactivated (56 °C, 30 min) fetal bovine serum (Flow Laboratories, McLean, VA) at a density of 0.5–1 × 10^6 cells/ml and subcultured every second day. Human lymphocytes were separated on discontinuous Ficoll-Hypaque gradient from leukapheresed blood obtained from healthy volunteers and cultured in 8% fetal bovine serum-RPMI-1640 medium in the presence of 1 μg/ml PHA for different periods of time.

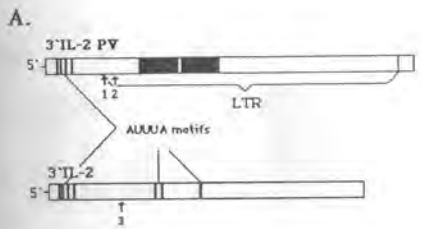
Transcriptional Inhibition Experiments, RNA Isolation, and Detection of IL-2 and GM-CSF Transcripts—0.5–1 × 10^8 cells were seeded in 250-ml tissue culture flasks (Falcon) at 10^6/ml density and treated either with ActD (5 μg/ml) or DRB (100 μM) for different periods of time. This concentration of DRB was shown to inhibit [^{3}H]uridine incorporation by MLA 144 cells over 95% within 5 min while having no effect on cell viability for up to 6–8 h. Cells were harvested, and total cellular RNA was isolated by the single step guanidium thiocyanate-phenol-chloroform extraction method (22) with a modification of increasing the amount of β-mercaptoethanol (Sigma) from 0.1 to 0.7 M in the 5 M guanidium thiocyanate (Fluka Biochemica) denaturing solution. Poly(A)$^+$ RNA was prepared by oligo(dT) column (Collaborative Biomedical, Bedford, MA) chromatography. For Northern blot analyses, equal amounts (5–10 μg) of RNA samples were size fractionated by 0.8% agarose-formaldehyde gel electrophoresis, blotted on Hybond nylon membrane (Amersham Corp.) overnight in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Membranes were air dried and baked at 80 °C, 2 h *in vacuo*, then prehybridized for at least 12 h in 50% formamide, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 200 μg/ml sheared salmon sperm DNA at 42 °C. RNA was detected by hybridization of the membranes at 42 °C overnight in prehybridization mix containing 10% dextran sulfate and 1 × 10^6 cpm/ml of [^{32}P[dCTP-labeled cDNA probes generated by the random priming method (23). Blots were then washed three times at 56 °C in 0.1 × SSC containing 0.02% sodium pyrophosphate and 0.5% Sarkosyl. Blots were air dried and exposed to Kodak XOmat film at −80 °C.

Nuclear Run-on Analysis—Nuclei were prepared by lysing 2–3 × 10^8 cells in a buffer (10 mM HEPES, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.05 % Nonidet P-40 detergent and centrifuged at 1000 × *g* through a 30% sucrose cushion at 4 °C. Nuclei were then resuspended in 50 mM HEPES, pH 7.9, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA at a density of 10^8/ml, snap frozen in liquid nitrogen in 100-μl aliquots, and stored at −80 °C. *In vitro* transcription reactions were carried out at room temperature by adding equal volume (100 μl) of two times transcription buffer (50 mM HEPES, pH 7.9, 120 mM KCl, 20 mM dithiothreitol, 30 mM β-mercaptoethanol, 50 mM magnesium acetate, 2 mM $MnCl_2$, 1 mM EDTA, 8 mM phosphoenolpyruvate, 6 μg/ml pyruvate kinase, 2 mM fructose-1,6-diphosphate, 1% Tween-20, 2 mM thymidine diphosphate, 1 mM ATP, CTP, and GTP each, 10 μM UTP, 1000 units/ml RNasin, and 0.4 mg/ml heparin) to 100 μl of nuclear suspension and incubated for 15 min at room temperature in the presence of 125 μCi of [^{32}P]UTP. Following DNase and proteinase K treatment, the mixture was phenol-chloroform extracted twice and precipitated in ethanol. Labeled RNA was purified by size-exclusion column chromatography (Bio-Spin 30, Bio-Rad). Following prehybridization in a mix containing 50 mM sodium phosphate, 50% formamide, 500 μg/ml yeast tRNA, 10% SDS, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1% Ficoll, 0.1% polyvinylpirrolidone, and 0.1% bovine serum albumin, results were detected by linearized plasmids containing the cDNA blotted onto Hybond nylon membranes (5 μg/slot) using a slot-blot apparatus (Schleicher & Schuell). The purified run-on transcripts were added directly to the prehybridization mix (10^7 cpm/ml final) and incubated at 42 °C for 48–72 h. After washing three times in 2 × SSC containing 0.02% sodium pyrophosphate and 0.5% Sarkosyl at 56 °C, membranes were dried and exposed to Kodak X-Omat film at −80 °C.

Cytoplasmic Lysate Preparation—Cytoplasmic lysates were prepared as described (24) with minor modifications. Briefly, 20 × 10^6 cells were washed in ice-cold serum-free medium, pellets were resuspended in buffer A containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and lysed on ice for 3 min by addition of 1% Triton X-100. Following centrifugation for 3 min at 900 × *g*, supernatants were collected, aliquoted, and frozen immediately. Exclusion of 1 mM $CaCl_2$ from the lysis buffer did not influence the detected levels of cytoplasmic AUBPs in this system. The Triton-insoluble pellets (cytoskeleton and associated components) were extracted in buffer B containing 10 mM HEPES, pH 7.4, 15 mM NaCl, 1.5 mM $MgCl_2$, 1% Tween-20, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride for 3 min on ice. Supernatants (referred to as INSOL) were collected after centrifugation with 1200 × *g* for 3 min at 4 °C.

RNA Probes and AUBP Assay—All the RNA probes in our experiments were transcribed with the T7 or T3 RNA polymerases (as indicated in Fig. 1*B*) from cDNA templates that had been subcloned into the pT7/T3-α19 vector and prepared for *in vitro* transcription as follows. A 338-base 3′-IL-2 probe was generated from an *Eco*RI linearized plasmid which contained the 3′-UTR (270 base pairs) of the human IL-2 cDNA downstream from the *Stu*I restriction site and therefore contained. The 126-base 3′-IL-2/B probe was transcribed from the same plasmid which was linearized with *Bgl*I. 3′-IL-2 PV, 3′-IL-2 PV/P, and 3′-IL-2 PV/E were transcribed from a plasmid that contained a 565-base pair fragment of the original retrovirally modified MLA gibbon IL-2 cDNA (CM-9) downstream from the *Stu*I site (20) that had been digested with *Bgl*I and *Bam*HI, *Pst*I, and *Eco*RV prior to transcription, yielding RNA probes with 597, 96, and 171 bases in length, respectively. The 90-base Δ2R1 probe was generated by the transcription of an *Eco*RI linearized plasmid that contained a 30-base pair portion of the GM-CSF 3′-UTR containing four consecutive AUUUA pentamers (10). The AUUUA motifs in each of these RNA probes are indicated in Fig. 1*B*. Comparable AUBP binding intensities were observed with 3′-IL-2 PV and its truncated forms that had been transcribed with T7 RNA polymerase from appropriate constructs, thereby excluding any potential contribution of the MCS segment encoded in the transcript in altering binding. ^{32}P-Labeled probe mRNAs (specific activity > 10^8 cpm/μg RNA) were generated by *in vitro* transcription where 50 μCi of [^{32}P]UTP (3000 Ci/mmol), 0.0125 mM UTP, and 2.5 mM each ATP, CTP, GTP were present. 8 × 10^4 cpm probe RNAs (≈1–15 fmol) were incubated with 2.5–10 μg of cytoplasmic lysate protein or 0.05 or 0.25 μg of recombinant C1 and A1 or La proteins, respectively, in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol for 10 min at 30 °C. Cold competitors where needed were added simultaneously to the binding reaction tubes. Protein-nucleic acid complexes were UV cross-linked on ice using Stratagene UV Stratalinker model 1800 or 2400 (5 min, 3000 microwatts/cm^2 or 3.5 min, 4000 microwatts/cm^2), then exposed to RNase digestion (15 units of RNase T1 and 30 μg of RNase A/sample) for 15 min at 37 °C. Samples were then separated by 15% SDS-PAGE under denaturing conditions, and gels were dried and analyzed by autoradiography.

Northwestern Analysis—15 μg of MLA 144 lysate protein was loaded and separated by 15% SDS-PAGE under reducing conditions. Gels were then equilibrated in transfer buffer (TB, 48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 15 min at room temperature and electrotransferred onto Hybond C nitrocellulose membrane (Amersham Corp.) for 25 min at room temperature at constant 15 V using a semidry transfer system (Bio-Rad). Membranes were soaked in PBS and hybridized in solution A (10% glycerol, 15 mM KCl, 12 mM HEPES, 0.2 μg/ml yeast tRNA, 0.2 μM dithiothreitol, pH 7.9) in the presence of 8 × 10^5 cpm/ml each of the *in vitro* transcribed RNA probes indicated in Fig. 5, for 30 min at room temperature. Membranes were then washed gently three



B.

3'IL-2 5' -CCCU-a30-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-a110-ACUUAUUUAUCCCAAAAUAUUUAUUA-a45-AACUAUUUAAUA-a77 (T7)

3'IL-2/B 5' -CCCU-a30-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-a75-CUUUUUGUAAG (T7)

3'IL-2 PV 5' - GGGA-a10-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-a42-AGAGCUGCAGUAAGGCC-a486 (T3)

3'IL-2 PV/P 5' -GGGA-a10-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-a30-UAUUAUUCUUAAAGAGC (T3)

3'IL-2 PV/E 5' -GGGA-a10-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-a105-CAGGGCCAAACAGGAU (T3)

Δ2R1 5' -CCCU-a40-CCAUUUAUUUAUUUAUUUAAGCUU-a22 (T7)

FIG. 1. *A*, schematic illustration of the 3′-UTRs of MLA 144-IL-2 and normal human T lymphocyte IL-2 mRNA. A 495-nucleotide long viral LTR is inserted within this portion of the MLA IL-2 mRNA with the deletion of three of the seven AUUUA pentamers (*dark bars*). There are two identical 94-nucleotide long direct repeat elements within the LTR (*dark areas*). *Arrows* indicate unique restriction sites where the cDNA was cut in order to generate templates for transcription of the corresponding RNA probes *in vitro* (*1*, *Pst*I → 3′-IL-2 PV/P; *2*, *Eco*RV → 3′-IL-2 PV/E; *3*, *Bgl*I → 3′-IL-2/B). *B*, sequences of *in vitro* transcribed RNA probes with the AUUUA motifs outlined within their AU-rich 3′ regions. The fragments of non-vector origin bordering the first four AUUUA motifs for six bases upstream and 28 bases downstream of the 3′-IL-2 or 3′-IL-2 PV and related probes are identical. A four-base deletion is then found in 3′-IL-2 PV (GUAA in 3′-IL-2) and related probes, with identity resumed for another 12 bases prior to the beginning of the LTR. RNA polymerases used to generate the RNA probes are indicated in *parentheses*.

times in solution B (15 mM KCl, 12 mM HEPES, pH 7.9) at room temperature, air dried, and analyzed by autoradiography.

Western Blotting—Cytoplasmic lysates from MLA 144 cells were size separated by denaturing SDS-PAGE and electrotransferred onto nitrocellulose membranes (Schleicher & Schuell) as described for Northwestern analysis. Membranes were incubated with either anti-hnRNP C antibody (4F4) or anti-hnRNP A1 (4B10) and visualized by using the ECL chemiluminescence detection method (Amersham Corp.).

RESULTS

Comparison of IL-2 and GM-CSF mRNA Expression in MLA 144 Cells—Comparison of IL-2 and GM-CSF mRNA levels in MLA 144 cells demonstrated markedly higher levels of IL-2 mRNA relative to GM-CSF (Fig. 2*A*, *lane 1*). Northern blot analysis indicated additionally that IL-2 mRNA was readily detectable with total cellular RNA preparations while GM-CSF mRNA required analysis of poly(A)$^+$ RNA for detection (Fig. 2, *A* and *B*). This finding suggested at least a 10–100-fold greater level of IL-2 mRNA relative to GM-CSF. The role of gene transcription was analyzed to define the potential mechanism behind the differential levels of IL-2 and GM-CSF mRNA accumulation in MLA cells. Nuclei were assayed for *in vitro* rates of transcription by nuclear run-on analysis (Fig. 2*C*). Equivalent rates of constitutive transcription of both IL-2 and GM-CSF genes were present, suggesting that transcription could not account for the increased levels of IL-2 mRNA relative to GM-CSF in MLA 144 cells.

Different Rates of IL-2 and GM-CSF mRNA Turnover—The

FIG. 2. **Northern blot analysis of IL-2 and GM-CSF mRNA production by MLA 144 cells.** *A*, following transcriptional inhibition by ActD and RNA extraction, 5 μg of poly(A)$^+$ RNA was analyzed in each lanes. GM-CSF mRNA is very unstable ($t_{1/2}$ < 15–30 min) while IL-2 mRNA is exceptionally stable with $t_{1/2}$ > 8 h as determined by Northern blotting of 15 μg/lane total cellular RNA extracted from cells that have been exposed to DRB (*B*). Time of transcriptional inhibition prior to RNA isolation is indicated at the *top* of each lane. β-2-Microglobulin mRNA served as loading control. Result of a nuclear run-on experiment indicates comparable levels of constitutive transcription from IL-2 and GM-CSF genes (*C*). pBR322 was used as negative control.

in vitro transcription data suggested that post-transcriptional mechanisms might account for the observed differences in IL-2 and GM-CSF mRNA accumulation. The rate of decline in mRNA levels in response to treatment with the transcriptional inhibitor, ActD, was examined using poly(A)$^+$ RNA (Fig. 2*A*). GM-CSF mRNA levels decreased rapidly and were undetectable after 30 min of ActD treatment. In contrast, IL-2 mRNA was readily detectable even after 1 h of transcriptional inhibition. In a separate experiment, treatment with the RNA polymerase II-specific inhibitor, DRB, revealed that IL-2 mRNA levels did not significantly decline for up to 16 h of DRB treatment, indicating its property as an unusually stable mRNA in this cell line (Fig. 2*B*).

Detection of Cytoplasmic Proteins with AUUUA Sequence Binding Capability in MLA 144 Cells—Reiterated AUUUA sequences (AU-rich sequences) have been demonstrated to confer, or contribute to, rapid cytoplasmic turnover of cytokine, lymphokine and proto-oncogene mRNAs (6–7), perhaps through the interactions with specific cytoplasmic binding proteins (9–12). The potential role of an altered AUBP-ARE interaction in mediating the observed differential lymphokine mRNA turnover in MLA 144 cells was examined utilizing *in vitro* transcribed, radiolabeled RNA probes containing the 3′-UTR of either human IL-2 (3′-IL-2) or the retrovirally infected gibbon IL-2 (3′-IL-2 PV) as well as an AU-rich region of GM-CSF mRNA (Δ2R1). Cytoplasmic lysates were incubated in the presence of the labeled RNA, followed by UV cross-linking, RNase digestion, and SDS-PAGE. Six major RNA-protein complexes were detected by label transfer. Single bands with molecular masses of approximately 36, 41, 50, 70, and 85 kDa and a double band of 90 kDa were detected (Fig. 3, *lanes 1–3*). Previous studies have established that under these conditions, these proteins are specific for reiterated AUUUA sequences (25). The binding of these proteins (AUBP) to 3′-IL-2 PV occurred at a significantly higher level relative to 3′-IL-2 and to Δ2R1. The observed differential binding between RNA probes was also apparent with lysates from activated human T lymphocytes (Fig. 3, *lanes 6–8*), although cell-dependent differences in overall cytoplasmic AUBP-binding patterns were seen. The dominant AUBP in MLA 144 cytoplasmic lysates were the 90- and 36-kDa complexes, while in activated lymphocyte lysates the 41-, 50-, and the 85-kDa complexes were more prominent than the 36 kDa and equal to that of the 90 kDa (Fig. 3, *lanes 6–8*). Analysis of the detergent-resistant cytoplasmic lysates (INSOL, that include cytoskeleton-associated proteins)

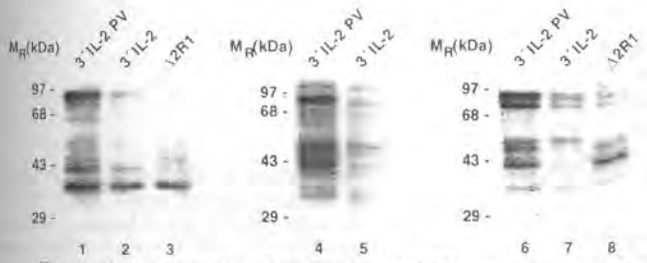
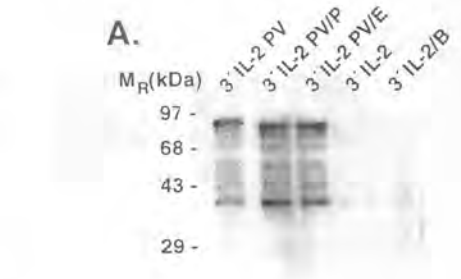


FIG. 3. **Detection of cytoplasmic AUUUA sequence-binding proteins by AUBP assay.** *In vitro* transcribed and radiolabeled RNA probes were incubated with cytoplasmic lysates from MLA 144 cells (*lanes 1–3*) or 8 h of PHA-activated lymphocytes (*lanes 6–8*) or INSOL cytoplasmic lysates from MLA 144 cells (*lanes 4* and *5*). Following UV cross-linking and RNase treatment, the samples were analyzed by SDS-PAGE under denaturing conditions. Radiolabeled probes are indicated at the *top* of each lane.

revealed additional AUBP (32–34 kDa and 100 kDa) with the same differences in binding (Fig. 3, *lanes 4* and *5*).

Effect of Removal of Proviral Sequences from 3′-IL-2 PV on AUBP Binding and Its Affinity—To determine whether the presence of the viral LTR itself specifically influenced AUBP binding to 3′-IL-2 PV distinct from its increase in RNA length, RNA probes were transcribed from templates in which the 3′-IL-2 PV had been truncated using the *Pst*I (3′-IL-2 PV/P) and *Eco*RV (3′-IL-2 PV/E) restriction sites (Fig. 1*B*). 3′-IL-2 PV/P (96 bases) and 3′-IL-2 PV/E (171 bases) generated a similar AUBP pattern to 3′-IL-2 PV, but with an approximately 1.6 or 1.2 times higher intensity (Fig. 4). In contrast, truncation of the human 3′-IL-2 using the *Bgl*I restriction site (3′-IL-2/B) to a comparably sized transcript (126 bases) did not influence AUBP binding relative to 3′-IL-2 (Fig. 4). These data correlated well with relative AUBP affinity for their RNA ligand as demonstrated by cross-competition experiments (Fig. 5). 3′-IL-2 PV/P binding was nearly completely competed by a 50-fold molar excess of the cold 3′-IL-2 PV/P. In contrast, incomplete competition of 3′-IL-2 PV/P binding was evident with a 250-fold molar excess of cold 3′-IL-2 PV (*center left panel*), whereas cold 3′-IL-2 failed to substantially reduce AUBP binding even at this high concentration (*center right panel*). This latter result further confirms the capability of 3′-IL-2 PV to associate with AUBP in a selectively stronger manner relative to 3′-IL-2.

Northwestern Analysis of AUBP Binding to Labeled RNA Probes—During the course of our AUBP experiments, the RNA-protein binding occurs in solution, and may be mediated through a complex of proteins interacting with a specific RNA ligand. Transfer and consequent immobilization of the electrophoresed AUBP of cytoplasmic lysates onto a membrane support enabled investigation whether the differential binding of AUBPs to the various RNA probes was dependent on protein-protein interactions. As demonstrated in Fig. 6*A*, all of the indicated RNA probes bound to proteins of 36 and 43–45 kDa on the membrane support. Binding of each proteins was four times greater to 3′-IL-2 PV relative to 3′-IL-2 (Fig. 6*C*). The binding intensity of 3′-IL-2 PV/E appears to be slightly higher relative to 3′-IL-2 PV. These observations suggest that specific interaction and differential binding of these RNA probes to AUBP occur independently of protein-protein interactions. In contrast, comparison of the binding of 3′-IL-2 *versus* 3′-IL-2/B yielded different results than that observed with AUBP assay, *i.e.* 3′-IL-2/B seemed to bind approximately two times more intensely than 3′-IL-2 to the membrane-bound AUBP. Subsequent Northwestern analyses, however, did not show enhanced AUBP binding to 3′-IL-2/B relative to 3′-IL-2, whereas the increased binding of 3′-IL-2 PV and 3′-IL-2 PV/E was consistently observed (data not shown). Previous work demonstrated



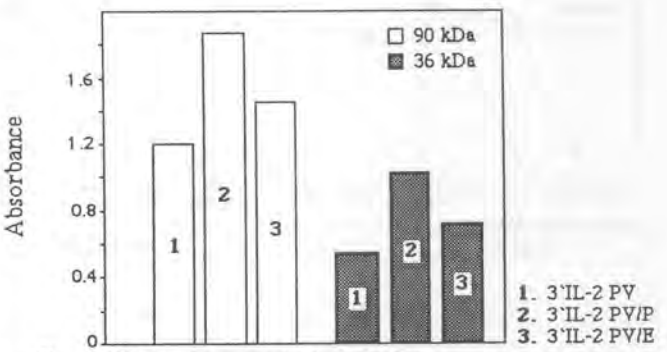


FIG. 4. **Effect of removal of viral LTR sequences from the 3′-IL-2 probe on the level of AUBP binding.** *A*, cytoplasmic lysates were incubated in the presence of each of the indicated radiolabeled RNA probes, UV cross-linked, RNase treated, and analyzed by SDS-PAGE. 3′-IL-2 PV/P, PV/E and 3′-IL-2/B probes were transcribed from DNA templates generated as indicated in Fig. 1*A*. Binding intensity of the 36- and 90-kDa proteins were evaluated by quantitative densitometric analysis (*B*).

that two AUBP found in cytoplasmic lysates of activated human T lymphocytes were members of the hnRNP family (25). A 36-kDa protein (hnRNP A1) and a 43-kDa protein (member of the hnRNP C protein family) were identified as capable of specific binding to reiterated AUUUA motifs. In order to ascertain whether the hnRNP A1 and C proteins can be among the complexes formed with the labeled RNA probes, we analyzed the membranes by immunoblotting. As shown in Fig. 6*B*, the band detected with antibody to hnRNP C appears to comigrate with the 43–45-kDa complex detected by Northwestern analysis. Additionally, the higher molecular mass isoform of hnRNP A1 appears colocalized with the 36-kDa size band on the Northwestern blot. Given the knowledge that hnRNP A1 undergoes considerable post-translational modification (reviewed in Ref. 26), the presence of two immunoreactive proteins suggests isoforms of the same gene product, with different capacity to interact with reiterated AUUUA sequences when immobilized. Alternatively, the doublet may represent highly homologous or nearly identical forms of hnRNP A1 generated from closely related genes or differential processing or translation of the hnRNP A1 mRNA (27, 28).

Differential Binding of Recombinant A1, C1, and La Proteins to in Vitro Transcribed RNA Probes—In order to correlate the immunoblotting and Northwestern data as well as determine the specificity of the observed increase in RNA binding of provirally modified IL-2 3′-UTR with known RNA-binding proteins in solution, the binding of recombinant human hnRNP A1, C1, and La proteins to labeled RNA probes in AUBP assays was examined. The autoantigen SS-B/La, a 47-kDa protein, was chosen because it has been shown to bind 3′-terminal oligouridine residues found in RNA polymerase III transcripts (29).

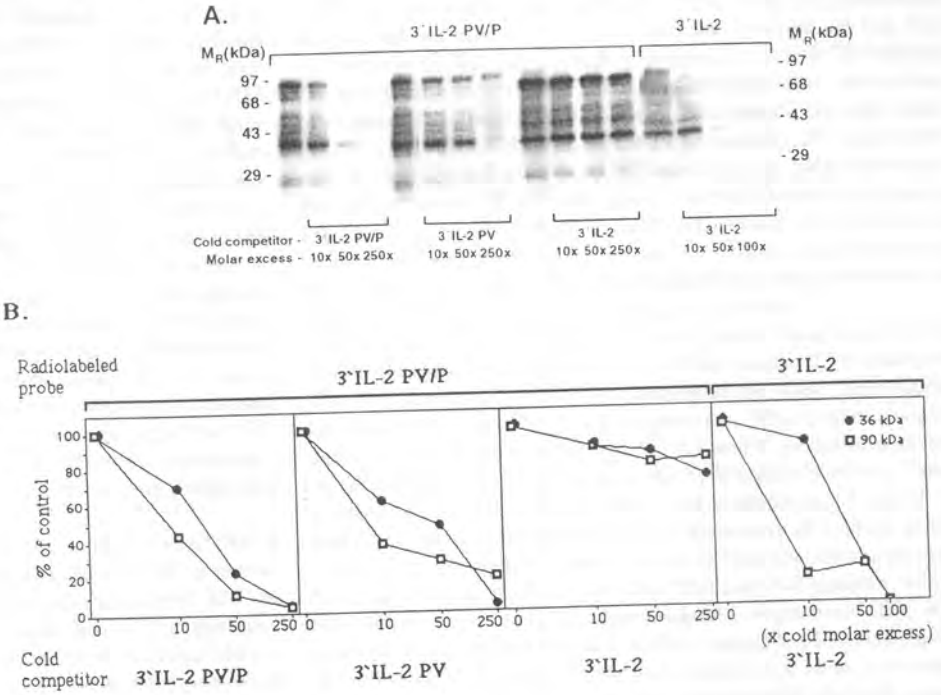


FIG. 5. **Analysis of AUBP binding affinity of *in vitro* transcribed RNA probes derived from virally altered and native IL-2 3′-UTRs.** Competition experiments were performed with ^{32}P-labeled and cold RNA transcripts added simultaneously in the binding reaction together with cytoplasmic lysates from MLA 144 cells (*A*). Labeled RNA probes are shown *above* the lanes, while cold competitors together with molar excesses of the cold probes are indicated at the *bottom*. Diagrams conclude the data from the densitometric analysis of the binding of the 36- and 90-kDa proteins from the same experiments (*B*).

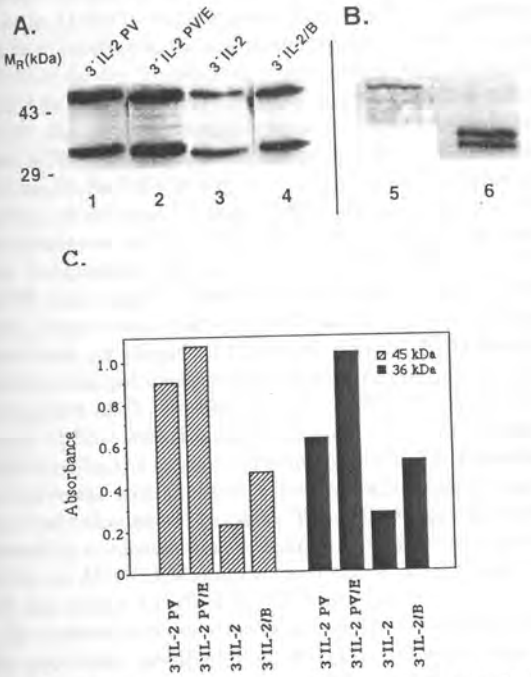


FIG. 6. **Northwestern analysis of AUBP binding to radiolabeled probes.** *A*, AUBPs were immobilized on membrane support prior to interaction with probe RNAs (indicated at the *top* of each lane) at 5 × 10^5 cpm/ml binding solution. The same membranes that are shown in *lanes 2* and *4* were examined by immunoblotting utilizing antibodies 4F4 and 4B10 directed against hnRNP C (*lane 5*) and A1 (*lane 6*), respectively (*B*). Intensity of binding in the Northwestern experiment was quantitated by densitometric analysis (*C*).

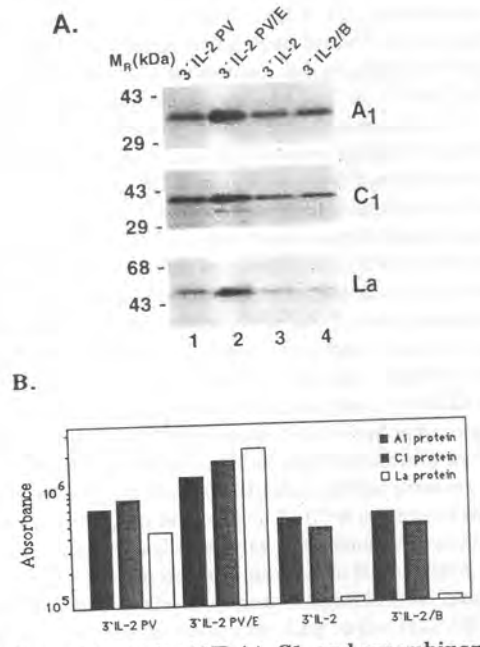


FIG. 7. **Recombinant hnRNP A1, C1, and recombinant La proteins were analyzed in *in vitro* AUBP assay for their ability of binding to the indicated RNA probes.** *A*, 0.25 μg of A1 and La and 50 ng of C1 proteins were incubated in the presence of 8 × 10^4 cpm probes, and following UV cross-linking and RNase treatment, samples were analyzed on SDS-PAGE under reducing conditions. Quantitative evaluation of binding differences of each of these proteins to the RNA ligands was made by densitometric measurements, with longer exposures used for La, relative to hnRNP A1 and C1 (*B*).

Both recombinant hnRNP A1 and C1 proteins demonstrated a hierarchy of RNA binding identical to that observed by both AUBP assay and Northwestern blotting (Fig. 7). In contrast, although recombinant La yielded a comparable binding profile of 3′-IL-2 PV and 3′-IL-2 PV/E (Fig. 7), very little binding to 3′-IL-2 or 3′-IL-2/B was observed, perhaps indicating recognition of an RNA sequence distinct from that bound by hnRNP A1 or C1.

DISCUSSION

The MLA 144 T cell line has been known to constitutively produce cytokines such as IL-2 and GM-CSF (18). In this report, we demonstrate that MLA 144 cells selectively accumulate at least a >10-fold greater level of IL-2 mRNA relative to GM-CSF despite comparable rates of gene transcription, suggesting that considerable differences in the regulation of their respective mRNA turnover exist. The rate of IL-2 mRNA turnover was found to be markedly prolonged relative to that of

GM-CSF mRNA, which was rapidly degraded with kinetics similar to those reported by others (6, 30, 31). These findings indicated differential post-transcriptional regulation of lymphokine mRNA, both of which contained reiterated AUUUA motifs (ARE) in their 3′-UTR. Thus, these effects might have been mediated by altered expression of AUBP that can differentially bind to the 3′-UTR of IL-2 and GM-CSF. Alternatively, the presence of a 495-base long proviral insertion in the IL-2 mRNA might have altered the ability of the AUBP to bind to the ARE present in the 3′-UTR. Correlation of this modification of the 3′-UTR of IL-2 with AUBP binding *in vitro* was of considerable interest because the retroviral insertion resulted in loss of the 3′ 180 nucleotides including three AUUUA motifs, while preserving four nearly continuous reiterations of AUUUA with one AUUUUA sequence, each of which have been shown to bind similar proteins (4). This sequence is very similar to the reiterated ARE within the GM-CSF 3′-UTR, which has been demonstrated to confer instability in heterologous gene constructs (6).

To address these issues, we examined the ability of AUBP to bind radiolabeled RNA probes containing the 3′-UTR of native and provirally modified IL-2 as well as the reiterated ARE of GM-CSF in different cell types. Comparable AUBP patterns were demonstrated in the detergent-soluble and -insoluble cytoplasmic fractions of MLA cells as well as of PHA-activated human lymphocytes, indicating no major qualitative differences in AUBP binding or their subcellular localization in MLA 144 cells relative to those (normal human T) cells in which IL-2 mRNA is labile. These data suggest that expression of a novel AUBP in MLA 144 cells cannot account for the altered IL-2 mRNA turnover. Although quantitative differences in specific RNA-AUBP interactions in different cell types were observed and cannot be definitively excluded as possible factors, it seems unlikely to account for the marked change in IL-2 mRNA turnover observed in MLA 144 cells. More striking was the finding that independent of cell type, the ARE of 3′-IL-2 PV bound AUBP with higher intensity compared to those of 3′-IL-2 or Δ2R1, suggesting that AUBP binding *in vitro* directly correlated with the abnormal IL-2 mRNA stability. Therefore, AUBP binding was not dependent on the absolute number of AUUUA pentamers of the RNA ligands: 3′-IL-2 PV with four motifs bound AUBP with higher intensity than 3′-IL-2 with seven. Additionally, the number of continuous AUUUA pentamers did not correlate with increased binding either (Δ2R1 has four continuously aligned pentamers). These findings were further supported by truncation experiments in which removal of the distal three AUUUA elements from the 3′-IL-2 probe (3′-IL-2/B) did not appear to affect AUBP binding.

In contrast to truncation of native IL-2 3′-UTR, shortening of the provirally modified IL-2 3′-UTR yielded enhanced AUBP binding affinity in cross-competition experiments. These experiments indicated that the increased affinity of AUBP binding of the IL-2 PV probe appeared to be mediated within the first 20 bases of the proviral insertion. The remaining approximately 500 bases (which contained the majority of proviral insertion) appeared to play little role in the increased AUBP binding of 3′-IL-2 PV relative to 3′-IL-2. As stated above, the effects of truncation appeared to be specific for 3′-IL-2 PV, as comparable binding of 3′-IL-2 and 3′-IL-2/B were observed. Thus, altered AUBP binding was unaffected by random shortening or lengthening of RNA probes, which was consistent with a previous report (4). Nevertheless, the enhanced ability of AUBP to bind 3′-IL-2 PV indicates something distinct about the modification of the 3′-IL-2 UTR by the proviral insertion. Because this finding correlates with increased IL-2 mRNA stability, it is therefore of considerable functional interest in understanding ARE-dependent mRNA turnover. Our data further indicate that either the addition of the first 20 nucleotides (contained within 3′-IL-2 PV/P) or the deletion of the four nucleotides (GTAA) at the site of proviral insertion (20) altered the secondary structure of the ARE-RNA ligand in such a way to enhance binding by AUBP. These data would therefore suggest that the secondary structure and context of a particular ARE sequence can modify AUBP-binding and ARE-dependent mRNA turnover. Finally, it raises concerns about the ability to examine the effects of ARE on mRNA turnover and translation in heterologous constructs, where the context and secondary structure may be considerably different from what is present in the 3′-UTR of cytokine/proto-oncogene mRNA.

In previous work, hnRNP A1 and C have been shown to be AUBP found in cytoplasmic lysates from human T lymphocytes (25). Northwestern blotting demonstrated that ARE-AUBP binding paralleled that seen in solution. This technique also permitted colocalization of hnRNP A1 and C proteins with the 36- and 43-kDa AUBP, respectively, by Western blotting. These studies prompted demonstration that recombinant hnRNP A1 and C1 proteins exhibited a similar hierarchy of binding as did the AUBP detected by cross-linking or Northwestern analysis, indicating their probable identity. The La/SS-B protein, which has been shown to bind oligouridylate sequences (29), was markedly less able to bind the ARE in native 3′-IL-2 or 3′-IL-2B than hnRNP A1 and C. Thus, the uridine-rich stretches recognized by the La/SS-B antigen appear to be differentially accessible for binding in 3′-IL-2 PV and 3′-IL-2, relative to the ARE-containing RNA ligand recognized by hnRNP A1 and C. These data provide further evidence, independent of ARE binding, that the secondary structure of the 3′-UTR of IL-2 has been substantially altered by the proviral insertion.

The observed correlation between AUBP binding and IL-2 mRNA turnover in MLA cells appears different from the finding of a cytoplasmic AU-rich element-binding factor of an erythroleukemia cell line which exhibited destabilizing activity in the analysis of *c*-myc mRNA in a cell-free mRNA decay system (12). Additionally, Bohjanen *et al.* (9) reported a 30-kDa AUBP protein whose disappearance correlated with increased lymphokine mRNA stability. It is therefore likely that there are several AUBP that might conceivably bind to a specific ARE, which may either stabilize or destabilize a specific mRNA or influence its translation. Our data was not designed to conclusively identify the AUBP which alters IL-2 mRNA stability *in vivo*. Rather, these data suggest that modification of the primary sequence in a very small region of the IL-2 3′-UTR enhances mRNA stability *in vivo* as well as its ability to be bound by several different AUBP (both known and unknown). In this regard, it should be noted that two of the proteins in cytoplasmic lysates (hnRNP A1 and C), whose increased binding to the IL-2 ARE correlated with the proviral insertion, have been implicated with the regulation of pre-mRNA processing (25). Thus, these proteins appear to have an established role in RNA metabolism that may vary as a consequence of intracellular location. Furthermore, with the recognition that hnRNP A1 can shuttle between the nucleus and cytoplasm (32), our correlation of its binding with altered mRNA stability suggests an additional role in cytoplasmic mRNA turnover.

In conclusion, we propose that the combination of a low level of constitutive transcription of an abnormally stable mRNA encoding IL-2 in the MLA 144 cell line (both events retrovirally mediated) played a major role in malignant transformation of a T cell. Production of abundant levels of IL-2 induced autocrine growth stimulation, thereby permitting eventual growth factor-independent cell growth and tumorigenesis. A similar oncogenic role of altered mRNA turnover is suggested by the finding of increased stability of proto-oncogene and cytokine mRNA in several tumors (15–17). Our results additionally offer a direct

correlation between the intensity and affinity of specific AUBP binding with mRNA stability. These studies highlight the complexity of examining both the interaction of *trans*-acting proteins with sequence-specific RNA ligands independent of their natural context. The corollary of this observation is that studies of putative destabilizing sequences in chimeric gene constructs may be limited in application due to the confounding influence of altered secondary structure. Finally, although our studies demonstrate a correlation between ARE-dependent mRNA turnover *in vivo* and AUBP binding *in vitro* in the differential regulation of two cytokine mRNA stability, they do not establish causality between these observations. The comparable patterns and intensity of AUBP binding of the native and provirally modified 3′-IL-2 UTR in normal human T cells (in which IL-2 and GM-CSF are each unstable) would seem to support the view that these effects are mediated in an ARE-dependent manner. Comparison of the normal and provirally modified IL-2 allele mRNA turnover in the MLA cell would help resolve this question. Unfortunately, induction of transcription of the non-virally modified allele has been unsuccessful. Therefore, future studies will be directed at identifying the nature of the proviral sequence which appears to modify AUBP binding. As a result, we hope to gain new insights into both retroviral induction of the malignant T cell phenotype as well as mechanisms of eukaryotic mRNA turnover regulation.

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Selective Modulation of IFN-γ mRNA Stability by IL-12/NKSF

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We investigated the role of IL-12 in regulating IL-2 and IFN-γ production in primary culture of human T cells. Addition of neutralizing antiserum against the 40-kDa subunit of IL-12 to PHA-stimulated PBMC markedly reduced both IFN-γ protein production and mRNA accumulation and stability. Moreover, concurrent treatment of partially purified T cells (>90% $CD3^+$) with PHA and rIL-12 selectively enhanced IFN-γ mRNA stability and protein production, while IL-2 protein and mRNA levels were unaffected. These studies also show that IFN-γ and IL-2 mRNA stability are temporally dissociated during the course of T cell activation, and we propose that this dissociation may be mediated through the production of IL-12. The effect of IL-12 on modulation of IFN-γ mRNA turnover is not associated with detectable changes in either the levels or affinity of cytoplasmic RNA-binding proteins capable of recognizing AU-rich sequences in the 3′ UTR of IFN-γ mRNA.

INTRODUCTION

The lymphokine IFN-γ is produced by activated T and NK cells in response to mitogen or antigen and has a broad range of immunoregulatory activity. Interleukin 12 (NK cell stimulatory factor, NKSF) has been described as a strong inducer of IFN-γ production (1, 2) as well as an enhancer of lymphocyte cytotoxicity and proliferation (3). The demonstration of high-affinity receptors for IL-12 on T lymphocytes suggested that IL-12 production by accessory cells could directly modulate IFN-γ production by T cells (4, 5). Activating stimuli (antigens, lectins, phorbol esters, etc.) have been shown to induce lymphokine gene transcription as well as modulate lymphokine mRNA turnover (reviewed in 6). Despite coordinate induction of gene transcription following T cell activation, lymphokine mRNAs can be differentially regulated in the same cell. Considerable differences in the involvement of transcriptional and post-transcriptional mechanisms in the regulation of gene expression for individual lymphokines have been demonstrated (7, 8).

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Assay of IFN-γ and IL-2 production. PBMC and purified T cells were activated for 20 hr with PHA in the presence or absence of neutralizing anti-IL-12 antiserum and rIL-12, respectively. Cell-free culture supernatants were analyzed for the level of IFN-γ by solid-phase radioimmunoassay (RIA) (Centocor Corp., Malvern, PA) that utilized two murine monoclonal antibodies to different epitopes of biologically active IFN-γ and for IL-2 activity as described (17), by the IL-2 concentration-dependent stimulation of a cloned murine IL-2-dependent T lymphocyte line (CTLL-2), which is insensitive to human IL-12.

Analysis of IFN-γ, IL-2, and HLA-B7 mRNA stability. Cells were activated with PHA for the indicated lengths of time, actinomycin D (5 μg/ml) was added, and total cellular RNA was extracted by a guanidium isothiocyanate method (18) at various time points. Equal amounts of RNA were analyzed by Northern blot hybridization. Briefly, the RNA samples were fractionated in a 1% agarose–formaldehyde gel, transferred to a Hybond-N nylon membrane (Amersham) by capillary action, and baked under vacuum at 80°C for 2 hr. The human IL-2, IFN-γ, and HLA-B7 cDNA probes (19–21) were prepared by the random priming method as described (22) using ~3000 Ci/mmol [α^{32}P]dCTP and added to filters at 10^6 cpm/ml concentration. Filters were subsequently hybridized with IL-2, IFN-γ, and HLA-B7 cDNA probes as described previously (23). Blots were exposed at −70°C to Kodak X-OMAT AR film using an intensifying screen. Extent of hybridization was evaluated by scanning densitometry.

Cytoplasmic lysate preparation. Cells were washed twice in ice-cold PBS or serum-free medium, and gently resuspended in lysis buffer containing 10 m*M* Pipes, pH 6.8, 100 m*M* KCl, 2.5 m*M* $MgCl_2$, 300 m*M* sucrose, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml each of leupeptin and pepstatin A (Boehringer Mannheim) as described previously (24). After resuspension of cell pellet Triton X-100 was added (1% final), incubated for 3 min on ice followed by a 3-min centrifugation at 900*g*, 4°C. The protein concentrations of supernatants was determined by using Micro BCA Protein Assay Reagent (Pierce).

RNA probes and AUBP assay. The IFN-γ 3′ UTR RNA probe was prepared by T3 RNA polymerase transcription of *Bam*HI linearized pT7/T3α19 plasmid with the 665-bp *Alu*I fragment of human IFN-γ containing 62 bp from the coding region and the full-length 3′ UTR (20) inserted into the *Sma*I site of the multiple cloning site (MCS). The IL-2 3′ UTR RNA probe was prepared by T7 RNA transcription of *Eco*RI-linearized pT7/T3α19 plasmid with the 270-bp *Stu*I–*Eco*RI fragment of human IL-2 (19) inserted into the *Bam*HI site of the MCS. α-^{32}P-labeled mRNAs were prepared by *in vitro* transcription in the presence of 50 μCi of [α-^{32}P]UTP (~3000 Ci/mmol), 0.0125 m*M* UTP, 2.5 m*M* ATP, GTP, and CTP. RNA probes (8×10^4 cpm; 1–5 f*M* of RNA) were incubated with 2–5 μg of protein from lysates in 12 m*M* Hepes, pH 7.9, 15 m*M* KCl, 0.2 μ*M* dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol for 10 min at 30°C. The RNA–protein interaction was stabilized by uv cross-linking, which was performed on ice using Stratalinker 1800 (Stratagene) (5 min, 3000 μW/cm^2) followed by RNase digestion (10 U of RNase T1 and 20 μg of RNase A for 30 min at 37°C). The samples were then analyzed under denaturing conditions by 12.5% SDS–PAGE, followed by autoradiography.

RESULTS

Effect of anti-IL-12 antiserum and rIL-12 on the production of IFN-γ. The effects of neutralizing anti-IL-12 antiserum and exogenous rIL-12 on IFN-γ production in

primary culture were examined. PBMC and purified T cells (from the same donor) were activated for 20 hr with PHA in the presence or absence of neutralizing anti-IL-12 antiserum and rIL-12, respectively. Supernatants were analyzed for the level of IFN-γ by solid-phase radioimmunoassay. In a representative experiment, shown in Fig. 1, treatment of PBMC with anti-IL-12 antiserum diminished IFN-γ levels by 75%, from 421 ± 19 to 103 ± 9 U/ml. A corresponding observation was made with partially purified T cells. Addition of rIL-12 augmented IFN-γ production by $CD3^+$ lymphocytes from 740 ± 56 to 1180 ± 107 U/ml. Similar findings were made in three experiments. In contrast, neither antibody-mediated neutralization of IL-12 nor exogenous rIL-12 modulated IL-2 protein levels (measured by bioassay) relative to controls (data not shown).

Neutralizing anti-IL-12 antiserum destabilizes IFN-γ mRNA in activated PBMC. To characterize the effect of IL-12 on IFN-γ mRNA turnover, neutralizing anti-IL-12 antiserum was added together with PHA to PBMC. Total cellular RNA was extracted after 24 hr before and after treatment with actinomycin D, and subjected to Northern blot analysis (Fig. 2). In PHA-activated PBMC, the half-life of IFN-γ mRNA was >2 hr at 24 hr of activation. Addition of neutralizing antiserum to IL-12 decreased the IFN-γ mRNA turnover to $t_{1/2} \sim 35$ min. These data imply that IL-12 is produced in response to PHA activation and modulates IFN-γ production at the post-transcriptional level.

rIL-12 selectively stabilizes IFN-γ mRNA in activated T cells. Accessory cells (monocyte/macrophages, B cells) are shown to produce IL-12 (25, 26). Therefore, partial purification of T cells was utilized to deplete endogenous IL-12 production, permitting study of the possible effect of exogenous IL-12 on IFN-γ mRNA levels and turnover in T cells. Partially purified (>90% $CD3^+$) cells were stimulated for 6 or 24 hr with PHA in the presence or absence of rIL-12. Total cellular RNA was then isolated both prior to, and at various times after actinomycin D treatment and analyzed by Northern blotting (Fig. 3).

To determine if there is coordinate regulation of IFN-γ and IL-2 mRNA turnover, we followed the dynamics of stability changes of these lymphokine mRNAs during the course of T cell activation. IFN-γ mRNA was less stable ($t_{1/2} \sim 30$ min), relative to IL-2 ($t_{1/2} > 90$ min) at 6 hr of PHA activation. Over time (24 hr of activation), the

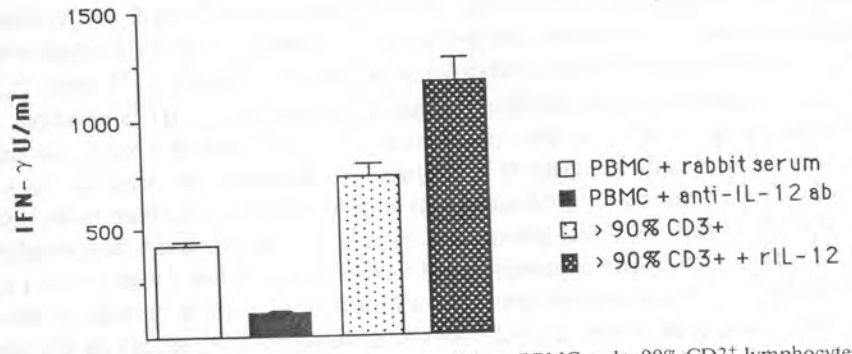


FIG. 1. Effect of rIL-12 and anti-IL-12 antiserum on IFN-γ. PBMC and >90% $CD3^+$ lymphocytes were stimulated with PHA for 20 hr in the presence or absence of anti-IL-12 antiserum and rIL-12, respectively, as shown. IFN-γ was measured by RIA from the cell-free supernatant of the cultures.

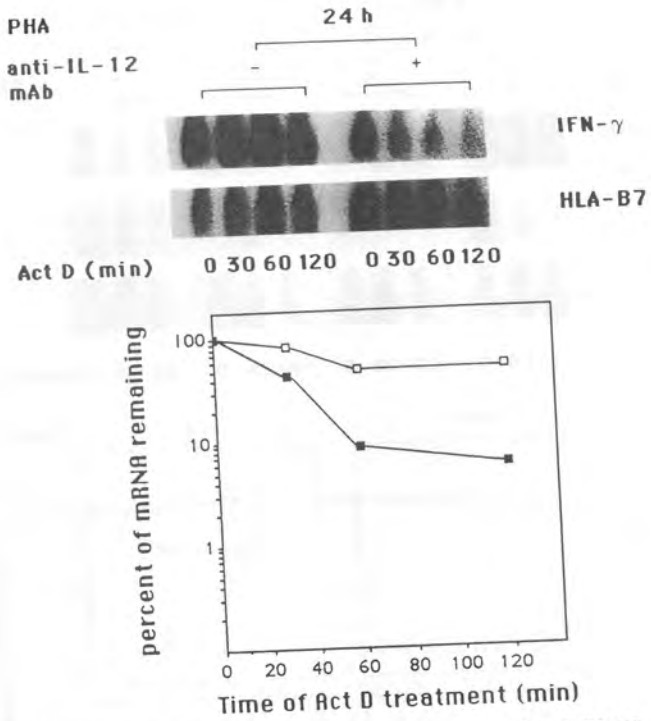


FIG. 2. Anti-IL-12 antiserum destabilizes IFN-γ mRNA in activated PBMC. PBMC were stimulated with PHA in the presence or absence of neutralizing anti-IL-12 antiserum (1:10 000) for 24 hr. Total cellular RNA was isolated at Time 0, or after treatment with actinomycin D for the specified number of minutes. RNA (20 μg) was analyzed in each lane for IFN-γ and HLA-B7 by Northern blotting (top). Blots were overexposed for sake of photography. The rate of mRNA degradation of IFN-γ was determined by measuring the hybridization intensities by scanning densitometry. The amount of mRNA at the various times is expressed as a fraction of the mRNA level at Time 0 (bottom). The densitometric measurements were performed on autoradiograms with exposure resulting in sensitivity in the linear range of the X-ray film. Open symbols represent data points from control; closed symbols represent data points from anti-IL-12 antiserum-treated cultures.

IL-2 mRNA became more labile ($t_{1/2} \sim 30$ min), while IFN-γ mRNA became more stable ($t_{1/2} > 2$ hr). These results suggest that the $t_{1/2}$ of IL-2 and IFN-γ mRNA vary as a function of time following T cell activation. Moreover, these data indicate that IFN-γ and IL-2 mRNA stability are temporally dissociated in activated T cells.

Addition of rIL-12 to partially purified T cells markedly increased IFN-γ mRNA half-life at the 6-hr time point ($t_{1/2}$ from 35 to >90 min), while no apparent effect at 24 hr was seen. One possible explanation of the lack of effect of rIL-12 at the later time point might be the production of significant levels of endogenous IL-12 in control cultures by either activated T cells or contaminating accessory cells after 24 hr, resulting in IFN-γ mRNA stabilization. Given the comparable effects of anti-IL-12 on IFN-γ mRNA stability at 24 hr, this would seem likely. Interestingly, IL-2 mRNA stability showed no change in T cells cultured with rIL-12 at either time point. The results of Northern blot analysis with anti-IL-12 antiserum and rIL-12, respectively, indicate that endogenously produced IL-12 selectively stabilizes IFN-γ mRNA and may there-

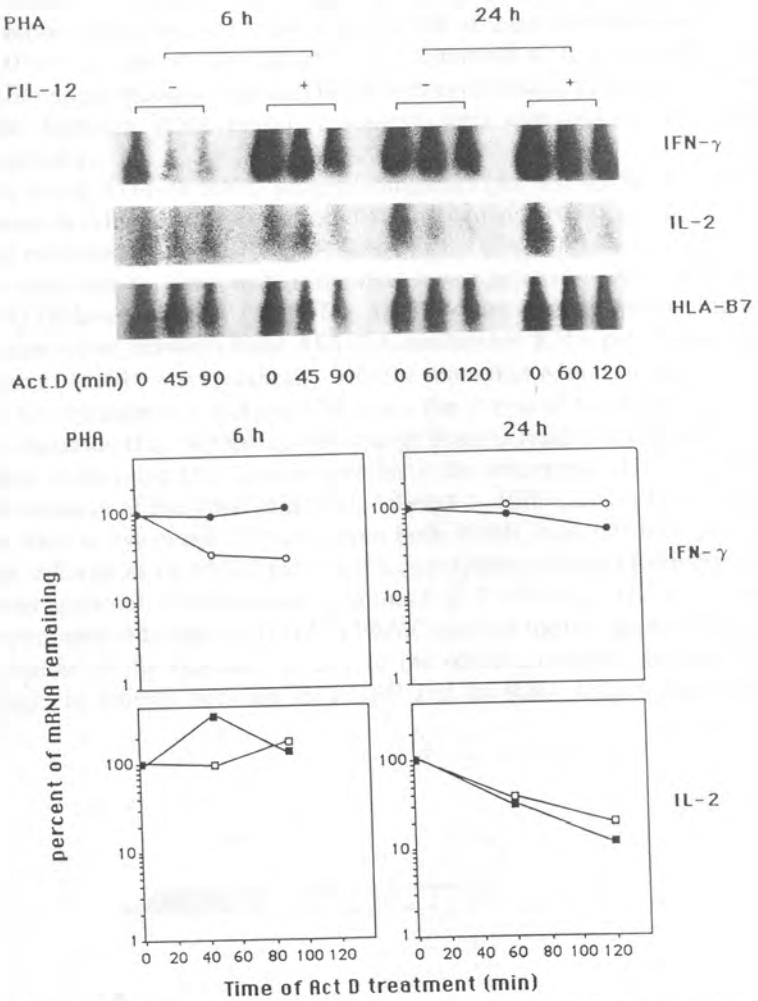


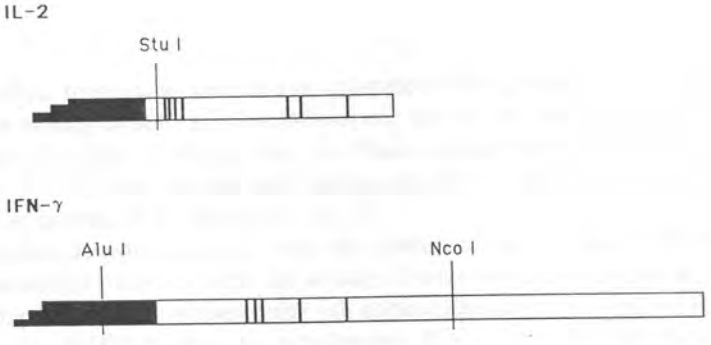
FIG. 3. rIL-12 stabilizes IFN-γ but not IL-2 mRNA in activated T cells. Cells (>90% $CD3^+$) were activated with PHA in the presence or absence of rIL-12 (10 U/ml) for 6 or 24 hr at which time actinomycin D was added. Total cellular RNA was analyzed at various times for IFN-γ, IL-2, and HLA-B7 using Northern blot analysis. Each lane contained 20 μg RNA (top). Blots were overexposed for sake of photography. The rate of mRNA degradation of IFN-γ and IL-2 was determined by measuring the hybridization intensities by scanning densitometry. The amount of mRNA at the various times is expressed as a fraction of the mRNA level at Time 0 (bottom). Open symbols represent data points from control; closed symbols represent data points from rIL-12-treated cultures. Hybridization of blots with HLA-B7 cDNA was used to ensure that comparable amounts of RNA were used for each experimental point.

fore play a role in the differential post-transcriptional regulation of IFN-γ and IL-2 during T cell activation.

Cytoplasmic AUBPs are not the IL-12 response elements in the selective stabilization of IFN-γ mRNA. Lymphokine mRNA commonly contain ARE which confer cytoplasmic instability in heterologous gene constructs. In an attempt to clarify the mechanism of action of IL-12, we characterized cytoplasmic proteins recognizing AU-rich sequences in the 3′ UTR of IFN-γ and IL-2 mRNAs. Cytoplasmic lysates were assayed

for binding to *in vitro* transcribed radiolabeled 3′ UTR of IFN-γ and IL-2 mRNAs. The probes contained the full-length 3′ UTR of these messages with variable number of AU-rich sequences, among which are reiterated AUUUA motifs (depicted in Fig. 4). After coincubation of labeled RNA with cytoplasmic proteins, uv crosslinking, and RNase digestion, RNA–protein complexes were analyzed by SDS–PAGE and autoradiography.

Six major AU-rich RNA–protein complexes (36, 41, 45, 50, 68, and 86 kDa) were detected in cytoplasmic lysates from PHA-activated partially purified T cells and PBMC using radiolabeled IFN-γ 3′ UTR RNA probe (Fig. 5A). A similar pattern of AUBP that appeared to bind with somewhat lower intensity was observed utilizing the [^{32}P]UTP-labeled IL-2 3′ UTR. (Fig. 5B). In other studies, we have demonstrated that the interaction between these AUUUA-containing RNA probes and the AU-binding proteins (AUBP) was specifically with the reiterated AUUUA sequence (16). Deletion of a 320-bp segment, lacking ARE from the 3′ end of the IFN-γ mRNA 3′ UTR by *Nco*I digestion (Fig. 4, top) did not change binding relative to the full-length transcript, further confirming the binding specificity for reiterated AUUUA (data not shown). The intensity of the 86-kDa doublet labeled by IFN-γ 3′ UTR was slightly increased over time in cytoplasmic lysates from both PBMC and partially purified T cells. No other differences in AUBP pattern between lysates isolated from cultures at 6 and 22 hr was apparent. Furthermore, treatment of T cells with rIL-12 or addition of neutralizing antibody against IL-12 to PBMC did not induce appearance, disappearance, or change in the intensity in any of the observed bands. Examination of relative changes in affinity between an AUBP and its RNA ligand was addressed through



3′IL-2 5′-T_7- CCTTCTATTTATTTAAATATTTAAATTTTATATTTA
$T(N)_{115}$TATTTATCCCAAAATATTTATTATTA$(N)_{45}$T
ATTTAATAAATTTTGATAAATAT$(A)_{12}$C$(A)_{12}$-3′

3′IFN-γ 5′-T_3- CTAAA$(N)_{98}$TATTTATTAATATTTAACATTATTTAT
AT$(N)_{35}$TATTTATAATA$(N)_{41}$TATTATTTATAATT
$(N)_{413}(A)_{18}$-3′

FIG. 4. RNA probe sequences. (Top) Schematic illustration of IFN-γ and IL-2 3′ UTR RNA probes and positions of AUUUA pentamers (indicated with bars). Shaded area, coding region; white area, 3′ UTR. (Bottom) Sequence of RNA probes with AUUUA sequences underlined are shown.

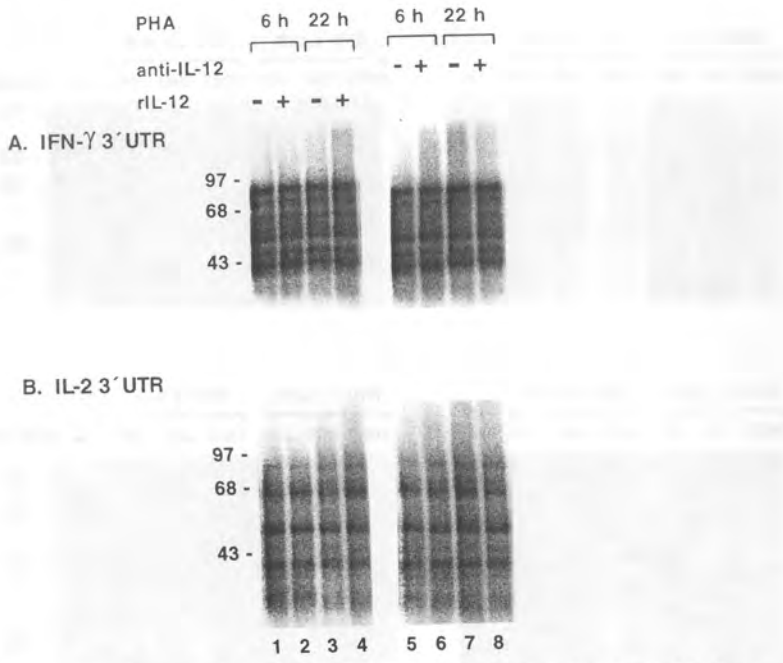


FIG. 5. The lack of effect of IL-12 on cytoplasmic AU-rich sequence-binding proteins detected in lymphocytes. *In vitro* transcribed, [^{32}P]UTP-labeled IFN-γ 3′ UTR (A) and IL-2 3′ UTR (B) RNA probes were incubated with cytoplasmic lysates (5 μg) prepared from >90% CD3$^+$ T cells (lanes 1–4) and from PBMC (lanes 5–8) activated with PHA for 6 or 22 hr in the absence or presence of rIL-12 and antiserum to IL-12, respectively, as shown. Following uv-cross-linking and RNase treatment, the samples were analyzed by SDS–PAGE.

competition studies. Increasing amounts of unlabeled IFN-γ and IL-2 3′ UTR were assayed for their ability to self- and cross-compete specific AUBP binding to radiolabeled RNA as a function of rIL-12 (Fig. 6). These experiments demonstrated that although IFN-γ 3′ UTR was bound with higher affinity by AUBP than by IL-2 in general, it did not appear to be affected by IL-12.

Previous work has demonstrated (27) that the relative affinity of protein binding to nucleic acids correlates inversely with the ability of increasing concentration of salts to reduce binding. The effects of increasing salt concentration in the binding reaction confirmed that the AUBP binding to radiolabeled IFN-γ 3′ UTR was diminished proportionally by increasing KCl concentration. No effect of modulating IL-12 levels was seen (data not shown). In other experiments, we also examined the binding of proteins to [^{32}P]ATP, [^{32}P]CTP, [^{32}P]GTP-labeled 3′ UTR of IFN-γ. The binding pattern for each of the different radiolabeled probes differed to varying degrees, but no effect of IL-12 or neutralizing anti-IL-12 antibody was observed with any of these RNA probes (data not shown).

DISCUSSION

Two approaches were used to examine the effects of IL-12 upon IFN-γ mRNA stability. First, addition of neutralizing antiserum against the 40-kDa subunit of IL-

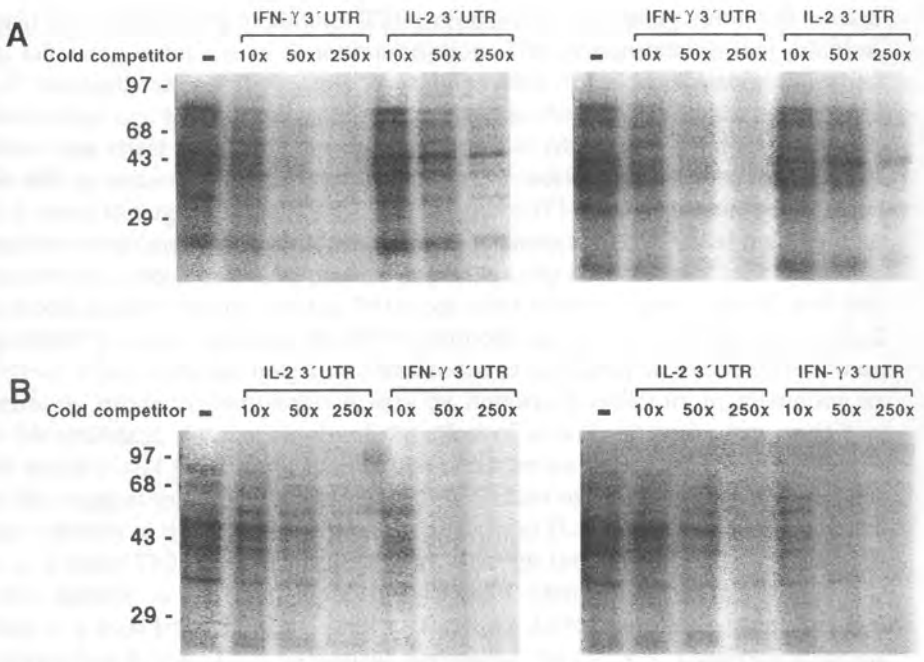


FIG. 6. IL-12 does not alter the affinity of protein binding to AU-rich sequences. Lysates from partially purified T cells activated with PHA for 6 hr in the absence (left panels) or presence (right panels) of rIL-12 were incubated with [^{32}P]UTP-labeled IFN-γ 3′ UTR (A) or IL-2 3′ UTR (B) RNA probes in the presence of 0–250× molar excess of unlabeled probes, as indicated. Lysates were analyzed as described above.

12 to PHA-stimulated PBMC significantly decreased the $t_{1/2}$ and accumulation of IFN-γ mRNA. Second, in partially purified (>90% CD3$^+$) T cells addition of rIL-12 markedly stabilized IFN-γ mRNA at 6 hr following activation, in contrast to its lack of effect on IL-2 mRNA turnover. At later times (24 hr) IFN-γ mRNA half-life increased as a consequence of activation, presumably through endogenous production of IL-12 by accessory cells (monocyte–macrophages, B cells, and adherent cells) and activated T cells (25, 26). This selective modulation of IFN-γ mRNA turnover by IL-12 was accompanied by corresponding and specific changes in IFN-γ protein production, while IL-2 mRNA and protein were unaffected. The lack of effect of IL-12 on the production of IL-2 in activated PBMC has also been confirmed by others (28). These data suggest that endogenously produced IL-12 has an important role in the regulation of IFN-γ production during the normal immune response, and additionally this regulation preferentially involves post-transcriptional mechanism(s). We also observed that the turnover rate of IL-2 and IFN-γ mRNAs are not uniformly regulated and change in an antiparallel fashion during T cell activation, although the genes for these two lymphokines are coordinately transcribed (29). IL-2 mRNA is more stable ($t_{1/2} > 120$ min), relative to IFN-γ ($t_{1/2} \sim 30$ min) in the first 6–8 hr following activation. Over time (20–24 hr of activation), the IL-2 mRNA become more labile ($t_{1/2} \sim 30$ min), while IFN-γ mRNA is stabilized ($t_{1/2} > 2$ hr).

Others have reported that IL-12 exerted its effect on IFN-γ mRNA stability in PHA-activated T cell blasts and in a T leukemia cell line only in the presence of simultaneously added IL-2 (9). Our data are consistent with the view that IL-12 may serve

as a signal which selectively modulates IFN-γ production in primary culture of human T cells, following induction of gene transcription. The demonstration that addition of rIL-12 markedly altered IFN-γ mRNA stability after only 6 hr of activation, when IL-2 production can be expected to be minimal, raises the possibility that under these conditions this effect of IL-12 may not require IL-2. Alternatively, other cytokines may be able to enhance the effects of IL-12. Recent work demonstrated that TNF-α and IL-2 acted synergistically with IL-12 to augment IFN-γ production, while IL-10 inhibited the stimulatory effect of IL-12 in SCID splenocytes (30). Post-transcriptional mechanisms have been shown to play an important role in these interactions (9, 31). In our model system (freshly isolated, PHA-activated normal human PBMC and partially purified T cells) several known IFN-γ inducers are also produced, such as IL-2 and TNF-α. Thus, although only IL-12 was added exogenously to the cultures, other, endogenously produced lymphokines may be important cofactors in mediating its effects. Nevertheless, the magnitude of the effect of anti-IL-12 antiserum on IFN-γ mRNA stability and protein production demonstrates its dominant role.

Our data suggest that there are substantial differences in susceptibility of these lymphokine mRNAs to degradation. Although IFN-γ and IL-2 are produced in the same subset of T cells (Th0, Th1), it would appear, through the action of IL-12, that IFN-γ mRNA stability is increased more than fourfold. Hence, production of IFN-γ is regulated at a post-transcriptional level to a greater degree than IL-2, implying that IL-2 production is more transcriptionally dependent than IFN-γ. Understanding the molecular basis of the differential post-transcriptional modulation of these genes has the great potential to determine how the normal immune response is shaped in terms of T cell specificity and differentiation.

Several studies have implicated the presence of reiterated AUUUA motifs (ARE) in the 3′ UTR of mRNAs as an important determinant in the post-transcriptional regulation of lymphokine gene expression. The mechanism by which reiterated AUUUA sequences modulate mRNA stability and translation is unclear. Studies exploring these issues have defined cytoplasmic proteins (AUBP) capable of recognizing and binding ARE (13–16). Whereas the presence of the conserved ARE in 3′ UTR of lymphokine mRNAs suggests that these sequences may play a role in regulating stability, our data would indicate that the ARE alone do not mediate selective and differential modulation of IFN-γ mRNA stability by IL-12. Both the 3′ UTR of IFN-γ and IL-2 mRNAs contain ARE, but IL-2 mRNA is not stabilized by IL-12. Moreover, IL-2 and IFN-γ are differentially regulated as a function of duration of activation. These findings suggest that the ability of IL-12 stimulation to stabilize IFN-γ mRNA most likely does not result from a property shared with IL-2. It may be that the ARE alone are not sufficient to confer mRNA stability or that the context and secondary structure in which the ARE exists is important in conferring stability, or its ability to engage *trans*-acting factors.

For these reasons, we examined the effect of IL-12 on cytoplasmic AUBPs. No correlation between the AUBP binding to *in vitro* transcribed IFN-γ or IL-2 3′ UTR could be demonstrated during the course of PHA activation in the presence or absence of rIL-12 or anti-IL-12 antiserum. Similarly, AUBP binding affinity for the ARE in IFN-γ mRNA was unaffected by IL-12 treatment as measured by cross-competition and salt dependency of binding. This lack of effect of IL-12 on AUBP was equally evident with T lymphoblasts maintained in IL-2 (data not shown). Finally, these studies were extended to include analysis of AUBP pattern under nondenaturing conditions

as well as with Northwestern blotting (data not shown). No difference was observed as a function of IL-12 treatment. Thus, we conclude the effects of IL-12 on IFN-γ mRNA stability cannot be attributed to altered AUBP binding to the IFN-γ ARE. It may be that the effects of IL-12 are mediated through sequences unique to IFN-γ mRNA or through IFN-γ mRNA-specific AUBP which cannot be discerned amid many other cellular proteins capable of specifically binding to reiterated ARE (16). These are currently areas of active investigation.

In summary, these studies have demonstrated that IL-2 and IFN-γ gene expression in T cells is differentially regulated at the post-transcriptional level as a function of duration of activation. Our data would suggest that this effect is mediated, at least in part, by IL-12. A correlation of these effects with altered cytoplasmic AUBP expression or affinity could not be demonstrated by *in vitro* binding. Nevertheless, these findings extend the observation that lymphokine mRNA expression can be differentially regulated through changes in mRNA turnover. These observations indicate that this activity of IL-12 provides a convenient model system in which the mechanisms which transduce these effects can be characterized. Through its study, considerable insight into the regulation of T cell activation and differentiation will be achieved.

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Glyceraldehyde-3-phosphate Dehydrogenase Selectively Binds AU-rich RNA in the NAD$^+$-binding Region (Rossmann Fold)*

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A 36-kDa protein that binds AU-rich RNA was purified from human spleen and identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH has been previously demonstrated to bind tRNA with high affinity. Competition studies suggested that cytoplasmic GAPDH binds the AU-rich elements (AREs) of lymphokine mRNA 3′-untranslated regions with higher affinity than tRNA. The AUUUA-specific RNA binding activity of GAPDH was inhibited by NAD$^+$, NADH, and ATP in a concentration-dependent manner, suggesting that RNA binding of GAPDH might involve the NAD$^+$-binding region, or dinucleotide-binding (Rossmann) fold. This hypothesis was supported by experiments that localized RNA binding to the predicted N-terminal 6.8-kDa peptide, known to be involved in the formation of the NAD$^+$-binding domain. The direct demonstration of ARE-specific binding protein activity localized to the NAD$^+$-binding region of GAPDH supports the general concept that enzymes containing this domain may exhibit specific RNA binding activity and play additional roles in nucleic acid metabolism. Finally, cytoplasmic GAPDH was found in the polysomal fraction of T lymphocytes. Thus, the RNA binding specificity of GAPDH as well as its localization within the cell merit its strong consideration as a protein important in the regulation of ARE-dependent mRNA turnover and translation in addition to its well described role in glycolysis.

Post-transcriptional regulation of gene expression has been demonstrated to play a major role in the regulation of cell growth and differentiation in eukaryotic cells (reviewed in Ref. 1). In T lymphocytes, mRNA turnover represents an important mechanism by which lymphokine production is modulated following activation (2–4). The post-transcriptional regulation of lymphokine and proto-oncogene gene expression has resulted in the discovery of highly conserved AU-rich sequences in the 3′-untranslated region that function in *cis* as destabilizing determinants (Refs. 5–7; reviewed in Ref. 8). These AU-rich elements (AREs)[1] consist of reiterations of the pentanucleotide AUUUA alone or oligo(U)$_{4-7}$ sequences in an AU-rich context. AREs have been shown to serve as binding sites for cytoplasmic and nuclear proteins that may function as *trans*-acting factors in regulating ARE-dependent mRNA turnover and translation. A variety of ARE-specific binding proteins (AUBPs) have been described (9–18), together with the recent identification of hnRNP A1 as a cytoplasmic AUBP in activated T lymphocytes (19).

Despite these findings, the molecular and cellular mechanisms by which these AUBPs modulate the turnover of labile mRNAs remain unclear. Recent work correlated a marked increase in the stability of provirally modified IL-2 mRNA with the enhanced binding of hnRNP A1 to AREs *in vitro* (20). These findings suggested that hnRNP A1 may serve multiple roles in RNA metabolism in addition to its role in RNA processing, perhaps as a function of its location (nuclear *versus* cytoplasmic) in the cell (19, 21). Indeed, many of the proteins important in the regulation of the turnover and translation of mRNA may serve additional and quite dissimilar roles as demonstrated with cellular iron metabolism and the reciprocal regulation of the iron response element-binding protein and aconitase activity (22, 23). In this regard, the number of enzymes with specific RNA binding activity is steadily growing, including glutamate dehydrogenase (24), NAD$^+$-dependent isocitrate dehydrogenase (25), thymidylate synthetase (26), dihydrofolate reductase (27), catalase (28), and thiolase (15). Thus, abundant metabolic enzymes may be involved in or regulate many processes involved in cellular homeostasis independent of the activity that characterized their initial description (reviewed in Ref. 29).

In an attempt to identify novel AU-rich RNA-binding proteins, a 36-kDa AUBP purified from human spleen was identified by N-terminal microsequencing as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a key glycolytic enzyme, utilizing NAD$^+$ as a coenzyme for the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate when assembled as a tetramer of identical 36-kDa subunits. Of interest, numerous non-glycolytic activities have been attributed to GAPDH, including nucleic acid binding (30–34), helicase activity (31), DNA repair (35), and interaction(s) with actin cytoskeleton (36) and microtubules (37). Of particular pertinence is the report that the 36-kDa subunit of GAPDH was purified from the nuclei of HeLa cells as a tRNA-binding protein implicated in nuclear export of tRNA (38). In this study, we characterize the AUBP activity of GAPDH and demonstrate that RNA binding occurs in the Rossmann fold or the NAD$^+$-binding region, a structural feature highly conserved among NAD$^+$-dependent dehydrogenases (39). This observation thus permits a rationale for the

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[1] The abbreviations used are: AREs, AU-rich elements; AUBPs, ARE-specific binding proteins; hnRNP, heterogeneous nuclear ribonucleoprotein; IL-2, interleukin-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region; IFN-γ, interferon-γ; GM-CSF, granulocyte-macrophage colony-stimulating factor; ptRNA, precursor tRNA.

RNA binding activity recently attributed to several dehydrogenases and other enzymes.

MATERIALS AND METHODS

Reagents—The monoclonal anti-human uracil-DNA glycosylase/GAPDH antibody (40.10.09) was generously provided by Michael Srivastava (Temple University School of Medicine, Philadelphia). Plasmid vectors containing tRNA were a gift from Sidney Altman (Yale University School of Medicine, New Haven, CT). Rabbit muscle GAPDH (G 2267), NADH, CAPS, 5,6-dichlorobenzimidazole riboside, and poly(U)-Sepharose beads were purchased from Sigma. NAD^+, Tween 20, protease inhibitors, and unlabeled nucleotides were from Boehringer Mannheim, while [α-^{32}P]UTP (~3000 Ci/mmol), Hybond-C nitrocellulose membrane, and the ECL chemiluminescence kit for Western blotting were obtained from Amersham Corp. Heparin-Sepharose CL-6B, protein A-Sepharose, and poly(I) were purchased from Pharmacia (Uppsala, Sweden). High and low molecular weight ^{14}C-labeled and low molecular weight prestained protein standards were obtained from Life Technologies, Inc.

Purification of the 36-kDa Protein—Human spleen obtained at elective splenectomy for hemolytic anemia was minced and then lysed in a Waring blender in homogenization buffer (50 mM HEPES/NaOH, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). Crude lysate was passed through successive layers of cheesecloth to remove unblended material and connective tissue. Nuclei were separated by centrifugation at 1800 × *g* for 7 min. The supernatant fraction was sequentially precipitated with the addition of 30, 60, and 80% ammonium sulfate. Pellets and the final supernatant fraction (nonprecipitable with 80% $(NH_4)_2SO_4$) were dialyzed against 0.5 × phosphate-buffered saline, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride. Subsequent purification of the 80% $(NH_4)_2SO_4$-precipitated fraction that contained the 36-kDa AUBP was performed using heparin-Sepharose chromatography. The fraction eluted from heparin-Sepharose with 0.25 M NaCl was subjected to poly(U)-Sepharose column chromatography with 0.1 M NaCl in 10 mM sodium phosphate buffer, pH 7.5, and stepwise salt elution. Chromatographic fractions were concentrated and equilibrated in a Centriprep-3 microconcentrator (Amicon, Inc., Beverly, MA) and stored in aliquots in ~50 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, at −70 °C. Protein concentrations were determined using the Micro BCA protein assay reagent (Pierce). RNA binding activity of the fractions was determined by *in vitro* label transfer assay throughout purification (see below). For microsequence analysis, the 0.25 M NaCl/poly(U) elution fraction was separated by Tris/Tricine-PAGE (40); transferred to Problott polyvinylidene difluoride membrane (Applied Biosystems, Inc., Foster City, CA) in 10 mM CAPS, pH 11.0, with 10% methanol; and stained with 0.1% Coomassie Brilliant Blue R-250 in 1% acetic acid, 40% methanol. The 36-kDa band was cut out and analyzed on a Model 476A protein sequencer (Applied Biosystems, Inc.) at the Dartmouth Protein Sequencing Facility.

Cytoplasmic Lysate Preparation—Human peripheral blood mononuclear cells were obtained from healthy volunteers by leukapheresis and isolated by Ficoll-Hypaque density gradient centrifugation. Cells were cultured at 4 × 10^6 cells/ml in RPMI 1640 medium (KC Biologicals, St. Louis, MO) supplemented with 8% heat-inactivated (56 °C, 1 h) fetal bovine serum (Flow Laboratories, Inc.) and 50 μg/ml gentamycin sulfate (U. S. Biochemical Corp.) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were washed twice in ice-cold phosphate-buffered saline or serum-free medium. Preparation of polysomal fractions was according to Brewer and Ross (41).

Preparation of in Vitro Transcripts—RNA transcripts of high specific activity (>10^8 cpm/μg of RNA) were synthesized from linearized plasmid DNA as a template in the presence of 50 μCi of [α-^{32}P]UTP, 20 μM UTP, and 4 mM each ATP, GTP, and CTP in 25 μl and incubated at room temperature for 2 h. The complete 3'-UTR of IFN-γ RNA was prepared by T3 RNA polymerase transcription of *Bam*HI-linearized plasmid pT7/T3α19 containing the 665-base pair *Alu*I fragment of the human IFN-γ gene containing 62 base pairs from the coding region and the full-length 3'-UTR (42) inserted into the *Sma*I site of the multiple cloning site. The 350-base pair 3'-fragment of the IFN-γ 3'-UTR containing all the AUUUA sequences was prepared by T3 RNA polymerase transcription of *Nco*I-linearized plasmid pT7/T3α19. This latter shorter IFN-γ 3'-UTR probe was used in all the experiments, except during the purification and identification of the 36-kDa protein, where the longer 3'-UTR probe was used. The c-*myc* 3'-UTR probe was prepared by SP6 RNA polymerase transcription of *Ssp*I-linearized plasmid pRK5 with the 400-base pair *Nsi*I-*Afl*II human c-*myc* fragment inserted into the *Sma*I site of the multiple cloning site (43). The *Xho*I fragment of the pXM vector containing the human GM-CSF DNA (provided by the Genetics Institute) was subcloned into the multiple cloning site of the pT7/T3α19 plasmid at the *Bam*HI site. The GM-CSF RNA probe was generated by T7 RNA polymerase transcription of this plasmid linearized with *Eco*RI. The 1, 2, 3, or 4 × AUUUA pentamer-containing probes were generated from a 108-base pair synthetic DNA template containing the T7 promoter (generously provided by James Malter), which was digested with the *Pst*I, *Bam*HI, *Sac*II, or *Eco*RI restriction enzyme, respectively. The IL-2 3'-UTR RNA probe was prepared by transcription of *Eco*RI-linearized plasmid pT7/T3α19 with the 270-base pair *Stu*I-*Eco*RI fragment of human IL-2 (44). The Δ2RI probe, which contains four consecutive AUUUA sequences (9), was prepared by T7 RNA polymerase transcription of *Eco*RI-linearized plasmid pT7/T3α19 with four reiterated AUUUA sequences in the *Bam*HI site of the multiple cloning site. The Δ2H3 RNA probe was transcribed by T3 RNA polymerase using the above plasmid as template, linearized with *Hin*dIII. The ptRNA^{Ser} RNA was transcribed by T7 RNA polymerase using *Ava*I-linearized plasmid pT7/T3α19 containing the human ptRNA^{Ser} gene (generously provided by Sidney Altman). The ptRNA$_f^{Met}$ RNA probe was prepared by SP6 RNA transcription of the *Bst*NI fragment of plasmid SP64 containing the yeast mitochondrial ptRNA$_f^{Met}$ gene (45). Unlabeled probes were prepared in the presence of 4 mM each ATP, GTP, CTP, and UTP at 30 °C for 2 h.

RNA Binding Assay—RNA probes (4–8 × 10^4 cpm, 0.2–0.8 ng) were incubated with the 0.25 M NaCl/poly(U)-Sepharose chromatographic fraction from human spleen containing the 36-kDa protein/GAPDH or the commercial rabbit muscle GAPDH preparation in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol for 10 min at 30 °C. In experiments using radiolabeled tRNA probes and in competition experiments, 0.5–2.5 μg/ml poly(I) was used instead of yeast tRNA to block nonspecific binding. In competition experiments, unlabeled competitors were added simultaneously with the radiolabeled RNA probe. The calculations of molar excess of unlabeled competitors were based on the molecular weights of the different probes relative to that of the radiolabeled probe. UV cross-linking of RNA-protein complexes was performed on ice using Stratalinker 1800 (Stratagene; 5 min, 3000 microwatts/cm^2), followed by RNase digestion (7.5 units of RNase T1 and 15 μg of RNase A for 30 min at 37 °C). Samples were analyzed under reducing and denaturing conditions by 12 or 15% SDS-PAGE according to Laemmli (46) and by autoradiography.

Immunoprecipitation—Purified spleen GAPDH was incubated with ^{32}P-labeled IFN-γ 3'-UTR RNA probe, UV-cross-linked, and digested with RNases as described above. Immunoprecipitation of RNA-protein complexes was performed using monoclonal antibody prepared against GAPDH (40.10.09) or control antibody (P3, parent hybridoma supernatant) in buffer IP (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 1 μM Pefabloc, 1 μg/ml each leupeptin and pepstatin) and incubation for 1 h on ice. Rabbit anti-mouse whole IgG (Cappel) and protein A-Sepharose beads were incubated for 1 h on ice and then added to RNA-protein-primary antibody complexes and incubated on ice with occasional mixing for 30–45 min. Beads were pelleted by centrifugation; the supernatants (depleted fraction) were collected; and then the beads were washed twice with 1 ml of buffer IP, boiled in Laemmli SDS sample buffer (46), and analyzed by 12% SDS-PAGE.

Immunoblotting—Proteins were separated by 12% SDS-PAGE and electrotransferred to Hybond-C nitrocellulose membrane in 48 mM Tris, 39 mM glycine buffer, pH 9.1, or in CAPS, pH 11.0, with 15% methanol. The membranes were washed with Tris-buffered saline, 0.075% Tween 20; 5% ECL blocking reagent was used for blocking nonspecific binding. Specific antigen-antibody reaction was detected by chemiluminescence.

Fingerprinting of the GAPDH RNA-binding Site with Staphylococcus aureus V8 Protease—2 μg of rabbit muscle GAPDH was digested with sequencing-grade *S. aureus* V8 protease in 50 mM NH_4HCO_3 buffer, pH 7.8, 15 mM KCl, 10% glycerol at different protein/protease ratios for 20 or 240 min at 37 °C, before or after RNA binding in the same buffer at 30 °C, followed by UV cross-linking and RNase digestion as described above. Under these conditions, V8 protease cleaves only at the C-terminal side of glutamic acid residues (47). Digestion was stopped by boiling the samples in Laemmli SDS sample buffer. The peptides were analyzed on 17% Tris/Tricine gels and visualized by silver nitrate staining (Bio-Rad) and autoradiography.

Northwestern Blotting and Microsequencing—20 μg of rabbit muscle GAPDH was incubated with or without 10 μg of sequencing-grade V8 protease in 50 mM NH_4HCO_3 buffer, pH 7.8, 15 mM KCl, 10% glycerol for 4 h at 37 °C. Undigested and digested GAPDHs were separated on

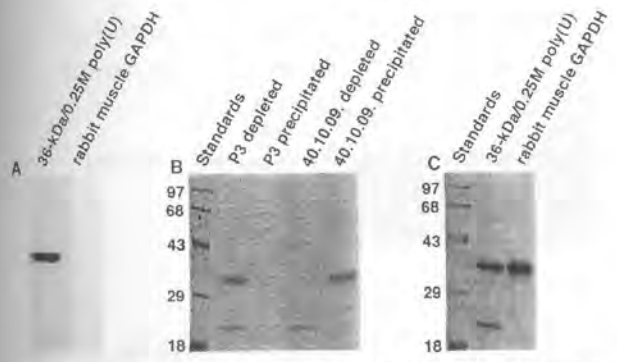

FIG. 1. **Identification of the 36-kDa AU-rich RNA-binding protein as glyceraldehyde-3-phosphate dehydrogenase.** *A*, Western blotting. 1.5 μg of protein of the 0.25 M NaCl/poly(U) fraction and 1.5 μg of rabbit muscle GAPDH (Sigma) were analyzed by Western blotting using the anti-uracil-DNA glycosylase/GAPDH antibody (40.10.09). *B*, immunoprecipitation. 1.5 μg of protein of the 0.25 M NaCl/poly(U) fraction was UV-cross-linked to the [^{32}P]UTP-labeled IFN-γ 3′-UTR, and the RNA-protein complex was immunoprecipitated with control (P3) or specific antibody (40.10.09) as described under "Materials and Methods." The depleted supernatants and the immunoprecipitated complexes were analyzed by SDS-PAGE and autoradiographed. *C*, binding of rabbit muscle GAPDH to the IFN-γ 3′-UTR. 0.5 μg of commercial GAPDH or 0.5 μg of the 0.25 M NaCl/poly(U) fraction was assayed for binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR.

17% Tris/Tricine gels and transferred to Hybond-C nitrocellulose or polyvinylidene difluoride membrane in 10 mM CAPS, pH 11, containing 20% methanol. The nitrocellulose membrane was preincubated in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and 15% glycerol for 10 min at room temperature. RNA binding was performed by adding fresh incubation buffer containing 2×10^5 cpm/ml ^{32}P-labeled IFN-γ 3′-UTR RNA probe and incubating for 15 min at room temperature with continuous agitation. After washing the blot with binding buffer, it was stained with 0.1% Coomassie Brilliant Blue R-250 in 1% acetic acid, 40% methanol; dried; and subjected to autoradiography. The polyvinylidene difluoride membrane was stained in the same way, and the 6-kDa peptide corresponding to the 6-kDa RNA-binding fragment detected by Northwestern blotting was excised and microsequenced.

RESULTS

Purification of the 36-kDa AUBP and Its Identification as GAPDH—A 36-kDa AUBP was purified from the cytoplasmic fraction of human spleen by following IFN-γ 3′-UTR RNA binding activity. Sequential ammonium sulfate precipitations substantially enriched the 36-kDa protein in the 80% fraction. This was followed by heparin-Sepharose and poly(U)-Sepharose chromatography, resulting in the presence of a single band on Coomassie Brilliant Blue-stained gels, although additional proteins with lower molecular weights were present upon silver nitrate staining (data not shown). The protein displayed insensitivity to trypsin in a limited proteolysis assay (in contrast to hnRNP A1) (19) and showed no immunological cross-reactivity with human hnRNP A1 (data not shown).

Sequencing of the N-terminal 23 amino acids of the 36-kDa AUBP revealed identity with the N terminus of human GAPDH. Immunoblotting with the 40.10.09 monoclonal antibody raised against human GAPDH reacted with the purified 36-kDa protein, but not with rabbit GAPDH (Fig. 1*A*). To demonstrate that the RNA binding was mediated by GAPDH, we determined that the anti-human GAPDH monoclonal antibody immunoprecipitated the radiolabeled RNA-protein complex (Fig. 1*B*). A 25-kDa protein with AUBP activity that was copurified with the 36-kDa protein/GAPDH was not recognized by the anti-GAPDH antibody. Its identity is unknown. Finally, purified 36-kDa AUBP and rabbit muscle GAPDH bound the ARE of the IFN-γ 3′-UTR with similar activity (Fig. 1*C*). In other studies, we were able to specifically immunoprecipitate

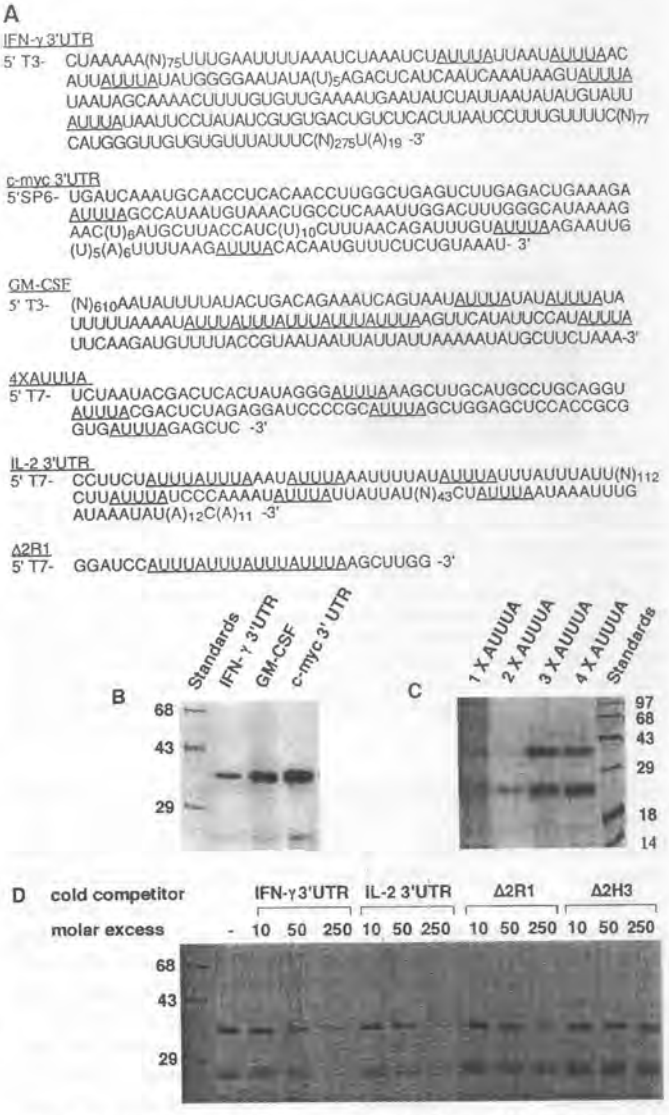

FIG. 2. **Specificity of binding of GAPDH to AU-rich RNA.** *A*, schematic representation of the sequences of the RNA probes used in RNA binding assays. *B*, binding of GAPDH to different AU-rich lymphokine and cytokine RNAs. [^{32}P]UTP-labeled IFN-γ 3′-UTR, GM-CSF, and c-*myc* 3′-UTR RNA probes were incubated with 0.75 μg of splenic GAPDH (0.25 M NaCl/poly(U) fraction), UV-cross-linked, and analyzed by SDS-PAGE. *C*, binding of GAPDH to separated AUUUA motifs. 0.2 μg of splenic GAPDH was incubated with ^{32}P-labeled RNA probes containing one, two, three, or four AUUUA pentamers as described under "Materials and Methods." (Equal molars amount of radioactive probes were added, corrected for the number of U nucleotides.) *D*, competition of lymphokine-cytokine mRNA 3′-UTRs with binding of GAPDH to the IFN-γ 3′-UTR. 0.2 μg of splenic GAPDH was assayed for binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR in the presence of the indicated unlabeled RNA sequences. 2.5 μg/ml poly(I) was used to block nonspecific RNA binding.

GAPDH complexed to radiolabeled RNA by UV cross-linking from cytoplasmic lysates of phytohemagglutinin-activated human lymphocytes (data not shown), indicating the activity of GAPDH as an RNA-binding protein in these cells.

Sequence-specific Binding of the 36-kDa Protein/GAPDH to AU-rich RNA—The 36-kDa protein/GAPDH was purified by measuring IFN-γ 3′-UTR RNA binding activity. To characterize the specificity of this binding activity, GAPDH was examined for its ability to bind ARE-containing mRNA from other proto-oncogene and cytokine mRNAs (sequences depicted in Fig. 2*A*). GAPDH bound radiolabeled GM-CSF and the c-*myc* 3′-UTR to a greater degree than the IFN-γ 3′-UTR (Fig. 2*B*).

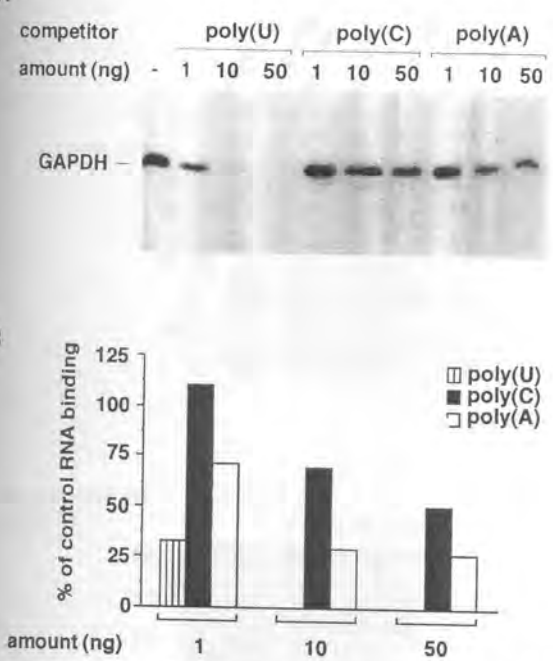

FIG. 3. **Competition of binding with ribohomopolymers.** *A*, 0.2 μg of rabbit muscle GAPDH was assayed for binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR in the absence or presence of the indicated amounts of poly(U), poly(C), or poly(A) RNA. *B*, the RNA-protein complexes were analyzed as described under "Materials and Methods," and the results of densitometric reading of the autoradiogram are expressed as percent binding to GAPDH in the presence of the indicated competitors relative to control.

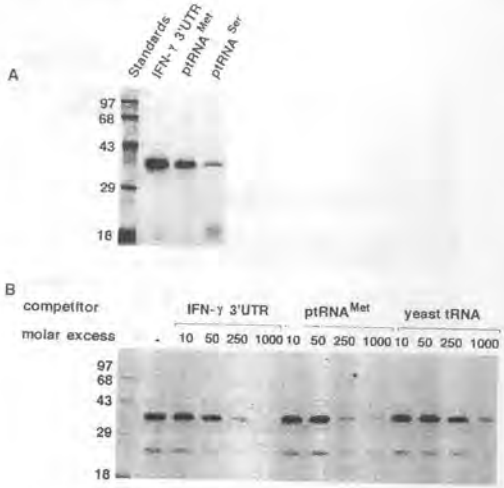

FIG. 4. **Comparison of binding of GAPDH to the ARE-containing 3′-UTR and tRNA.** *A*, binding of GAPDH to the radiolabeled IFN-γ 3′-UTR, ptRNA$_f^{Met}$, and ptRNA^{Ser}. 0.2 μg of rabbit muscle GAPDH was incubated with the indicated [^{32}P]UTP-labeled RNA probes in the presence of 0.5 μg/ml poly(I). (Equal molar amounts of radioactive probes were added, corrected for the number of U nucleotides of the different probes.) *B*, competition of binding of GAPDH to the IFN-γ 3′-UTR with tRNAs. 0.2 μg of splenic GAPDH was assayed for binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR in the presence or absence of the indicated molar excess of the unlabeled *in vitro* transcribed IFN-γ 3′-UTR and ptRNA$_f^{Met}$ and yeast tRNA (Sigma). 0.5 μg/ml poly(I) was used to block nonspecific RNA binding.

The number and proximity of reiterated AUUUA pentamers necessary for GAPDH binding were examined using radiolabeled RNA probes that contained one, two, three, or four AUUUA reiterations separated by 20 unrelated nucleotides (Fig. 2*C*). Strong binding of GAPDH to RNA required at least three reiterations of AUUUA, although a single AUUUA motif generated detectable signal. Thus, GAPDH binds several AREs that differ considerably in both their sequence as well as the proximity of AUUUA pentamers. This is further supported by the finding of equivalent binding between the sense and antisense IFN-γ 3′-UTR probes, the latter containing two AUUUA and one AUUUUA motif (data not shown).

The specificity of the GAPDH-ARE interaction was further studied in competition studies (Fig. 2*D*). The AUUUA sequence specificity was confirmed by demonstrating that the unlabeled *in vitro* transcribed IFN-γ and IL-2 3′-UTRs as well as a ribo-oligonucleotide containing the sequence 5′-AUUUAUUUA-UUUAUUUA-3′ (Δ2R1 probe) effectively competed for binding, while the addition of the nonspecific inhibitor Δ2H3 (antisense Δ2R1) had little or no effect on complex formation. In other studies, GAPDH binding to the IFN-γ and c-*myc* 3′-UTRs was found to greatly exceed that found in a ribo-oligonucleotide with five continuous reiterated AUCUA sequences, further indicating specificity for oligouridine or AUUUA sequences (data not shown). Further information on the specificity of GAPDH binding to RNA was obtained with the testing of the ability of unlabeled ribohomopolymers to compete for binding to the ^{32}P-labeled IFN-γ 3′-UTR. Poly(U) was superior to either poly(C) or poly(A) at reducing binding, with poly(A) demonstrating an intermediate level of inhibition (Fig. 3).

GAPDH was recently identified as a nuclear tRNA-binding protein (38). The binding of the two types of RNA ligands [tRNA and the ARE-containing 3′-UTR) by GAPDH was examined with selected tRNAs, ptRNA^{Ser} (human precursor serine tRNA) and ptRNA$_f^{Met}$ (yeast mitochondrial precursor methionine tRNA). GAPDH bound the IFN-γ 3′-UTR more strongly than the tRNA probes, exhibiting a rank order of binding of IFN-γ 3′-UTR > ptRNA$_f^{Met}$ > ptRNA^{Ser} (Fig. 4*A*). Consistent with this observation, both unlabeled *in vitro* transcribed ptRNA$_f^{Met}$ and the yeast tRNA mixture were less efficient than the unlabeled IFN-γ 3′-UTR at competing with GAPDH binding to the radiolabeled IFN-γ 3′-UTR (Fig. 4*B*).

Regulation of the RNA Binding Activity of GAPDH—Previous studies have demonstrated that the binding activity of both the iron response element-binding protein and AUBP is modulated by the redox state of the proteins (48, 49). These observations prompted us to study the possible role of redox changes in the AUBP activity of GAPDH. Incubation of GAPDH with the reducing agent 2-mercaptoethanol enhanced the binding of both lymphokine 3′-UTR and tRNA probes (Fig. 5*A*). In contrast, treatment with the oxidizing agent diamide (10 mM) markedly decreased the binding of GAPDH to the [^{32}P]UTP-labeled IFN-γ 3′-UTR (Fig. 5*B*). This effect of diamide could be reversed by subsequent treatment with 2-mercaptoethanol. These data are very similar to those observed with the iron response element-binding protein (48) and an AUBP described previously (49), raising the possibility that SH groups in GAPDH may also function intracellularly as a "sulfhydryl switch" by which its RNA binding activity is regulated.

NAD^+ and NADH are necessary for the glycolytic function of GAPDH. It has been reported that incubation of GAPDH with NAD^+ reduces its RNA binding activity (30, 38). Experiments were therefore undertaken to determine if the same was true of AUBP binding activity. Increasing concentrations of NAD^+ and NADH decreased GAPDH binding of both [^{32}P]UTP-labeled IFN-γ 3′-UTR (Fig. 6*A*, *upper panel*) and ptRNA$_f^{Met}$ (*lower panel*) RNA probes. These data indicate that NAD^+ and NADH may regulate RNA binding by GAPDH to both types of RNAs. The specificity of this inhibitory effect on GAPDH-RNA interactions was supported by the lack of effect of these coenzymes on AUBP activity on the serendipitously copurified 25-kDa protein.

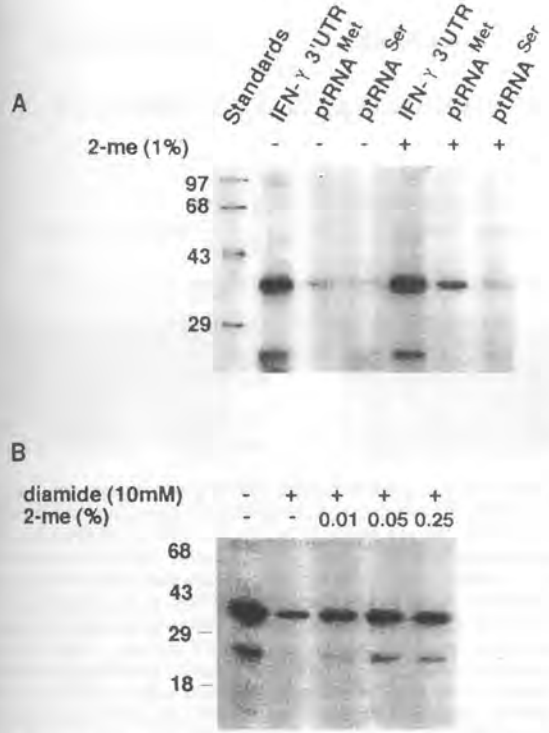

FIG. 5. **RNA binding activity of GAPDH is regulated by redox changes.** *A*, effect of 2-mercaptoethanol (*2-me*) on GAPDH binding to both the ARE and tRNA. 0.25 μg of splenic GAPDH was preincubated for 5 min at room temperature in the absence or presence of 1% 2-mercaptoethanol prior to incubation with the radiolabeled IFN-γ 3′-UTR, $ptRNA_f^{Met}$, and $ptRNA^{Ser}$ (as described in the legend to Fig. 4*A*). RNA-protein complexes were analyzed by nonreducing SDS-PAGE. *B*, effect of diamide on the AUBP activity of GAPDH. 0.2 μg of splenic GAPDH was preincubated with 10 mM diamide for 10 min at room temperature, and then the indicated amount of 2-mercaptoethanol was added for 5 min at room temperature. Radiolabeled GAPDH·IFN-γ 3′-UTR RNA complexes were analyzed as described under "Materials and Methods."

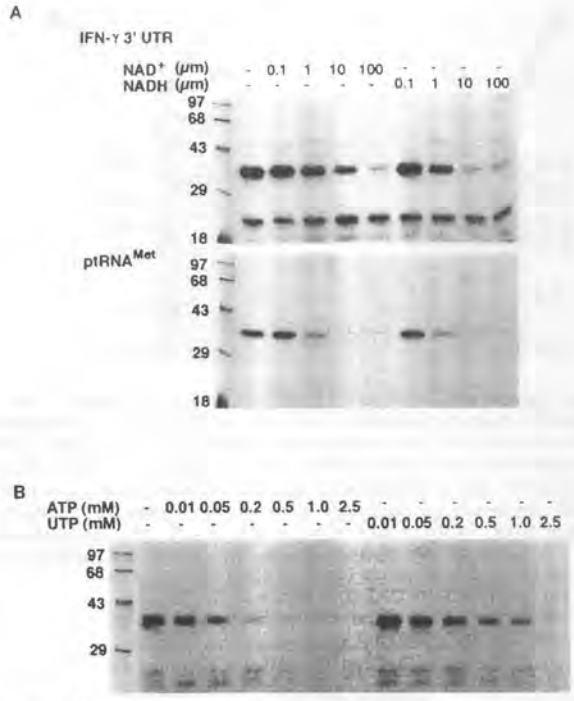

FIG. 6. **Inhibition of RNA binding of GAPDH by coenzymes NAD+ and NADH and by ATP.** *A*, 0.4 μg of splenic GAPDH was preincubated for 5 min at room temperature with the indicated concentrations of NAD^+ and NADH prior to binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR (*upper panel*) or $ptRNA_f^{Met}$ (*lower panel*). *B*, 0.25 μg of splenic GAPDH was assayed for binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR in the presence of the indicated concentrations of ATP or UTP. RNA-protein complexes were analyzed as described under "Materials and Methods."

It has been reported that GAPDH is capable of autophosphorylation *in vitro* (50). This prompted us to study whether the AUBP activity of GAPDH can be modulated by autophosphorylation. GAPDH was preincubated with ATP and/or Mg^{2+} under conditions to promote autophosphorylation (50) and then analyzed for binding to the [^{32}P]UTP-labeled IFN-γ 3′-UTR probe. Preincubation with 1 mM ATP alone or ATP + Mg^{2+} reduced the binding of GAPDH to AU-rich RNA dramatically (data not shown). This effect was equally apparent whether the incubation was carried out at 0 or 30 °C, further suggesting that ATP binding to GAPDH, and not phosphorylation, altered AUBP activity. To examine the specificity of this effect, ATP or UTP was added to the binding solution at increasing concentrations without preincubation or Mg^{2+}. ATP progressively decreased GAPDH binding to RNA, with half-maximal inhibition at ~50 μM, while a 20-fold higher concentration of UTP was needed to mediate comparable inhibition (Fig. 6*B*).

Localization of the RNA-binding Site on GAPDH—Our data demonstrate that the *in vitro* binding of GAPDH to the ARE-containing 3′-UTR and tRNA is decreased by the addition of NAD^+, NADH, and ATP. Since all of these agents can bind to the NAD^+-binding domain (51), we directly addressed if the site of RNA binding by GAPDH is in the Rossmann fold, a structural domain highly conserved among dehydrogenases (39). GAPDH was subjected to proteolysis by *S. aureus* V8 protease under nondenaturing conditions. For these experiments, the commercially available rabbit muscle GAPDH preparation was used as it demonstrated equivalent RNA binding specificity to human splenic GAPDH, unsurprising given the high level (94%) of amino acid identity. The expected fragments of GAPDH upon complete digestion with V8 protease are depicted in the peptide map shown in Fig. 7*A*. GAPDH was digested with V8 protease at different protease/protein ratios for various times (20 or 240 min), both prior to (Fig. 7*B*) and after (Fig. 7*C*) incubation with the radiolabeled IFN-γ 3′-UTR. Increasing V8 protease concentrations were associated with the progressive disappearance of the 36-kDa AUBP, which represents the intact subunit of GAPDH, and with the eventual generation of five peptides with AUBP activity ranging from 6 to 25 kDa (Fig. 7*B*, 20-min incubation at 1:30 and 1:5 ratios). With further digestion, the larger peptides (25, 20, and 16 kDa) with AUBP activity disappeared (Fig. 7*B*, 240-min incubation at a 1:5 ratio), while the smaller ones (10 and 6 kDa) increased. The progressive appearance and stability of the ~6-kDa fragment to further proteolysis suggest that this peptide might represent the N-terminal fragment of rabbit GAPDH with a calculated size of 6.8 kDa. Thus, the RNA binding activity of GAPDH appears to be localized to a peptide that is part of the NAD^+-binding domain, or Rossmann fold (39).

It is worth noting that the generation of peptide fragments by V8 protease digestion was influenced by GAPDH·RNA complex formation (Fig. 7*C*). V8 protease digestion after RNA binding (but prior to UV cross-linking) inhibited the generation of the 6-kDa peptide relative to that observed when V8 protease digestion was performed prior to RNA binding (Fig. 7, *B* and *C*, digestion, 1:5 ratio). This effect was accompanied by a corresponding change in the rate of loss of the 10-kDa peptide with AUBP activity, suggesting that RNA binding has made it more resistant to proteolytic attack by V8 protease. One interpretation of this observation is that access of the protease is blocked by RNA binding, thereby localizing the RNA-binding domain of GAPDH to the C-terminal end of the predicted N-terminal

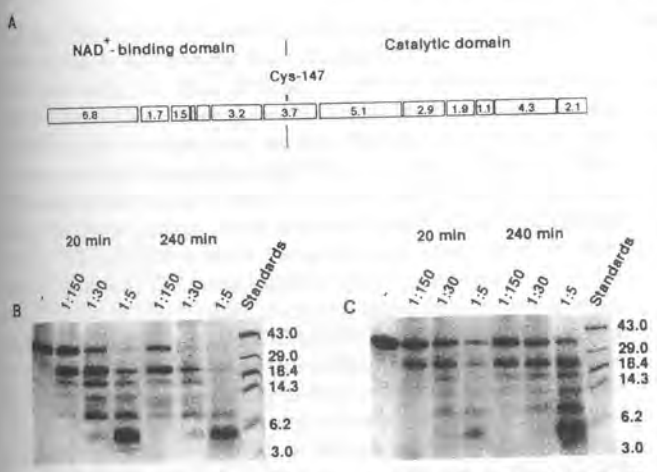

FIG. 7. **Fingerprinting of the RNA-binding domain of GAPDH.** Shown is schematic representation of the predicted V8 protease peptide map of GAPDH (*A*). The subunit of GAPDH consists of two domains. The first half of the subunit is the NAD^+-binding domain, and the second half is for substrate binding, specificity, and catalysis. Cysteine 147 (in rabbit muscle GAPDH) occurs at the junction between the two domains in the center of the subunit. Digestion of 2 μg of rabbit muscle GAPDH with *S. aureus* V8 protease at the indicated protein/protease ratios was performed before (*B*) or after (*C*) binding to the radiolabeled IFN-γ 3'-UTR for the indicated lengths of time as described under "Material and Methods." Following UV cross-linking and RNase digestion, the proteins and peptides were analyzed by 17% Tris/Tricine-PAGE.

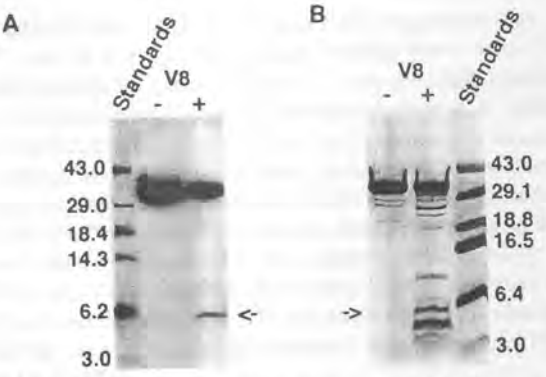

FIG. 8. **Identification of the IFN-γ 3'-UTR-binding peptide fragment of GAPDH as the NAD^+-binding region (Rossmann fold).** Rabbit muscle GAPDH was digested with V8 protease and then transferred to nitrocellulose membrane. RNA-binding proteins were detected by Northwestern blotting (as described under "Materials and Methods") (*A*); total proteins were visualized by Coomassie Brilliant Blue staining (*B*). The 6-kDa peptide (indicated by the *arrows*) was excised from the membrane for microsequencing.

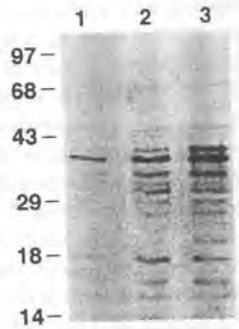

FIG. 9. **Polysomal location of GAPDH and its modulation by transcriptional inhibition.** Proteins of the polysomal fractions of 7×10^6 peripheral blood mononuclear cells (~1 μg) were separated by 12% SDS-PAGE and analyzed by immunoblotting with the anti-GAPDH antibody (40.10.09) as described under "Materials and Methods." *Lane 1*, resting human lymphocytes; *lane 2*, human lymphocytes activated with phytohemagglutinin for 16 h; *lane 3*, phytohemagglutinin-activated human lymphocytes treated with 5,6-dichlorobenzimidazole riboside (100 μM) for 1 h.

6.8-kDa peptide (Glu-61). The selectivity of the RNA binding activity of the five observed fragments of GAPDH is underscored by the complex digestion pattern demonstrated by silver staining, which reveals at least 15–20 peptides ranging from 3 to 33 kDa (data not shown).

Identification of the observed 6-kDa RNA-binding peptide as the N terminus of GAPDH cannot be made solely on the basis of the predicted size of a proteolytic fragment. Therefore, we determined the amino acid sequence of the observed 6-kDa RNA-binding peptide. GAPDH was digested with V8 protease, and the digestion mixture was separated by electrophoresis and transferred to nitrocellulose membrane. RNA binding performed *in situ* on the membrane-attached peptides (Northwestern blotting) revealed an ~6-kDa fragment (Fig. 8*A*). Microsequence analysis of the excised 6-kDa peptide identified by RNA binding and Coomassie Brilliant Blue staining (Fig. 8) yielded an amino acid sequence that is 100% identical to that of the N terminus of rabbit muscle GAPDH. These experiments unambiguously localize the RNA-binding domain of GAPDH to its NAD^+-binding region as well as demonstrate RNA binding by GAPDH in the absence of UV cross-linking.

Intracellular Localization of GAPDH in T Lymphocytes—Given the identification of GAPDH in this report as a cytoplasmic protein with mRNA binding activity, we examined its subcellular location in the cytoplasm of resting and activated T lymphocytes. Following cell fractionation (41), polysomal fractions were analyzed by immunoblotting (Fig. 9). In three separate experiments, GAPDH was found in the polysomal fraction of human T cells. Interestingly, polysomal levels of GAPDH appeared to be increased by the transcriptional inhibitor 5,6-dichlorobenzimidazole riboside, similar to data reported for other AUBPs (18, 19). Thus, the potential role of cytoplasmic GAPDH as a functionally relevant AUBP is strengthened by demonstrating both its polysomal location as well as a common pattern of modulation by RNA polymerase II inhibition relevant to other AUBPs.

DISCUSSION

The presence of the ARE in the 3'-UTR of labile cytokine, lymphokine, and proto-oncogene mRNAs has been shown to be important in the regulation of both mRNA stability (5–8) and translation (52–55). The ability of specific AUBPs to interact with these sequences has been correlated with changes in both nuclear and cytoplasmic mRNA stability (11, 20). In this paper, we report the identification of a 36-kDa AUBP purified from human spleen as glyceraldehyde-3-phosphate dehydrogenase based on its N-terminal amino acid sequence, immunoprecipitation of GAPDH·IFN-γ 3'-UTR complexes with the anti-GAPDH monoclonal antibody, and the demonstration that rabbit muscle GAPDH shares similar RNA binding activity.

Here we demonstrate that GAPDH binds to AU-rich RNA sequences present in the 3'-untranslated region of IFN-γ, c-*myc*, GM-CSF, and IL-2 mRNAs. The observation that GAPDH formed complexes with the 3'-UTR of c-*myc* very efficiently is intriguing because of the reported dysregulation of c-*myc* expression in Bloom's syndrome (56), a condition in which structural alterations in GAPDH have been described (57). GAPDH was able to bind RNA probes with discontinuous AUUUA pentamers, although three reiterations were necessary for optimal

binding. This is in contrast to observations with hnRNP A1, where little or no binding was observed.[2]

Of relevance to this finding is the previous report that GAPDH is a tRNA-binding protein implicated in nuclear export of tRNA (38). Competition studies indicate that both human splenic and rabbit muscle GAPDHs bind the 3'-UTR of IFN-γ with somewhat higher affinity than two selected tRNA probes. Although these assays do not permit the affinity of GAPDH for the 3'-UTR of IFN-γ to be measured, previous work demonstrated that GAPDH bound tRNA with a high affinity ($K_A \sim 1.8 \times 10^{-8}$ M for $tRNA^{Ser}$) (38). Based on these data, it would seem reasonable to infer that the GAPDH-ARE binding was also a high affinity interaction. In addition, $ptRNA^{Ser}$ and the $ptRNA_f^{Met}$ used in this study contain oligo(U) sequences (CUUUUUUUA and AUUUUA, respectively). In this regard, it is of interest that combinatorial selection studies have demonstrated that the HELN-1 protein, a member of the AUBP family, recognizes oligo(U) sequences (3, 4, or even 5 U nucleotides) flanked by other nucleotides than A (14). The stronger binding to lymphokine 3'-UTRs relative to tRNAs may therefore have been the result of higher avidity given the presence of reiterated AU-rich sequences that could serve as multiple binding sites for GAPDH, which exists as monomers, dimers, and tetramers in solution. Unfortunately, measurement of RNA binding affinity under native conditions in the absence of UV cross-linking was not successful because of the aggregation of GAPDH in the presence of RNA. However, these studies do permit the identification of AUUUA pentamers and U-rich sequences as sites at which GAPDH can specifically interact with RNA with high affinity. This is in contrast to studies of tRNA binding by GAPDH, where the nature of the sequence recognized by GAPDH was not established (38).

It is worth noting that multiple AUBPs between 32 and 40 kDa have been described in cytoplasmic and nuclear extracts (9–11, 13, 17, 18). Based on similar binding characteristics, one of these RNA-binding proteins may represent GAPDH. We have previously described a cytoplasmic 36-kDa AUBP activity in activated T lymphocytes that consisted of trypsin-sensitive (hnRNP A1) and trypsin-resistant components (19). Given their relatively comparable, but not identical AUBP activity (see above), and their similar size, it may be that the trypsin-resistant component of the 36-kDa AUBP represented GAPDH, instead of a closely related molecule or isoform of hnRNP A1 (58).

The demonstration that NAD^+, NADH, and ATP were able to diminish the specific AUBP activity of GAPDH suggested that the dinucleotide-binding (Rossmann) fold of GAPDH might serve as an RNA-binding domain. This was further supported by the finding that inhibition by UTP occurred at a 20-fold higher concentration, consistent with the lower binding affinity of this compound for the NAD^+-binding region of GAPDH (59). To date, we are unaware of direct evidence that identifies the Rossmann fold as an RNA-binding domain. Through V8 protease-mediated proteolysis, Northwestern blotting, and microsequencing, we have demonstrated that the AUBP activity of GAPDH was exhibited by the predicted N-terminal 6.8-kDa peptide. As RNA binding of GAPDH retarded generation of this predicted N-terminal 6.8-kDa peptide, it is likely that glutamate 61 was masked from proteolytic attack by the GAPDH·RNA complex. Thus, it can be inferred that a component of the RNA-binding domain extends beyond the predicted N-terminal 6.8-kDa peptide. This observation would be consistent with the structure of the NAD^+-binding domain, which is made up of two roughly identical mononucleotide-binding sites in the NAD^+-dependent dehydrogenases (39). In GAPDH, one of the mononucleotide-binding areas is in the predicted 6.8-kDa V8 peptide fragment, with the other residing in the C-terminal half of the dinucleotide-binding fold. Hydrophobic interactions have been shown to be important in coenzyme binding in the NAD^+-binding domain (39). Consistent with these findings is our observation that the AUBP activity of GAPDH is unaffected over a wide pH range (from 3.5 to 9.7) (data not shown), suggesting that specific ARE binding by GAPDH is not a consequence of its overall cationic nature (pI 8.1–8.7) or ionic interactions. This insensitivity to pH changes suggests the importance of hydrophobic interactions in RNA binding by GAPDH.

Concurrent with these studies, other members of the family of dehydrogenases (lactate dehydrogenase, yeast mitochondrial NAD^+-dependent isocitrate dehydrogenase, and glutamate dehydrogenase) and $NAD(P)^+$/NAD(P)H-binding enzymes (catalase and dihydrofolate reductase) have been demonstrated to have sequence-specific RNA binding capacity (reviewed in Ref. 29). Yeast isocitrate dehydrogenase binds the AU-rich 5'-untranslated region of all major yeast mitochondrial mRNAs (25), while glutamate dehydrogenase has a specific RNA binding activity for cytochrome *c* oxidase mRNA (24). Given the absence of RNA-binding domains with characteristic ribonucleoprotein consensus sequences (reviewed in Refs. 60 and 61) and the similarity in three-dimensional structure of the dinucleotide-binding regions of different dehydrogenases (39, 62), our data suggest that the Rossmann fold is capable of serving as an RNA-binding domain. The strong evolutionary conservation of the Rossmann fold and its presence in kinases, tRNA synthetases, and $NAD(P)^+$- and FAD-dependent dehydrogenases (39, 63) may therefore be relevant to the ability of these proteins to regulate RNA metabolism. Characterization of the NAD^+-binding region as an RNA-binding domain may therefore have broad implications for both mRNA metabolism and translation, as evidenced by the reciprocal identification of GAPDH as a tRNA-binding protein (38) and some tRNA synthetases as mRNA-binding proteins (64).

In human T lymphocytes, GAPDH can be localized to the polysomes, supporting the notion that GAPDH binds RNA *in vivo*. Furthermore, polysomal levels of GAPDH are increased by transcriptional inhibition, similar to data reported for other AUBPs (18, 19). Thus, the potential role of cytoplasmic GAPDH as a functionally relevant AUBP is strengthened by demonstrating both its polysomal location as well as a common pattern of modulation by RNA polymerase II inhibition. Based on this finding, the local concentration of NAD^+, NADH, and ATP in different subcellular compartments (polysomes, cytosol, and nucleus) may regulate GAPDH activity. Our data suggest that the Rossmann fold of GAPDH is reciprocally regulated between its RNA binding (inactive in glycolysis) and NAD^+ binding (active in glycolysis) states *in vivo*. This mechanism would favor glycolytic activity of GAPDH in the cytosol (where the concentration of NAD^+ and ATP is high) by inhibiting RNA binding. Conversely, GAPDH may bind RNA in the polysomal and nuclear microenvironments due to lower concentrations of NAD^+, NADH, and free ATP. This interpretation is supported by the finding that NAD^+ and NADH blocked IFN-γ binding to GAPDH at concentrations (~1–10 μM) at which they have been reported to associate with its Rossmann fold (65).

Alternatively, post-translational modifications (redox or otherwise) might influence the intracellular location of GAPDH as well as differentially regulate its RNA or NAD^+ binding activity. In this regard, it is worth noting that purified splenic GAPDH consisted of a single isoform on two-dimensional

[2] B. J. Hamilton and W. F. C. Rigby, unpublished observation.

PAGE, in contrast to what has been demonstrated for the multiple isoforms of total cellular GAPDH (30, 31). Thus, isoforms of GAPDH may differ considerably in their affinity for specific RNA ligands. Indeed, Karpel and Burchard (31) reported that only the most basic isoform of yeast GAPDH possesses poly(U) binding capacity and helix destabilizing activity *in vitro*.

GAPDH has been shown to possess a wide range of biological activities in addition to its well characterized role as a specific dehydrogenase essential in glycolysis. Our studies potentially extend these activities into mRNA metabolism. In this regard, it is intriguing that hnRNP A-type proteins have been shown to shuttle between the nucleus and cytoplasm and are implicated in mRNA export (66, 67). Given the relatively reciprocal distribution of GAPDH and hnRNP A1 between the nucleus and cytoplasm, their demonstrated AUBP activity, and the elevation of polysomal GAPDH levels with RNA polymerase II inhibition, GAPDH may play a similar role not only in nuclear tRNA export (38), but also in the export of AU-rich mRNAs, as reported for other AUBPs (19, 68). In addition to this potential role in nucleocytoplasmic export of tRNA and mRNA, the polysomal location of GAPDH and its RNA binding specificity suggest a role in ARE-dependent mRNA turnover and translation. Given the ability of GAPDH to decrease the melting point of RNA (31), GAPDH may be important for RNA unwinding during translation or accessibility of the 3′-UTR to endoribonuclease attack and may therefore influence the translatability or stability of mRNA through its binding to the ARE. In contrast to GAPDH, hnRNP A1 has been shown to have annealing activity (69). As the ability of hnRNP A1 to bind the ARE *in vitro* correlates with increased mRNA stability *in vivo* (20), these data suggest the intriguing possibility that GAPDH and hnRNP A1 may compete for similar, but not identical, RNA ligands *in vivo*, with profoundly different consequences in terms of mRNA stability and translation.

In conclusion, identification of the Rossmann fold of GAPDH as an RNA-binding site provides new insights into the regulation of this multifunctional protein. Moreover, this finding may be relevant to the RNA binding activity that has been recently described in other dehydrogenases, given the conservation of the Rossmann fold among these enzymes. The RNA binding specificity of GAPDH as well as its polysomal localization not only prompt consideration of this protein as a regulator of ARE-dependent mRNA turnover and translation, but also extend the conceptual framework of cytoplasmic mRNA metabolism to include proteins with previously defined roles in glycolysis.

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COMBINED APPLICATION OF *IN VIVO* UV-CROSSLINKING AND *IN VITRO* LABEL TRANSFER IN THE EXAMINATION OF AU-RICH SEQUENCE BINDING PROTEIN - RNA INTERACTIONS.

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Abbreviations: ARE (AU-rich element), AUBP (AU-rich sequence element binding proteins), 3′ UTR (3′ untranslated region), IL-2 (interleukin-2), FBS (fetal bovine serum), PHA (phytohemagglutinin), PMA (phorbol myristyl acetate), Iono (Ionomycin), DTT (dithiothreitol), SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), CAPS (3-[cyclohexamino 1-propanesulfonic acid]), hnRNP A1 (heterogeneous nuclear ribonucleoprotein A1), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), RER (rough endoplasmic reticulum).

ABSTRACT

*A combination of **in vivo** UV light-induced crosslinking of nucleic acids to proteins and **in vitro** label transfer assay was applied to investigate specific interactions between AU-rich sequences (ARE) in the 3′ UTR of lymphokine mRNAs and cytoplasmic AU-rich sequence element binding proteins (AUBP) in normal human lymphoblasts and MLA 144 gibbon lymphoid tumor cells. We demonstrate that a pool of cytoplasmic AUBP can be effectively crosslinked to RNA **in vivo**, suggesting a close association of these proteins with ARE sequences in the cytoplasm. We also show that the UV-crosslinked AUBP pool is markedly reduced in malignantly transformed MLA 144 cells compared with normal lymphoblasts, indicating weaker interaction between lymphokine ARE and AUBP in these tumor cells. Similar differences in AUBP-RNA associations were found between the membrane-bound polysomal subfractions of the two cell types where most of the AUBP activity was localized. We suggest that the decreased AUBP-mRNA association in MLA 144 cells might reflect a process concerned with disturbances of mRNA metabolism in the neoplastic phenotype.*

INTRODUCTION

Selective regulation of cytoplasmic mRNA turnover is an important control mechanism in the expression of a variety of eukaryotic genes (Ross, 1988; Atwater et al., 1990; Belasco and Brawerman, 1993). This mechanism has been shown to contribute to rapid and precise lymphokine, cytokine and proto-oncogene production in response to various environmental stimuli, such as cell activation. Many of these factors are encoded by shortlived mRNAs with a unique 3′ UTR containing reiterations of the motif AUUUA in a AU-rich context. The presence of AU-rich elements (ARE) has been associated with intrinsic cytoplasmic instability of a number of different mRNAs (Shaw and Kamen, 1986; Peppel et al., 1991). Although the exact mechanism by which ARE reduce the turnover rate of mRNAs is not yet clear, the identification of cytoplasmic AU-sequence binding proteins (AUBP) and the capability of AUBP to specifically bind to ARE sequences *in vitro*, emphasize the potential role of AUBP in selective mRNA catabolism (Malter, 1989; Bohjanen et al., 1991; Vakaloupoulou et al., 1991). Previous work in our laboratory has described cytoplasmic AUBP with specific lymphokine ARE-binding capability in activated normal human

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lymphocytes (Hamilton et al., 1993) and MLA 144 tumor cells (Henics et al., 1994). Among these, two 36-kDa proteins have been identified as heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and glyceraldehyde-3-phosphate dehydro- genase (GAPDH) and their *in vitro* RNA binding activities have been characterized (Hamilton et al., 1993; Nagy and Rigby, 1995). Intriguingly, higher *in vitro* AUBP binding affinity has been correlated with substantially increased stability of IL-2 mRNA in MLA 144 cells (Henics et al., 1994). Moreover, phorbol ester or calcium ionophore induced transient stabilization of lymphokine mRNAs might be mediated by AUBP binding to the 3′ ARE of the mRNA (Malter and Hong, 1991). Thus, selective control of the interactions between mRNA and various AUBP might be a critical mechanism which regulates the cytoplasmic turnover of labile mRNAs.

In an attempt to examine specific mRNA-AUBP interactions in lymphoid cells *in vivo*, we have combined the techniques of UV-crosslinking with subsequent label transfer assay. The exposure of cells to UV light allows the preservation of mRNA-AUBP complexes which exist *in vivo* at the time of UV irradiation. Subsequent analysis of these complexes by *in vitro* label transfer assay, using a radiolabeled AU-rich RNA probe, provides the detection of non-crosslinked AUBP as an indirect measure of the portion of AUBP complexed *in vivo* with ARE. We demonstrate that AUBP from normal human lymphoblasts and MLA 144 lymphoid tumor cells can be effectively crosslinked to RNA molecules *in vivo*. Our data also indicate differences in the association of cytoplasmic AUBP with RNA in the normal and neoplastic cytoplasm. We also show that cytoplasmic AUBP localizes predominantly in the membrane-bound polysomal compartment of lymphoid cells. Finally, we suggest that the combination of UV light-induced *in vivo* crosslinking and *in vitro* label transfer assay is a valuable means of studying sequence specific RNA binding protein-RNA interactions *in vivo*.

MATERIALS AND METHODS

Reagents: PHA was purchased from Wellcome Reagent Ltd. (Beckenham, England). PMA was from Sigma Chemical Co. (St. Louis, MO), and Ionomycin from Calbiochem (San Diego, CA). Recombinant human IL-2 was generously provided by Cetus Corporation (Emeryville, CA). [^{32}P]-UTP (~3000 Ci mmole^{-1}) was obtained from Amersham (Arlington Hts., IL), unlabeled nucleotides were purchased from Boehringer-Mannheim (Indianapolis, IN). 4B10 monoclonal antibody against hnRNP A1 was generously provided by Dr. Gideon Dreyfuss and the 40.10.09. Anti-GAPDH antibody was a gift of Dr. Michael Sirover.

Cell culture and activation: MLA 144 gibbon lymphoid tumor cell line (ATCC, Rockville, MD) was maintained in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 8% heat inactivated (30 min, 56°C) FBS (Flow Laboratories, McClean, VA) at a density of 0.5-1x10^6 cells/ml. Peripheral venous blood samples of healthy volunteers were separated individually on discontinuous Ficoll-Hypaque gradients and the lymphocytes were cultured in 8% FBS supplemented RPMI 1640 medium at a density of 0.5-1x10^6 cells/ml. Lymphoblasts were generated by subsequent maintenance of PHA activated (1 g/ml, 3 days) lymphocytes of individual donors in the presence of 25 U/ ml recombinant IL-2 in 8% FBS supplemented medium at a density of 10^6 cells/ml (referred to as IL-2 lymphoblasts). IL-2 lymphoblasts and MLA 144 cells were cultured in the absence or presence of 10 nM PMA and 1 M Ionomycin for 0, 4 or 20 h.

***In vivo* UV-crosslinking and preparation of cytoplasmic lysates:** Following activation, cells were pelleted, resuspended in cold PBS, distributed into 6-well tissue culture plates (1.6x 10^7 cells/well) and exposed to 254 nm UV light on ice in UV Stratalinker 1800 (Stratagene, La Jolla, CA), (3000 Wcm^{-2}) for 0, 2 and 8 or 0, 2.5 and 8.5 min. Cells were then harvested and lysates were prepared as described (Cervera et al., 1981). Briefly, cells were washed in ice-cold PBS, pellets were resuspended in a buffer containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and incubated on ice for 3 min. Following centrifugation for 3 min at 900xg, supernatants were collected, aliquoted and immediately frozen. To ascertain that the UV irradiation was specific only for

the fixation of close nucleic acid-protein complexes and that this procedure did not affect the extractability of proteins in the various lysate fractions, total protein concentrations (BCA Microprotein Kit, Pierce) of these cytoplasmic fractions were compared from control and UV-irradiated cells. There were no differences in the yields of total proteins extracted (data not shown), as would otherwise be expected if non-specific protein-protein crosslinking was formed.

RNA probe and *in vitro* label transfer (AUBP) assay: A 97 base ^{32}P-labeled probe (*PV-PstI*) was transcribed with T3 polymerase from a DNA template that had been generated by *BglI* and subsequent *PstI* digestion of the plasmid pT7/T3 19 containing the 3′ UTR of the IL-2 mRNA from MLA 144 cells (Henics et al., 1994). Probe molecules with specific activity > 10^8 cpm g^{-1} RNA were generated by using 50 Ci [^{32}P]-UTP (~3000 Ci $mmole^{-1}$), 0.0125 mM UTP and 2.5 mM e.a. ATP, CTP and GTP in the transcription reaction (Figure 1A). 8x 10^4 cpm of probe RNAs (~10 fmoles) were incubated with 5 g total protein from cytoplasmic lysates of polysomal preparations in 12 mM HEPES pH 7.9, 15 mM KCl, 0.2 M DTT, 0.2 g ml^{-1} yeast tRNA and 10% glycerol for 10 min at 30°C. RNA-protein complexes were then UV-crosslinked on ice (5 min, 3000 Wcm^{-2}), followed by digestion with RNase T1 (10 U) and A (20 g) for 15 min at 37°C. Samples were then separated by 12.5% SDS-PAGE, gels were dried and analyzed by autoradiography.

Western blotting: 20 g total proteins of crude cytoplasmic lysates or polysomal fractions were separeted by 12.5% SDS-PAGE. Gels were equilibrated in transfer buffer (10 mM CAPS pH 11.0, 15 % methanol) and transferred at 35 V onto nitrocellulose membrane (Hybond-C, Amersham). Blocking and all incubations were performed in TBS-T buffer (20 mM Tris-HCl pH 7.6, 140 mM NaCl, 0.05% Tween-20). For detection, the ECL Chemiluminescent Kit (Amersham) was used.

Preparation of polysomal subfractions: 10^8 IL-2 lymphoblasts or MLA 144 cells were placed in glass Petri dishes (d=20 cm) and exposed to UV light for 0, 2.5 or 8.5 min as described, under continuous gentle agitation. Cells were collected and incubated in 1.5 ml ice-cold PB-I (Polysomal Buffer I) (10 mM Tris-HCl pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.05% Triton X-100, 100 g/ml Heparin and 0.5 M Pefabloc) for 10 min followed by centrifugation at 200x g for 5 min (Hesketh et al., 1994). Supernatants were saved on ice (Sup I), pellets were washed once in the same buffer, then incubated in 1.5 ml PB-II (same as PB-I except with 130 mM KCl) for 10 min on ice and centrifuged at 800x g for 10 min. Supernatants were saved on ice (Sup II) and pellets were resuspended in ice-cold PB-III (same as PB-I except with 0.5% Triton X-100 and 0.5% Sodium-deoxycholate), lysed for 10 min and centrifuged at 1850x g for 10 min. Supernatants were stored on ice (Sup III) and pellets were discarded. Sup I-III were overlayered on 15 ml 40% sucrose cushions and centrifuged at 32 000x g at 4°C for 16 h. Polysomal pellets from Sup I, II and III were resuspended in a buffer containing 12 mM HEPES pH 7.9, 45 mM KCl and frozen immediately as "free" (FPS), cytoskeleton-associated (CYPS) and membrane-bound (MBPS) polysomal subfractions, respectively (Hesketh et al., 1994). Protein contents in each fraction were determined by the BCA Microprotein Kit (Pierce).

RESULTS

To examine the relationship of specific AUBP with the 3′ ARE of labile lymphokine mRNAs *in vivo*, we exposed normal human lymphoblasts and MLA 144 tumor cells to UV light *in vivo* prior to preparation of cytoplasmic lysates. AUBP activity was assayed using a radiolabeled RNA probe that contained 4 AUUUA pentamers (Figure 1A). The IL-2 *PV-PstI* probe was chosen since it is bound by cytoplasmic AUBP with high affinity (Henics et al., 1994). With this technique, we detected at least six cytoplasmic AUBP out of which the 90-, 50- and the 43-kDa proteins were labeled most intensely with the probe, but other proteins, including a 36-kDa one, also appeared (Figure 2). Increasing the time of UV exposure from 0 to 2, or from 2 to 8 min resulted in the reduction of the 90-, 50- and 43-kDa AUBP binding activity in both cell types (Figure 2). Substantial differences in the extent of the decline in AUBP activity was observed in normal and tumor cells. In normal IL-2 lymphoblasts, after 8 min of *in vivo* UV exposure,

A. 5`-GGGA-n_{10}-CU**AUUUAUUUA**AAU**AUUUA**AAUUUUAU**AUUUA**UUG-n_{30}-
-UAUUAUUCUUAAAGAGC-3`

B.

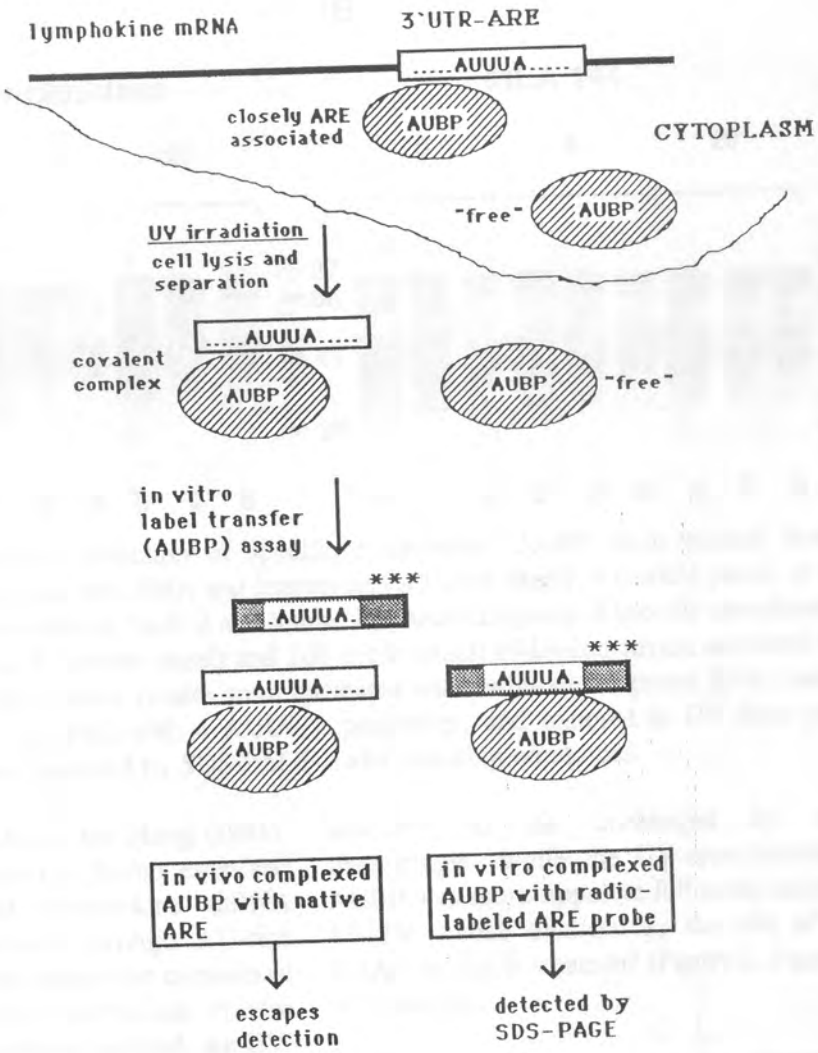

Figure 1. A) Sequence of *in vitro* transcribed *PV-PstI* RNA probe with the AUUUA motifs underlined. **B)** Combination of *in vivo* UV-crosslinking and *in vitro* label transfer techniques in the analysis of intracellular associations of lymphokine mRNA and specific 3′ UTR-ARE binding proteins (AUBP). Assuming a regulated balance in the distribution of ARE-associated and "free" AUBP, exposition of living cells to UV light renders ARE-associated AUBP to be covalently crosslinked to RNA. Subsequent detergent mobilization of ARE-AUBP complexes and "free" AUBP enables detection only of free AUBP by means of *in vitro* complex formation with radiolabeled probes containing specific lymphokine ARE.

AUBP activity of the 90-kDa protein was reduced by ~ 90%, whereas the 50-kDa and 43-kDa activity declined by ~50% and ~40% after 8 min, respectively (Figure 3, panel A, B and C). In contrast, analysis of lysates obtained from unactivated MLA cells showed that the 90- and 50-kDa proteins were reduced by only ~25 and ~30%, respectively (Figure 3, panel A, B). The *in vitro* detectable 43-kDa AUBP activity was reduced by ~50% after 8 min of UV exposure, comparable to that seen in IL-2 lymphoblasts (Figure 3, panel C). In general, UV-crosslinkable AUBP pools from MLA cells were always smaller than those from IL-2 lymphoblasts in multiple experiments.

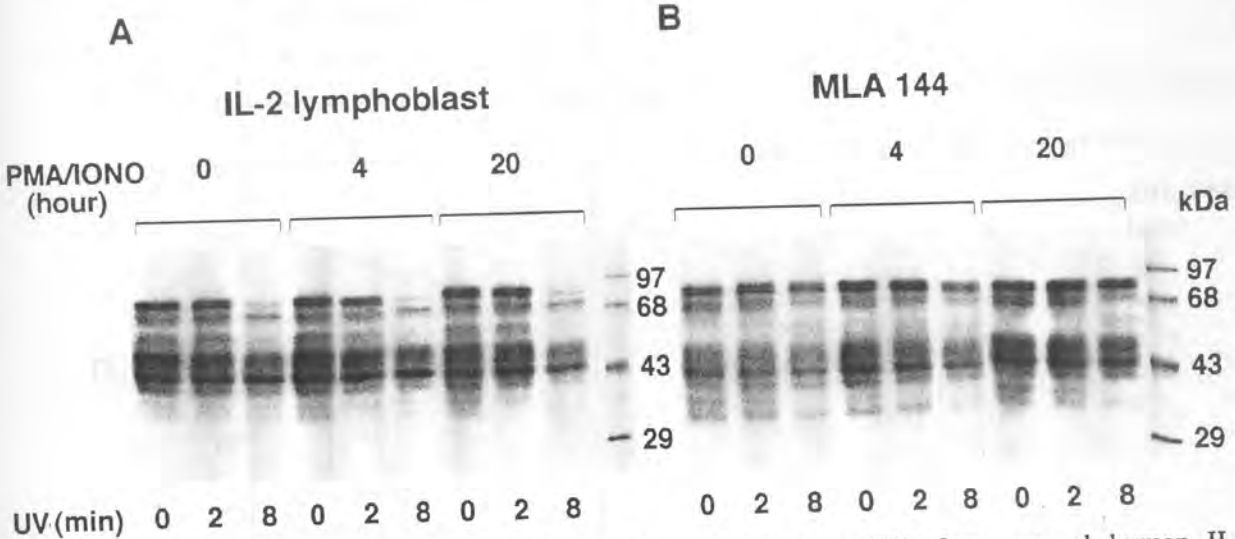

Figure 2. A) Autoradiographic detection of specific cytoplasmic AUBP from normal human IL-2 lymphoblasts. Cells were activated with PMA and Ionomycin for 0 (left panel), 4 (middle panel) or 20h (right panel) prior to *in vivo* UV-crosslinking for 0, 2 and 8 min. **B)** Autoradiogram of specific cytoplasmic AUBP from untreated (left panel) or 4 (middle panel) and 20h (right panel) PMA-Ionomycin activated MLA 144 cells. Detergent-extracted cytoplasmic lysates were incubated with radiolabeled probe RNA containing an MLA IL-2 ARE fragment (*PV-PstI*) with 4 AUUUA pentamers, and exposed to UV light and RNase treatment. Samples were then analyzed by SDS-PAGE under reducing conditions.

As has been proposed by Malter and Hong (1991), molecular mechanisms involved in phorbol ester- and calcium ionophore-induced lymphokine mRNA stabilization may be mediated through AU-rich element binding proteins. To address the question of whether altered ARE-AUBP associations *in vivo* could be detected by our combined method, we UV-crosslinked activated cells and monitored their extracted ARE-AUBP complexes. Analysis of lysates from PMA/ Ionomycin-activated cells revealed that, in IL-2 lymphoblasts, the 90-kDa UV-crosslinkable AUBP was unaffected by activation, whereas the same pool of the 50- and 43-kDa AUBP increased (Figure 2, Figure 3, panel A, B and C). In activated MLA cells, similarly to IL-2 lymphoblasts, the behaviour of the 90-kDa AUBP upon UV exposure appeared to be unchanged by activation. Interestingly, while the UV-crosslinkable 50-kDa AUBP was more apparent following activation, the 43-kDa AUBP declined by the end of the 20 h PMA/Ionomycin treatment (Figure 2, Figure 3, panel A, B and C).

In order to demonstrate that the absolute level of extracted RNA binding proteins was unchanged by UV crosslinking, we measured the amount of two known AUBPs, hnRNP A1 and GAPDH in the same cytoplasmic lysates as a function of UV exposure (Figure 4). The AUBP activity of these proteins has been described previously and characterized in lymphoid cells (Hamilton et al., 1993; Nagy and Rigby, 1995). *In vivo* UV exposure did not affect the

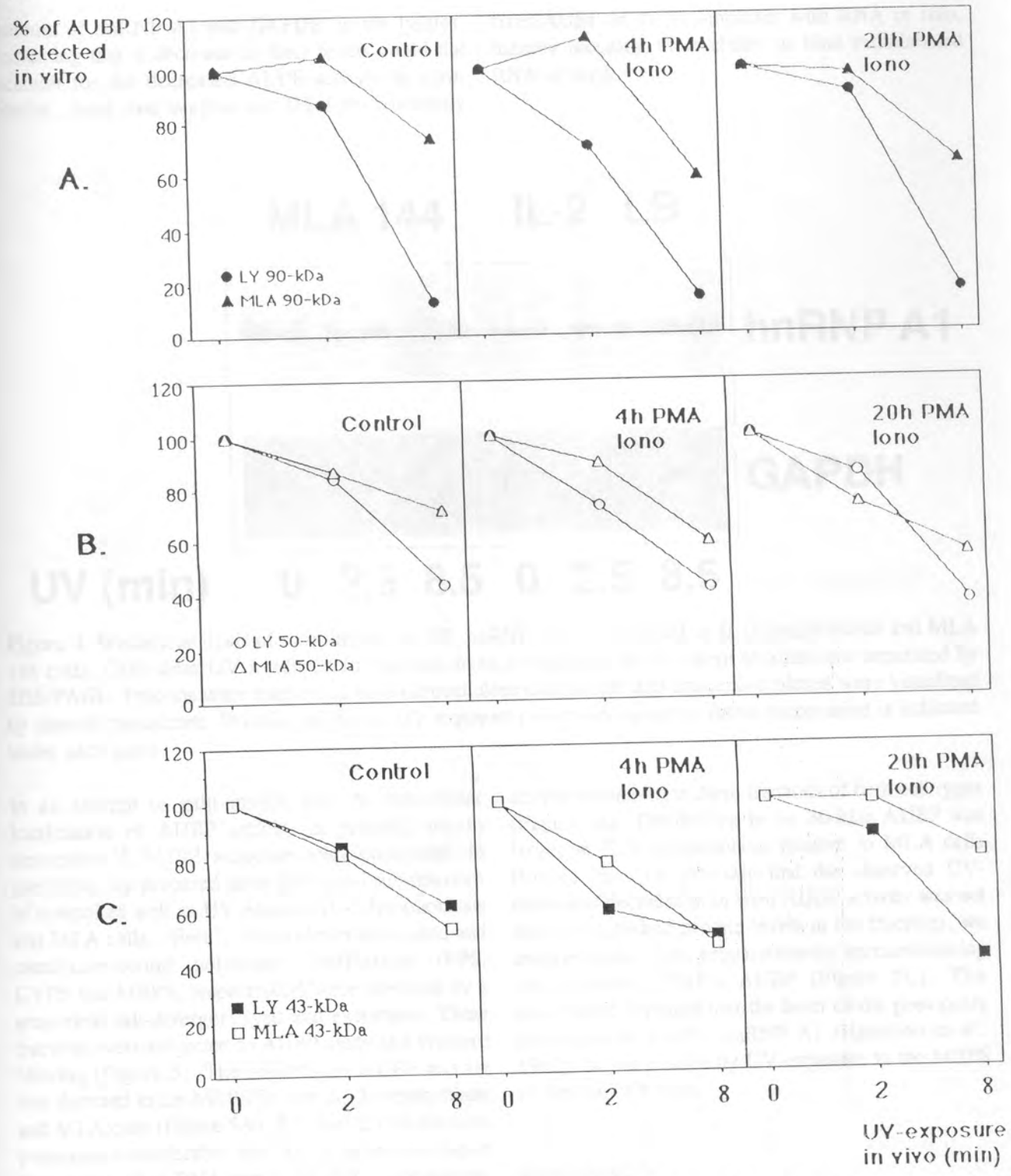

Figure 3. Changes in the detectable levels of cytoplasmic AUBP after various times of *in vivo* UV light exposure of normal human IL-2 lymphoblasts and MLA 144 lymphoid tumor cells. The indicated Mw complexes (90-, 50- and 43-kDa in panel A, B and C, respectively) were quantitated by densitometric analysis of the autoradiograms shown in Figure 2 and the relative values at various times of *in vivo* UV irradiation were expressed as % of the control (0 min UV exposure). Duration of PMA-Ionomycin activation is indicated within the boxes. Time of *in vivo* UV light exposure is shown at the bottom of the figure.

amount of hnRNP A1 and GAPDH in the lysates, indicating that a decrease in their levels could not account for the decreased AUBP activity *in vitro*. Rather, these data suggest that UV light covalently fixes AUBP in their complexes with RNA *in vivo*, thereby blocking their ability to bind radiolabeled RNA *in vitro*.

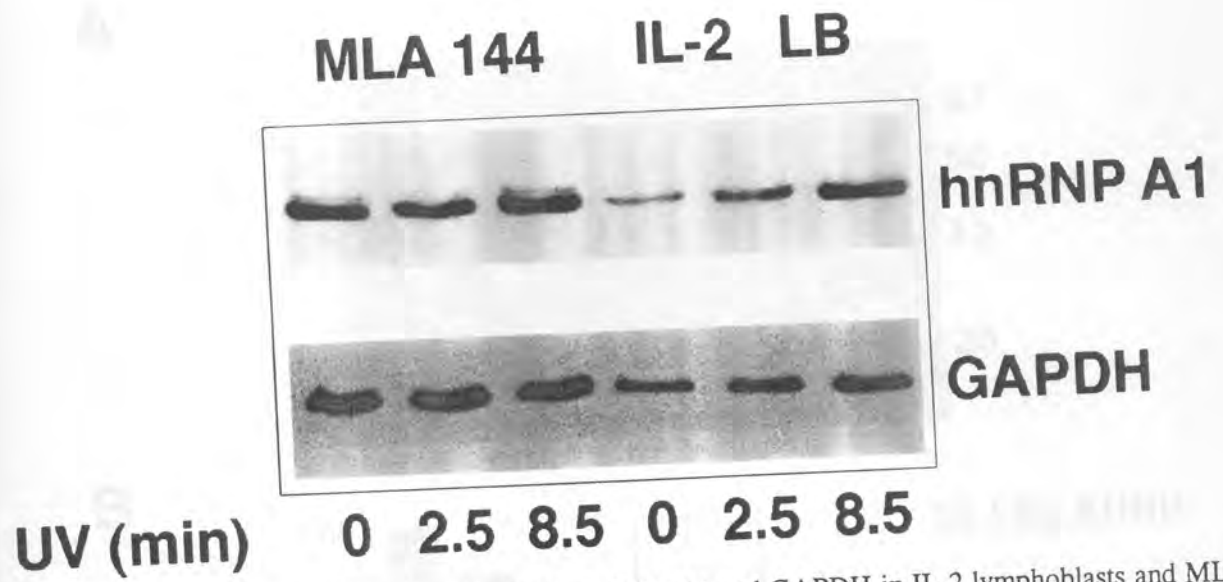

Figure 4. Western analysis of cytoplasmic AUBP, hnRNP A1 and GAPDH in IL-2 lymphoblasts and MLA 144 cells. Cells were UV irradiated for various times, cytoplasmic lysates were obtained and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and immuncomplexes were visualized by chemiluminescence. Duration of *in vivo* UV exposure prior to cytoplasmic lysate preparation is indicated under each lanes.

In an attempt to gain insight into the subcellular localization of AUBP activity in general, and to determine if AUBP associate with polysomes in particular, we prepared three polysomal sub-fractions of control as well as UV-exposed IL-2 lymphoblasts and MLA cells. "Free", cytoskeleton-associated and membrane-bound polysomal subfractions (FPS, CYPS and MBPS, respectively) were prepared by a sequential salt-detergent lysis and extraction. These fractions were subjected to AUBP assay and Western blotting (Figure 5). The majority of AUBP activity was detected in the MBPS in both IL-2 lymphoblasts and MLA cells (Figure 5A). FPS and CYPS fractions possessed considerably less AUBP activities despite their comparable RNA content by OD_{260} (not shown). Interestingly, in the MBPS, optimal UV- crosslinking and label transfer occured with the 36- and 43-kDa AUBP, while the larger complexes were less evident (Figure 5A). When MBPS were analyzed after *in vivo* UV exposure, the overall detected AUBP activity decreased in these fractions of both cell types (Figure 5A). The decline in the 36-kDa AUBP was larger in IL-2 lymphoblasts relative to MLA cells (Figure 5B). To ascertain that the observed UV-dependent decrease in *in vitro* AUBP activity was not due to diminished protein levels in the fractions, we analyzed each of the preparations by immunoblotting for a known 36-kDa AUBP (Figure 5C). This experiment revealed that the level of the previously characterized AUBP, hnRNP A1 (Hamilton et al., 1993) did not change by UV exposure in the MBPS of the two cell types.

DISCUSSION

The mechanism by which cytoplasmic AU-rich RNA binding proteins, found in many eukaryotic cells, contribute to the turnover regulation of labile mRNAs is unclear (Sachs, 1993). One difficulty in the

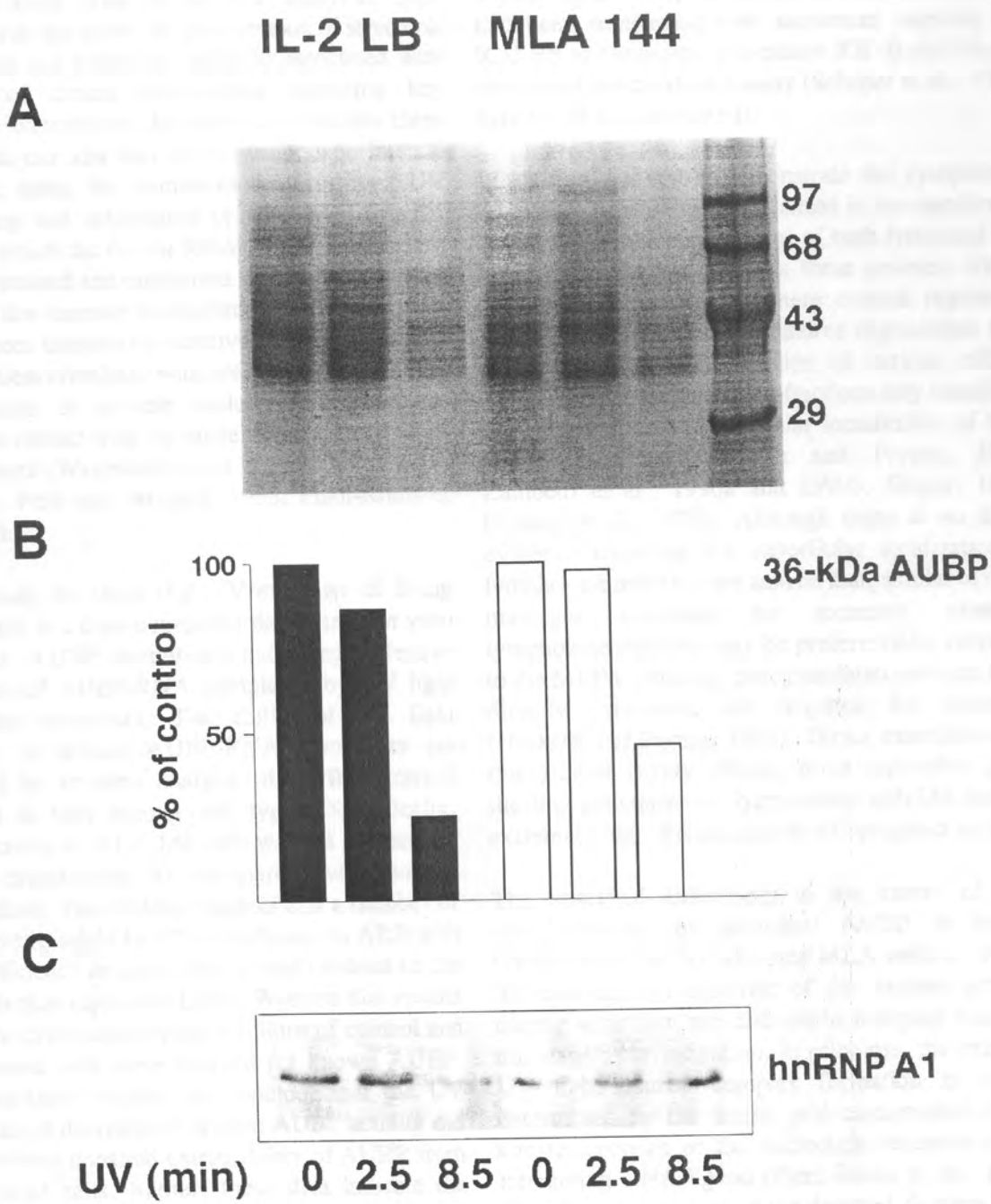

Figure 5. **A)** AUBP activities in the membrane-bound polysomal sub- fractions (MBPS) of UV-irradiated IL-2 lymphoblasts and MLA 144 cells. Each polysomal preparation (20 g of total protein) was analyzed in AUBP assay as described in the Methods section. Complexes were separated by 12.5% SDS-PAGE and visualized by autoradiography. Duration of *in vivo* UV exposure is indicated above each lanes. **B)** Densitometric analysis of the 36-kDa AUBP detected in the membrane-bound polysomal subfraction (MBPS) shown in Figure 5A. Values are shown as a percentage of the non-irradiated control. **C)** Detection of hnRNP A1 in the MBPS of IL-2 lymphoblasts and MLA 144 cells after various times of UV exposure *in vivo*.

examination of *in vivo* function of such cytoplasmic factors is the necessity for cell fractionation and extraction steps prior to *in vitro* analysis. Such procedures do not allow the preservation of structural architecture and therefore might be associated with the loss of critical information regarding key regulatory interactions. In order to overcome these difficulties, our aim was to design an experimental approach, using the combination of *in vivo* UV crosslinking and subsequent *in vitro* label transfer assay, in which the *in situ* RNA-AUBP associations could be retained and monitored. Irradiation of living cells with low intensity monochromatic (254 nm) UV light induces chemically reactive nucleotides which form covalent crosslinks with residues of virtually all amino acids of protein molecules that are in immediate contact with the nucleic acid at the time of UV exposure (Wagenmakers et al., 1980; Pashev et al., 1991; Pellé and Murphy, 1993; Pinol-Roma et al., 1989).

In this study we show that UV exposure of living cells results in a dose-dependent decrease of *in vitro* detectable AUBP activities, indicating effective generation of AUBP-RNA complexes by UV light within the cytoplasm. The ability of UV light exposure to induce AUBP-RNA complexes (as measured by *in vitro* analysis of AUBP activity) appeared to vary among cell types. Specifically, AUBP activity in MLA 144 cells was not as sensitive to UV crosslinking as compared with normal lymphoblasts. This finding suggests that a number of these proteins might be UV-crosslinked to ARE with lower efficiency *in vivo*. That it might indeed be the case is further supported by the Western blot results where the cytoplasmic lysate fractions of control and UV-exposed cells were assayed for known AUBP. Based on these results, we conclude that the UV light-induced decrease of *in vitro* AUBP activity did not arise from impaired extractability of AUBP from UV-exposed cells. Rather, these data indicate the presence of intrinsic RNA that covalently crosslinked to AUBP *in vivo*, masking its capacity to bind radiolabeled probe *in vitro*. The significance and practical applicability of our experimental approach is further strenghtened by recent studies of Scheper et al. They have demonstrated that an mRNA binding protein (Insulin-like growth factor II Cleavage Unit Binding Protein, ICU-BP) -with functional role in the cytoplasmic metabolism of IGF-II mRNAs- could be crosslinked to these RNAs *in vivo* and therefore depleted in the cytoplasmic lysates of those cells that had been transfected with sequences required for ICU-BP to recognize and cleave IGF-II mRNAs as monitored in a band-shift assay (Scheper et al., 1995; Scheper et al., submitted).

In addition, our results demonstrate that cytoplasmic AUBP are predominantly localized in the membrane-bound polysomal subfractions of both lymphoid cell types. This finding connects these proteins with a functional compartment, where critical regulatory steps of mRNA translation and/or degradation take place. Preferential distribution of various mRNA subsets among polysomal subfractions may contribute strongly to specific subcellular localization of their protein products (Hesketh and Pryme, 1991; Zambetti et al., 1990a and 1990b; Singer, 1992; Greene et al., 1976). Although there is no direct evidence resolving the subcellular localization of lymphokine mRNAs, we assume that, similar to other messages encoding for secretory proteins, lymphokine mRNAs may be preferentially recruited to the MBPS, allowing their translated proteins to be directly processed and targetted for excretion (Hesketh and Pryme, 1991). Direct examination of this question is very difficult, if not impossible, given the low abundance of lymphokine mRNAs and the extremely high RNase activity of lymphoid cells.

The observed differences in the extent of UV-crosslinkability of individual AUBP in normal lymphoblasts vs. transformed MLA cells as well as the non-uniform behavior of the various proteins during activation are difficult to interpret totally at this stage of investigation. In principle, the extent of UV light-induced complex formation is largely determined by the amino acid composition of the contact proteins or the nucleotide sequence of the interacting RNA ligand (Pinol-Roma et al., 1989). Therefore, evaluation of the detected changes in the UV-crosslinkability of individual AUBP cannot be directly addressed in the light of AUBP-ARE associations. On the other hand, fine alterations of AUBP-ARE interactions might well be part of a generally manifest abnormality in macromolecular associations in neoplastic cells (Ben-Ze'ev, 1985). Indeed, pathological stabilization of cytokine and

certain proto-oncogene mRNAs has been described in several tumor cell lines where alterations of regulatory *cis*-acting sequences of the messsenger RNA are not obvious (Schiavi et al., 1992; Ross et al., 1991). It might be interesting to note that in three additional tumor cell lines (Jurkat T cells, DP1727 erythroleukemia cell line and HEp-2 epithelial carcinoma cells) we found UV-crosslinkable pools of cytoplasmic AUBP similar in size to those of MLA cells, using other lymphokines as well as the *c-myc* proto-oncogene 3′ UTR-ARE as the RNA probe (not shown). Altogether, these results offer new aspects of the disordered mRNA metabolism in MLA cells where increased *in vitro* AUBP binding and affinity to a retrovirally altered ARE has been correlated to selective IL-2 mRNA stability *in vivo* (Henics et al., 1994).

In conclusion, these data indicate that association of cytoplasmic AUBP with RNA can be utilized to identify and examine these proteins as well as the functional significance of their molecular associations. Perturbations of AUBP-ARE interactions in tumor cells might contribute to pathologic mRNA metabolism. The advantages of this combined technique are that: i) it allows the preservation of intracellular nucleic acid-protein interactions apparent during complex regulatory processes and ii) it provides selective detection of the preserved alterations by means of specific RNA probes. Further data obtained through this approach might help us to estimate more accurately the *in vivo* interrelationships of RNA binding proteins with their RNA ligands during normal cellular processes and in tumorigenesis.

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INTERACTION OF AU-RICH SEQUENCE BINDING PROTEINS WITH ACTIN: POSSIBLE INVOLVEMENT OF THE ACTIN-CYTOSKELETON IN LYMPHOKINE mRNA TURNOVER

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SUMMARY

In this study we report that cytochalasin-induced disruption of microfilaments stabilizes lymphokine mRNAs in activated human peripheral lymphocytes. Parallel with this observation, a dose- and time-dependent increase in AUBP activities is apparent in the non-ionic detergent-resistant fractions of these cells, suggesting that cytochalasin-induced modulation of lymphokine mRNA stability might be mediated through cytoplasmic AUBPs. We provide evidence that some of the AUBPs can be immunoprecipitated with anti-actin antibodies, implicating that these proteins associate with the actin-based cytoskeleton *in vivo*. Moreover, disruption of the microfilament network by cytochalasins yields increased immunoprecipitable actin-AUBP complexes in the detergent-resistant cytoplasmic subfractions of lymphocytes. Additionally, we show that cytochalasin-induced changes in AUBP activities parallel with higher binding affinity of these proteins to ARE-containing RNA as judged by *in vitro* competition and *in vivo* UV-crosslinking analysis. Correlation of these findings with changes in mRNA stability indicates that the actin-cytoskeleton may play a physiologically important role in post-transcriptional regulation of lymphokine gene-expression during early lymphocyte activation.

Abbreviations: AUBP (AU-rich sequence binding protein), ARE (AU-rich sequence element), 3`-UTR (3`-untranslated region), PBMC (pheripheral blood mononuclear cell), HMG-CoA (3-hydroxy-3-methylglutaryl Coenzyme A), MBP (membrane-bound polysome), GAP (glyceraldehyde-3-phosphate), GAPDH (glyceraldehyde 3-phosphate dehydrogenase)

Regulation of eukaryotic gene-expression is a complex process that requires well-synchronized coupling of events from nuclear gene-activation to cytoplasmic synthesis of gene products. Post-transcriptional modulation in the overall expression of a gene is a potent mechanism by which cells adjust the level of many transiently expressed factors, including cytokines (1-3), lymphokines (4-6), proto-oncogenes (7-9) or certain transcription-factors (10-14). These proteins are key regulators of proper cell growth and differentiation and are encoded by intrinsically labile mRNAs with specific AU-rich instability determinant sequences (ARE) in their 3` untranslated region (3`UTR) (15-16). Transient modulation of the turnover and/or translation of such mRNAs have been shown to be mediated in part by interactions of their *cis*-acting ARE sequences with specific AU-rich sequence element binding proteins (AUBPs) (17-21). Recently, many proteins capable of specifically binding to ARE have been identified in various cell types (22-27). Although, a number of attempts has been made to uncover molecular details about the nature and consequences of AUBP-ARE interactions, it is likely that no uniform mode of action can be applied to fully explain the mechanisms upon which these proteins function in the post-transcriptional metabolism of short-lived mRNAs (reviewed in 28).

Several lines of evidence suggest that cytoplasmic compartmentalization may contribute to appropriate post-transcriptional processing of various mRNAs (29-34). For example, specific distribution of α- and β-actin and *c-myc* mRNAs on polysomes within a well-defined subcellular compartment has been linked to their site-specific translation (33-36). Specific sequences responsible for mRNA localization have been mapped within the 3`-UTR of these transcripts (33-36). The same portion of the mRNA has been implicated in specific polysomal and mitochondrial-cytoplasmic distribution of histone and the yeast tRNA processing enzyme, MOD5 mRNAs, respectively (37-38). Additionally, alternatively spliced HMG-CoA reductase mRNAs with various length 5`-UTR possess different

polysomal distribution, suggesting the involvement of 5`-UTR sequences in selective localization in addition to its known influence on translation (39). These studies as well as other reports have also revealed that localized polysome assembly as well as sorting of various mRNAs require intact cytoskeletal components (40-42). Specifically, it is well documented that cytochalasin-induced disruption of the microfilament network releases mRNA from the polysomes and ceases translation (40, 43). Interestingly, removal of the 5`cap structure or the poly $(A)^+$ tract does not affect the association of mRNA with microfilaments, implying the role of coding-, 5`-or 3`-UTR portions of the message in this process, possibly through specific RNA-binding proteins (41).

Little is known, however, of how cytoarchitecture interact with unstable mRNA-AUBP complexes in regulated mRNA turnover. This might be pertinent during lymphocyte activation, when transient stabilization of various lymphokine mRNAs coincides with cytoskeletal rearrangements (44-46). Recently, we have shown that analysis of polysomal subfractions from various lymphoid cells revealed considerably more lymphokine-ARE specific AUBPs in the membrane-bound polysomal subfraction (MBPs) than in the non-membrane-bound fractions (47). This, together with the observation that MBPs strongly associate with the actin based cytoskeleton, through a yet unclear mechanism (41), indicate that some of the AUBPs may play a role in post-transcriptional lymphokine mRNA metabolism in the context of microfilament-MBP complexes. In this study we test our hypothesis that the actin-cytoskeleton of lymphocytes should have links to ARE-AUBP mediated mRNA turnover. Our results indicate that the actin-based microfilament system may play a physiologically important role in post-transcriptional regulation of lymphokine gene-expression during early lymphocyte activation.

Reagents. Actinomycin D (Act D), Cytochalasin B and D, rabbit antiserum raised against human actin, purified chicken gizzard actin, 2-mercaptoethanol and RPMI 1640 medium base were purchased from Sigma. Act D was kept at 4 ^{0}C as a 5 mg/ml stock solution. Phytohemagglutinin (PHA) was obtained from Wellcome Reagent Ltd., Beckenham, United Kingdom or from Sigma. [^{32}P]-UTP (3000 Ci/mmol) was either from Amersham or Izotop Kft. (Budapest, Hungary) and unlabeled nucleotides were obtained from Boehringer Mannheim. Guanidium thiocyanate was purchased from Fluka Biochemica.

Cell culture. PBMCs were separated from whole blood of healthy volunteers on discontinuous Ficoll-Paque gradient (Pharmacia Biothec) and cultured immediately in RPMI 1640 medium supplemented with 8 or 10% fetal calf serum (Protein GMK, Hungary). PHA was added to the cultures at a final concentration of 1 μg/ml.

Determination of mRNA stability. 30-50 million PBMCs were seeded at a density of 4 x 10^6 cells/ ml and activated in the presence of 1 μg/ml PHA for various periods of time. Cells were then exposed to Act D and DMSO (vehicle control) or cytochalasins (2.5 - 20 μ M final concentration), harvested and their total cellular RNA was isolated by the single-step guanidium thiocyanate-phenol-chloroform extraction method (48) except that 0.1 volume of 2-mercaptoethanol was used in the denaturing solution. Equal amounts (5 - 10 μ g/ lane) of RNA samples were size fractionated in 0.8% agarose-formaldehyde gel, blotted onto Hybond-N nylon membrane (Amersham Corp.) in 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Membranes were air dried and baked at 80 ^{0}C, 2 h *in vacuo*, then prehybridized for at least 12 h in 50% formamide, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, 0.1 % bovine serum albumin and 200 μ g/ml sheared salmon sperm DNA at 42 ^{0}C. RNA was detected by hybridization of the

membrane at 42 ^{0}C overnight in prehybridization mix containing 10 % dextran sulfate and 10^6 cpm/ml of [^{32}P]dCTP-labeled specific cDNA probes specific for IL-2, TNF-α or β2-microglobulin and generated by the random priming method (49). Blots were then washed three times at 56 ^{0}C in 0.1 x SSC containing 0.02 % sodium pyrophosphate and 0.5 % Sarkosyl. Blots were dried, exposed to Fuji RX film at -80 ^{0}C and autoradiograms analyzed densitometrically.

Preparation of cytoplasmic subfractions. Cytoplasmic subfractions were prepared as described (50) with minor modifications. 2-3 x 10^7 cells were washed in ice cold serum-free medium, pellets were resuspended in buffer A containing 10 mM PIPES, pH 6.8, 100 mM KCL, 2.5 mM $MgCl_2$, 300 mM sucrose, 1mM phenylmethylsufonyl fluoride (PMSF) and lysed on ice for 3 min by addition of 1 % Triton X-100. Following centrifugation for 3 min at 900 x g, supernatants were collected, aliquoted and frozen immediately. Triton-insoluble pellets were extracted in buffer B containing 10 mM HEPES, pH 7.4, 15 mM NaCl, 1.5 mM $MgCl_2$, 1 % Tween-20, 0.5% sodium deoxycholate and 1 mM PMSF for 3 min on ice. Supernatants were collected after centrifugation with 1200 x g for 3 min at 4 ^{0}C.

RNA probes and in vitro label transfer assay. The 155-base RNA probe was generated as described (50) from a template encoding the proximal portion of IL- 2 mRNA 3`-UTR with four AUUUA motifs. The sequence of this probe is as follows: 5`-CCCU-n30-CU**AUUUAUUUA**AAU**AUUUA**AAUUUUAU**AUUUA**UUG-n75-CUUUUUGUAAG.
8 x 10^4 cpm [^{32}P]UTP-labeled probe RNAs (~10 fmol) were incubated with 2.5-10 μg proteins from cytoplasmic subfractions in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA and 10 % glycerol for 10 min at 30 ^{0}C. Protein-RNA complexes were fixed with UV light on ice using the UV Stratalinker model 1800 (Stratagene) (5 min, 3000 microwatts/cm^2) and exposed to RNase treatment (15 units of RNase T1 and 30 μg of RNase A/sample) for 15 min at 37 ^{0}C. Samples were then

separated by 12.5% SDS-PAGE under reducing conditions, gels were dried and analyzed by autoradiography.

Immunoprecipitation. Protein A-Sepharose was preincubated with BSA, washed at low pH extensively, regenerated and conjugated with either polyclonal antibody against actin or with rabbit preimmune serum. Beads were then washed with buffer IP (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF and 1 μg/ml pepstatin A). 10 μg of detergent insoluble cytoplasmic subfractions were incubated with 3.2 x 10^5 cpm [^{32}P]UTP-labeled RNA probe, UV-crosslinked and digested with RNases as described above. Immunoprecipitation of RNA-protein complexes was performed in buffer IP at 4^0C by incubating the reaction mixture with the prepared beads for 45 min under gentle agitation. Beads were then pelleted and the supernatants were collected (depleted fractions). After washing extensively with buffer IP, beads were analyzed by 12.5 % SDS-PAGE and autoradiography.

In vitro competition experiments. Equal amounts of detergent-insoluble cytoplasmic lysate fractions were incubated with 8 x 10^4 cpm [^{32}P]-labeled RNA probe (~10 fmol) in the presence of various molar excess of unlabeled competitor RNA transcripts that had been generated by the addition of 2.5 mM each ATP, UTP, CTP and GTP in the transcription reaction. Labelled and cold RNAs were added simultaneously to the label transfer reaction, incubated with the lysates for 15 min, UV-crosslinked and RNase-treated as described. Complexes were analyzed by 12.5 % SDS-PAGE and autoradiography.

In vivo UV-crosslinking analysis. UV-irradiation of intact cells was combined with subsequent label transfer analysis essentially as described (47). Briefly, following activation and Act D/cytochalasin treatment, cells were pelleted, washed once in PBS and resuspended in ice cold PBS. Cells were distributed into 6-well tissue culture plates (1.6 x 10^7 cells/ well) and exposed to 254 nm UV light on ice under continuous gentle agitation using the UV Stratalinker 1800 (Stratagene) (3000 microwatts/cm^2) for 0 and 8 min. Cells were then

harvested and cytoplasmic subfractions were prepared as described above. Samples were then analyzed in *in vitro* label transfer assay. We have demonstrated earlier that this technique is capable to distinguish between AUBP pools with various degree of *in vivo* association with specific RNA sequences based on the assumption that *in vivo* covalently complexed AUBPs escape detection in a subsequent label transfer assay (47).

RESULTS

Cytochalasin treatment stabilizes lymphokine mRNAs. To assess the effect of microfilament disruption on lymphokine mRNA stability, we activated freshly isolated PBMCs with PHA for 8 hours and exposed the cells to DMSO (vehicle control) or various concentrations of cytochalasins in the presence of Actinomycin D. We have tested Cytochalasin B and D and both agents were equally effective in our studies. Interleukin-2 and TNFα mRNA levels were monitored by Northern blot analysis. **Figure 1A** demonstrates a representative of multiple experiments which were performed with at least four different cell batches. After 30 min of transcriptional blocade, IL-2 and TNFα mRNA levels were declined markedly, indicating unstable RNA species. When cells were exposed to Cytochalasin B at 2.5 or 5 micromolar concentrations, Act D treatment failed to decrease mRNA levels under a certain value, suggesting that IL-2 and TNFα mRNAs became more stable. However, the two mRNA species showed different stabilization response upon cytochalasin treatment. As judged by densitometric analysis, IL-2 mRNA was stabilized more effectively as compared to that of TNFα, 2.5 x and 1.5 x longer respective half lives in response to cytochalasin as compared to Act D alone (**Figure 1B**). IFN-γ mRNA behaved similarly to that of IL-2 (not shown). β2 microglobulin mRNA was chosen as a control and no apparent change in its stability was detected in response to any treatment in the experiments. Interestingly, when PBMCs were activated for longer periods of time (> 20 h), IL-2 mRNA appeared to be already stable and we did not observe stabilization effect of cytochalasins (data not shown).

Effect of cytochalasin treatment on cytoplasmic AUBPs in activated PBMCs. To elucidate whether cytochalasin-induced lymphokine mRNA stabilization was accompanied by changes in the levels of cytoplasmic AUBPs in various subcellular fractions, we monitored the activity of these proteins in label transfer assays with a specific RNA probe encoding a portion of the 3`-UTR-ARE of IL-2 mRNA (see in Matherials and Methods). This RNA probe detected 4 major AUBPs in the Triton X-100-soluble fraction (90-, 70-, 50-, 41-kDa) and 4 in the detergent-insoluble compartment (70-, 50-, 41- and 36-kDa) of PBMCs activated for 8h (**Figure 2**). Marked dose and time dependent increase in AUBP activities was observed following Cytochalasin D treatment in the detergent-resistant fractions, but not in the detergent soluble fractions (**Figure 2A** and **B**). Among the detected AUBPs, the 36- and 41-kDa proteins appeared to be affected the most, and the appearance of a 60-kDa complex was observed after 2-3 h treatment (**Figure 2B**). We were interested to see if there were comparable changes in the activity of these proteins in similar subcellular fractions of PBMCs activated for longer periods of time. At >20 h of activation, cytochalasin treatment failed to cause any detectable changes in AUBP activities in either of the two subfractions (data not shown).

Immunoprecipitation of AUBP complexes with anti-actin antibodies. From the experiments described above, we concluded that cytoplasmic AUBPs might be in spatial and functional association with cytochalasin sensitive structures during specific phases of lymphocyte activation. To test this assumption, we attempted to immunoprecipitate RNA-AUBP complexes using specific antibodies to actin. **Figure 3** shows that a dominant 36-kDa AUBP complexed with anti-actin antibodies from the Triton X-100-insoluble cytoplasmic subfraction of activated PBMCs. The difference in AUBP activity in the control vs. cytochalasin-treated subcellular fractions was retained in the immunoprecipitated AUBP fractions (**Figure 3B, lanes 5-6**). At this point, it was important to ascertain that actin itself did not bind AU-rich RNA. Purified chicken gizzard actin was tested in a label transfer assay using various ARE-containing RNA probes and no complex formation was

detected in any case (not shown). However, in fractions, immunoprecipitated prior to the label transfer assay, we also detected efficient binding of a 36-kDa complex to AU-rich RNA (not shown). These data implicate that some RNA-AUBP complexes are in strong association with actin in the extracted subcellular fractions through the protein partner of the complex as well as demonstrate that this association is permissive for these AUBPs to bind ARE sequences *in vitro* (see Discussion).

Effect of cytochalasins on in vitro AUBP binding affinity and in vivo UV-crosslinkability to ARE. In order to gain more details of how cytochalasin-mediated effects influence AUBP-RNA interactions, first we monitored *in vitro* AUBP binding affinity to ARE-containing RNA probes in a competition assay. **Figure 4** demonstrates that cytoplasmic AUBPs from the detergent-resistant fractions of cytochalasin-treated lymphocytes bind IL-2 3`-UTR ARE with considerably higher affinity as compared to those of control cells. This result suggests that microfilament disruption *in vivo* facilitates *in vitro* association of AUBPs with specific ARE sequences within the extracted cytoplasmic fractions. Individual AUBPs, however, behaved disssimilarly in this context, indicating their different susceptibility to the treatment and/or association with the RNA *in vivo*. To test if these changes in *in vitro* AUBP binding affinities were reflected in the *in vivo* association of any of these proteins with ARE sequences, we exploited the capability of a technique, described recently in our laboratory, to detect such parameters (47). In agreement with our *in vitro* results, a dominant 36- and a less apparent 50-kDa AUBP complex could be UV-crosslinked to RNA in cytochalasin-treated lymphocytes with higher efficiency *in vivo*, suggesting their closer association with ARE following microfilament disruption (**Figure 5**).

In this study we made attempts to better characterize basic mechanisms of how lymphokine mRNA stability is modulated and to find potential links between ARE-AUBP mediated mRNA turnover and changes in cytoarchitecture upon lymphocyte activation. Two distinct lines of evidence prompted us to test possible microfilament involvement in mRNA-AUBP interactions and consequently, in lymphokine mRNA stability. First, coordination of cellular events brought upon by lymphocyte activation has been proposed to occur in part with rearrangement of cytoskeletal structures (51-54). Development of normal "capping" process, for example, can be prevented by microfilament disrupting agents such as cytochalasins (44-46). Moreover, key mediators in mitogenic stimulation-triggered signal transduction, such as protein kinase C, have been documented to associate with cytoskeletal components (53, 55-57). Thus, lymphocyte cytoarchitecture might be an essential element in normal progression through activation. Second, subcellular localization of many mRNA species has been implicated in proper spatial expression of the encoded gene product (29-32). *c-myc* protein, for example, appears to be synthesized more efficiently on polysomes located on the perinuclear cytoskeleton as it has to be transported back to the nucleus following translation (33-34). Similarly, lymphokine mRNAs are thought to be translated primarily on membrane-bound polysomes, from where the proteins can easily be recruited to the endoplasmic reticular and Golgi compartments for continuous processing and excretion (47). Polysomal anchorage to cytoskeletal structures seems to be generally important in efficient translation of mRNAs (41, 43). Since stability of many mRNA species is closely linked to their translation, it is conceivable to assume that specific association of the mRNA with cytoskeletal elements may, in turn, contribute to the regulation of its turnover. These observations raise a number of pertinent questions. Do lymphocyte cytoskeletal elements contribute to selective targeting, and possibly to specific turnover regulation of various mRNA species? Do these cells take advantage of structure-based regulatory functions evident in cell types that possess considerably more extensive cytoskeletal substance?

Our attempts to examine the effect of microfilament disruption on lymphokine mRNA turnover revealed that IL-2, TNFα and IFN-γ (not shown) mRNAs were stabilized when activated lymphocytes were exposed to cytochalasins (Figure 1). This effect was observable at early periods of activation (6-8 h) and not at later (> 20 h) time points. This would indicate that cytochalasin-sensitive structures are involved in regulated mRNA stability during early T cell activation, when lymphokine mRNAs are still unstable (58). After 20 h of mitogenic activation, when lymphokine mRNAs become transiently stabilized, we could not detect cytochalasin-directed effects at the mRNA or AUBP activity level. Therefore, it is possible that microfilament-based structures are parts of a mechanism which underlies the transition from transcriptional to post-transcriptional means of lymphokine production, leading eventually to down-regulation of lymphokine gene-transcription and manyfold stabilization of lymphokine mRNAs (58). Cytochalasins disorganize the actin bundles by reversibly binding to the barbed end of the filament, and possibly also sever preformed F-actin based structures. Disruption of the lymphocyte microfilament scaffold may result in unregulated early lymphokine mRNA stabilization and consequently, in abnormal lymphokine production by these cells. The relevance of this speculation may especially be emphasized in the case of hematopoietic tumor cells where aberrant stabilization of various cytokine mRNAs were observed without apparent sequence alterations in their 3`-UTR instability determinant elements (59). On the other hand, structural alterations in the organization of cytoskeletal matrix seem to be a general phenomenon apparent in transformed cells (60-61). Our results, showing that IL-2 mRNA became more stable upon cytochalasin-treatment -as compared to that of TNFα-, suggest different association of this two mRNA species with cytochalasin-sensitive structures and/or that their stability might involve different mechanisms at this stage of activation. The primary scope of this study was to test if lymphokine mRNA stability, thought to be mediated by ARE-AUBP interactions, involved any elements which were related to the actin-cytoskeleton. We found that cytochalasin-induced stabilization of various lymphokine mRNAs correlated with increased AUBP activities in the non-ionic detergent-resistant cytoplasmic subfractions and not in the detergent-soluble fractions of cells exposed to the microfilament disrupting agents

(Figure 2). Importantly, we detected these changes at<8 h of activation and not at later time points. Therefore, increased AUBP activities in these cytoplasmic fractions coincided with increased mRNA turnover.

Our immunoprecipitation experiments revealed a predominant 36-kDa complex in close association with actin or actin-containing structures in the detergent-resistant cytoplasmic fraction of activated lymphocytes. This finding indicates that AUBP activities co-localize with F-actin in lymphocytes (Figure 3). Moreover, this finding is novel in that it describes a molecular assembly in which a lymphokine 3`-UTR-ARE associates specifically with actin-containing structures through its *trans*-acting AUBP regulator(s). Two proteins with near identical molecular mass has been identified within this complex in activated T lymphocytes as being heteronuclear ribonucleo-protein A1 (hnRNP A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (24, 62). Additionally, GAPDH, along with additional members of the glycolytic cascade, is spatially localized to actin-based microfilaments in virtually all eukaryotic cells (63-65). Taken these observations together, it is tempting to speculate that certain locations of the actin-cytoskeleton may serve as regulatory switching points where multifunctional RNA-binding enzymes, such as GAPDH receive multiple metabolic signals to prompt the enzyme to perform one metabolic function over the other. Intriguingly, Bassell et al. demonstrated that visualization of single mRNAs by ultrastructural *in situ* hybridization reveals most of the poly $(A)^+$ mRNA at actin filament intersections in fibroblasts (66). As of importance, it has been shown that the substrate, glyceraldehyde-3-phosphate (GAP) dissociates GAPDH from actin (67). The *in vitro* competition results, in accordance with the *in vivo* UV-crosslinking data presented here, demonstrate that disruption of the actin-based component of the actin-AUBP-ARE assembly results in altered capability of some of these AUBPs to associate with the RNA (Figure 4 and 5) and may, therefore, influences the association of the mRNA itself with the structural scaffold. Nevertheless, these data strongly implicate cytoplasmic microfilaments in the regulation of ARE-AUBP interactions and consequently, in AUBP-mediated lymphokine mRNA turnover.

In conclusion, we demonstrated that disruption of the actin-based microfilament network in lymphocytes during early activation leads to stabilization of lymphokine mRNA species. This phenomenon is paralleled with increased AUBP activity in the Triton X-100-insoluble cytoplasmic subfraction of these cells. The association of these AUBPs with ARE is augmented as revealed in *in vitro* competition studies and by the combined *in vivo* UV-rosslinking - *in vitro* label transfer method. Increased AUBP activity within the dominant 36-kDa complex was apparent in immunoprecipitation experiments, indicating that cytochalasin-derived effects on lymphokine mRNA stability might, in part, be mediated by alterations of molecular associations between AUBPs and actin-based structures. Although, these results strongly suggest structural and functional connection between cytoplasmic microfilaments and lymphokine mRNA stability, further studies will be needed to uncover key aspects of the involved mechanisms.

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FIGURE LEGENDS

Figure 1. ***Effect of cytochalasin treatment on lymphokine mRNA stability.*** **A)** Northern blot analysis of total cellular RNA extracted from human PBMCs that had been activated with PHA for 8 h and exposed to the transcriptional inhibitor, Actinomycin D (5 μg/ml) and to the microfilament disorganizing agent, Cytochalasin B ("+" = 2.5 μM, "++" = 5.0 μM). 10 μg of total RNA was loaded in each lane. **B)** Densitometric analysis of the Northern blot shown in Figure 1A. Values were normalized for the loading control and expressed as percentage of the untreated control. **1:** untreated control, **2:** Act D (5 μg/ml), **3:** Act D and Cytochalasin B (2.5 μM), **4:** Act D and Cytochalasin B (5 μM).

Figure 2. ***Analysis of AUBP activities in cytoplasmic subfractions of cytochalasin-treated lymphocytes.*** 2.5 μg total proteins of Triton X-100-soluble (**A**) and insoluble (resistant)

(**B**) cytoplasmic fractions of PHA-activated (8 h) human PBMCs were incubated with ^{32}P-UTP-labeled AU-rich RNA probe (see in Materials and Methods) and UV-crosslinked. Following RNase treatment, samples were analyzed by 12.5% SDS-PAGE and autoradiography. **1:** untreated control, **2:** DMSO (vehicle control), 3h, **3:** 5 μM Cytochalasin D, 1h, **4:** 10 μM Cytochalasin D, 1h, **5:** 20 μM Cytochalasin D, 1h, **6:** 10 μM Cytochalasin D, 2h, **7:** 10 μM Cytochalasin D, 3h.

Figure 3. ***Immunoprecipitation of cytoplasmic fractions with anti-actin antibodies.***

A) Coomassie Blue-stained loading control of total Triton X-100-insoluble cytoplasmic fractions of activated human PBMCs. **1:** DMSO control, **2:** 10 μM Cytochalasin B-treatment for 2 h. One-fourth of the immunodepleted fractions after immunoprecipitation with Protein A-Sepharose immobilized anti-actin immuneserum (**3** and **4**), and immunoprecipitated fractions with anti-actin immuneserum or rabbit preimmuneserum (**5-6**

and **7-8**, respectively) were analyzed by 12.5% SDS-PAGE. **B)** Autoradiographic detection of immunoprecipitated ARE-AUBP complexes. Lanes are identical to those of Figuer 3A.

Figure 4. ***Analysis of the effect of cytochalasin treatment on AUBP binding affinity to AU-rich RNA in vitro.*** Triton X-100-insoluble cytoplasmic fractions (2.5 μg total protein) of activated and DMSO- (**left panel**) or Cytochalasin B-treated (10 μM, 2 h) (**right panel**) human PMBCs were analyzed in a competition label transfer experiment. Increasing molar excess of the unlabeled (cold) competitor is indicated on the top of each panel.

Figure 5. ***Effect of cytochalasins on the in vivo UV-crosslinkability of cytoplasmic AUBPs to ARE-containing RNA.*** **A)** 8 h PHA-activated, and DMSO (**left panel**) or Cytochalasin B-treated (10 μM, 2 h) (**right panel**) human PBMCs were exposed to monochromatic UV light (see in Materials and Methods) for 0 or 8 min. Triton X-100-insoluble cytoplasmic fractions were analyzed in a label transfer assay as described. **B)** Intensities of the dominant 36-kDa and a less apparent 50-kDa complex were quantitated by densitometry and relative values were expressed as the percentage of non-UV-treated samples. Duration of *in vivo* UV exposure is indicated below each panels.

FIG. 1.

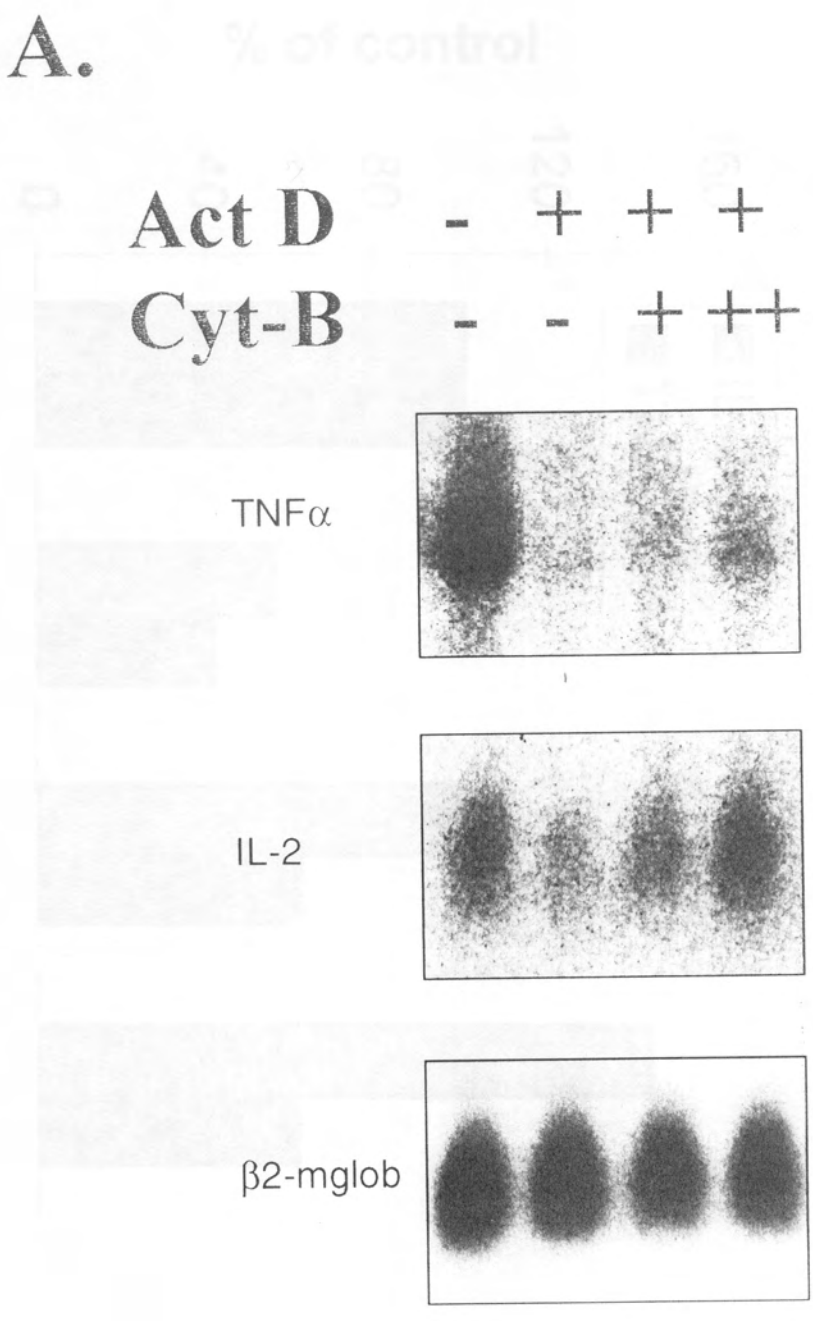
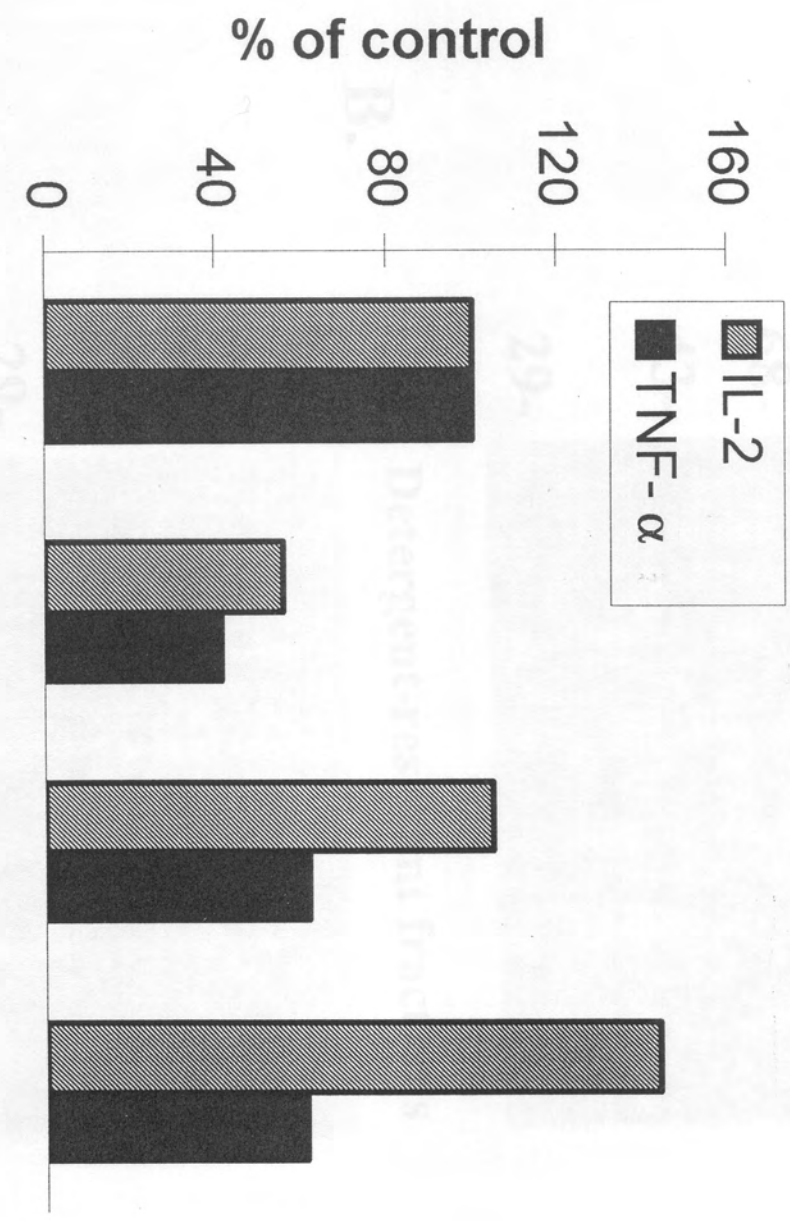


FIG. 1.



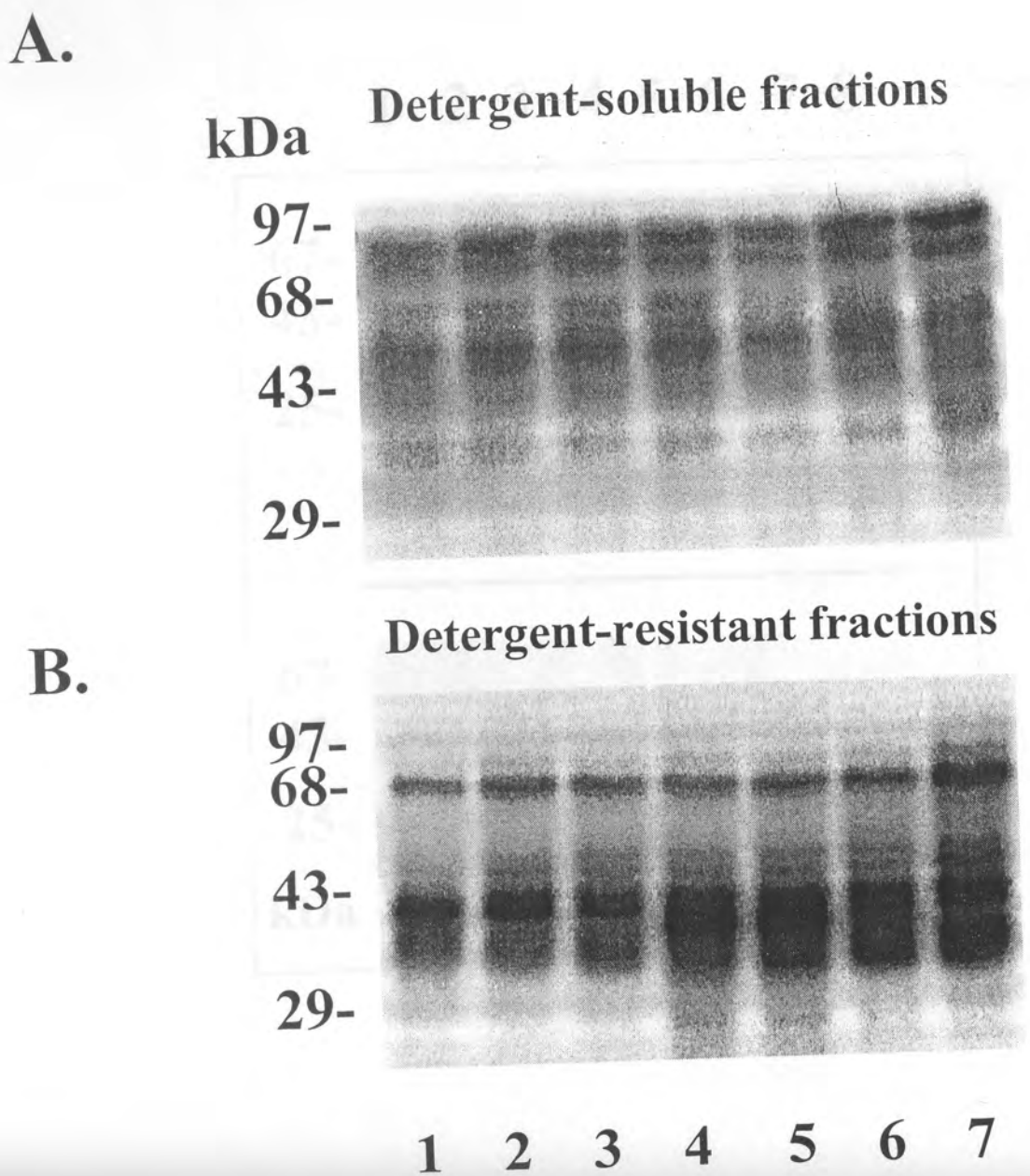

A.
Detergent-soluble fractions
kDa
97-
68-
43-
29-
B.
Detergent-resistant fractions
97-
68-
43-
29-
1 2 3 4 5 6 7

FIG. 2.

FIG. 3.

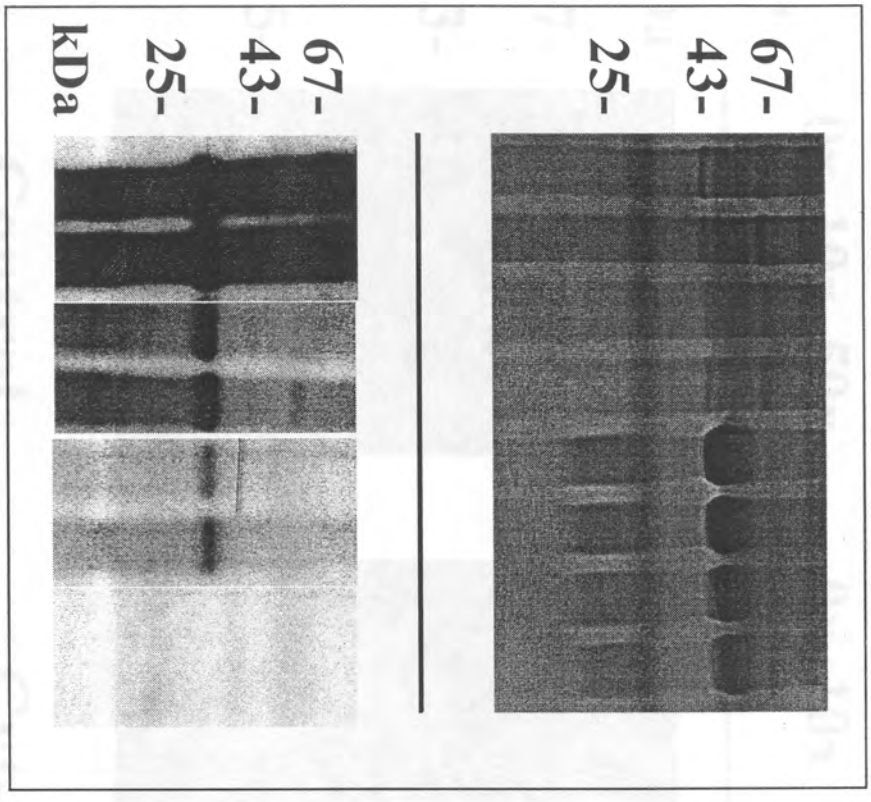
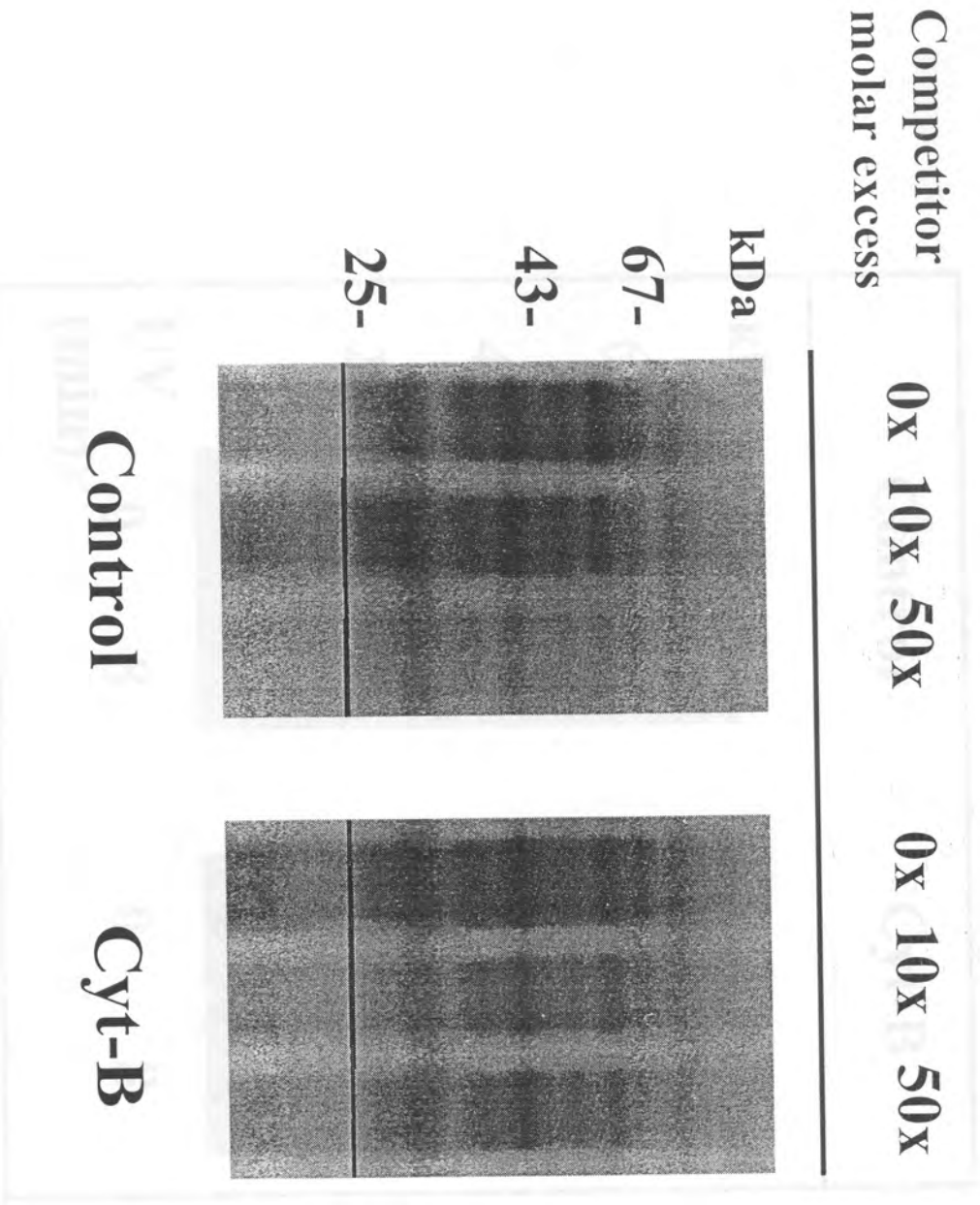


FIG. 4.


Competitor
molar excess
0x 10x 50x
0x 10x 50x
kDa
67-
43-
25-
Control
Cyt-B

A.

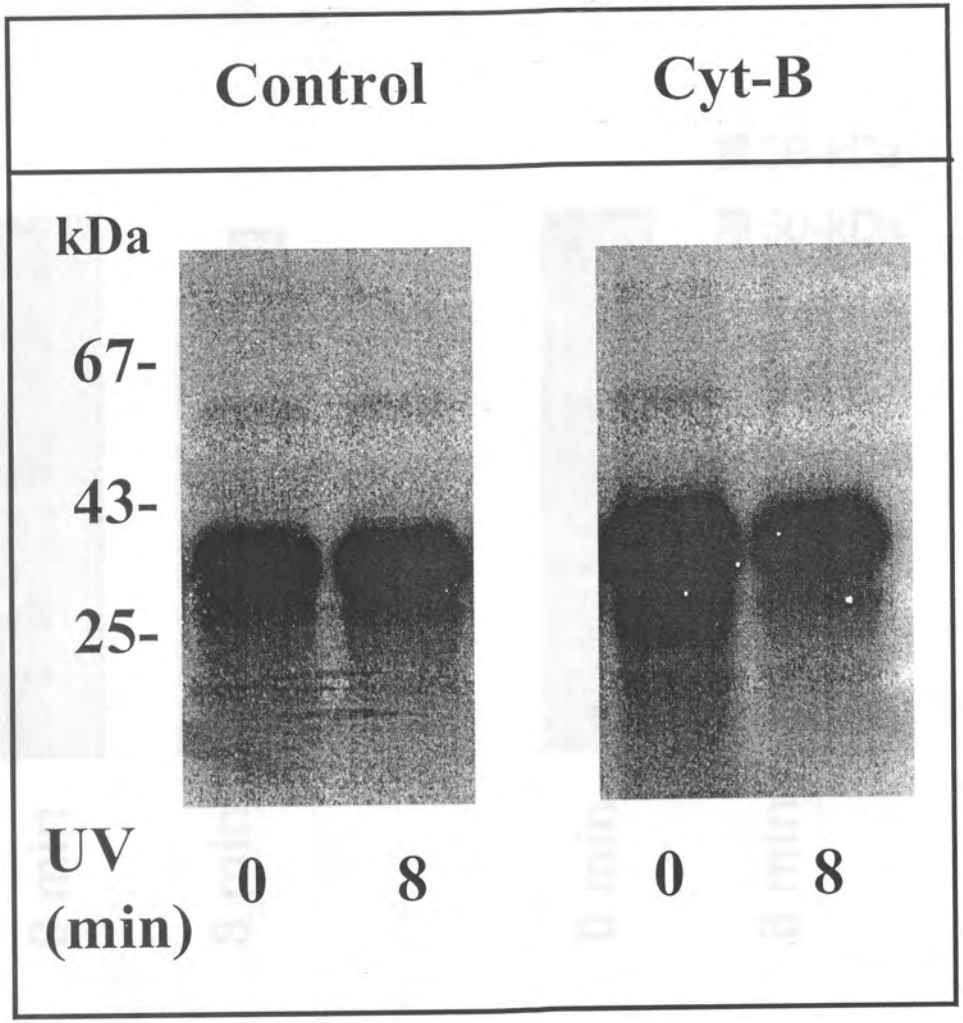
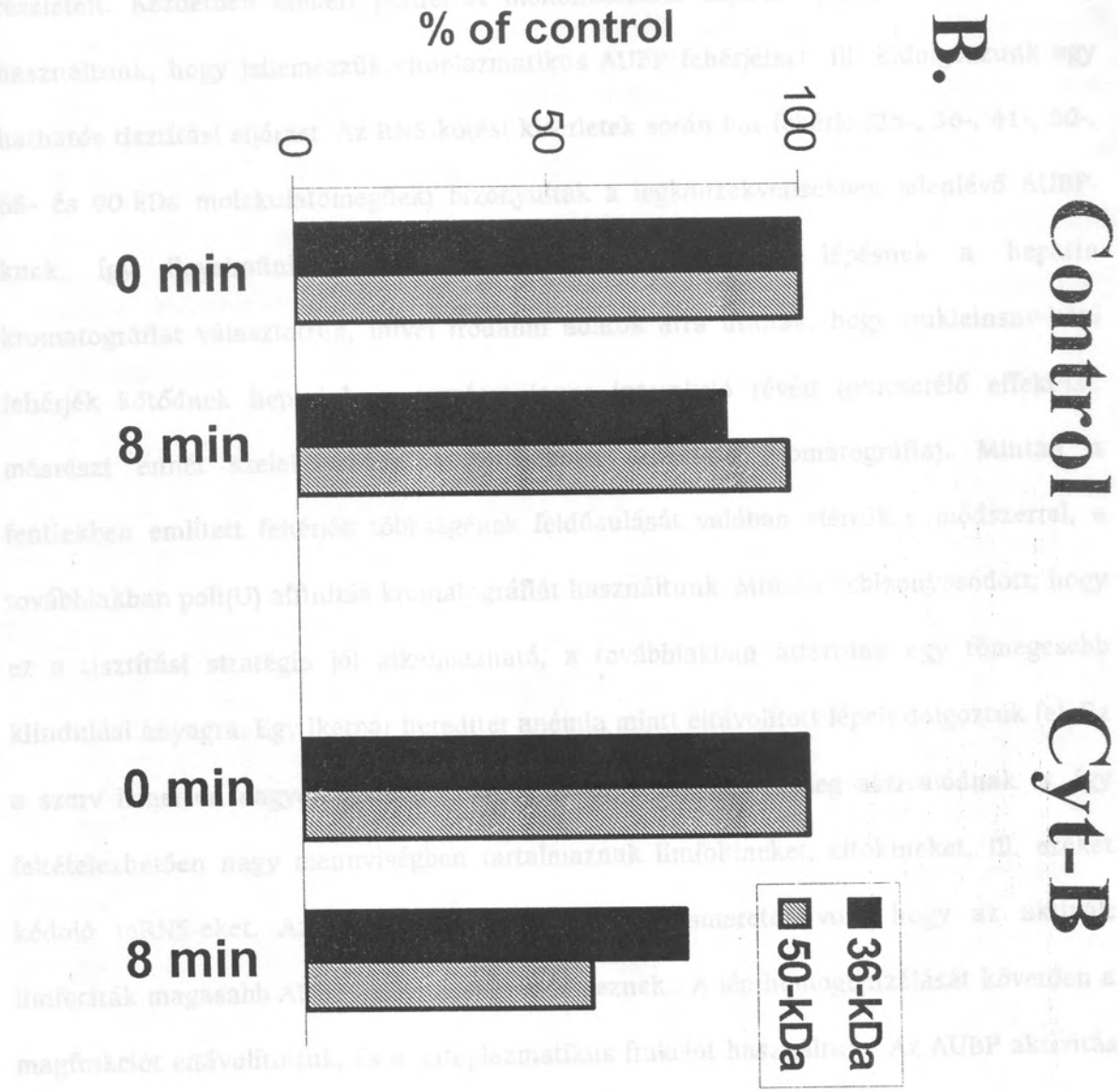


FIG. 5.

FIG. 5.

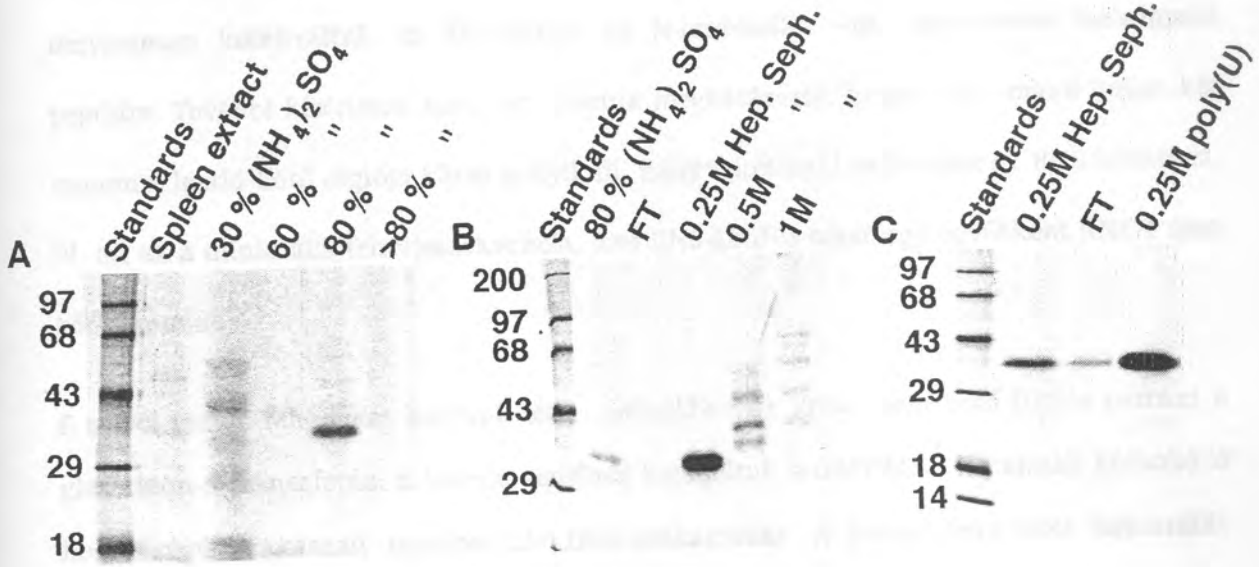

B.
Control
Cyt-B
% of control
0
50
100
0 min
8 min
0 min
8 min
36-kDa
50-kDa

B. Publikálatlan eredmények

I. AU-gazdag RNS-kötő fehérjék tisztítása.

Mivel a közleményekben nem szerepel, itt ismertetem az AUBP-k tisztítási lépéseinek részleteit. Kezdetben emberi perifériás mononukleáris sejteket (limfociták, monocita) használtunk, hogy jellemezzük citoplazmatikus AUBP fehérjéiket, ill. kidolgozzunk egy hathatós tisztítási eljárást. Az RNS-kötési kísérletek során hat fehérje (25-, 36-, 41-, 50-, 68- és 90-kDa molekulatömegűek) bizonyultak a legkonzekvensebben jelenlévő AUBP-knek, így figyelmünket ezekre fókuszáltuk. Előtisztítási lépésnek a heparin kromatográfiát választottuk, mivel irodalmi adatok arra utaltak, hogy nukleinsav-kötő fehérjék kötődnek heparinhoz, egyrészt ionos interakció révén (ioncserélő effektus), másrészt ennél szelektívebben és erősebben (affinitás kromatográfia). Miután a fentiekben említett fehérjék többségének feldúsulását valóban elértük e módszerrel, a továbbiakban poli(U) affinitás kromatográfiát használtunk. Miután bebizonyosodott, hogy ez a tisztítási stratégia jól alkalmazható, a továbbiakban áttértünk egy tömegesebb kiindulási anyagra. Egy ikerpár herediter anémia miatt eltávolított lépeit dolgoztuk fel. Ez a szerv ismerten nagyon gazdag T lifocitákban, amelyek némileg aktiválódnak is, így feltételezhetően nagy mennyiségben tartalmaznak limfokineket, citokineket, ill. ezeket kódoló mRNS-eket. Az már előző kísérleteinkből ismeretes volt, hogy az aktivált limfociták magasabb AUBP aktivitással rendelkeznek. A lép homogenizálását követően a magfrakciót eltávolítottuk, és a citoplazmatikus frakciót használtuk. Az AUBP aktivitás nyomonkövetésére radioaktívan jelzett AU-gazdag RNS (IFN-γ 3'UTR)-kötő képességet mértünk. Különböző telítettségű ammónium-szulfátos kicsapás során a 80% $(NH_4)_2SO_4$ frakcióban egy 36-kDa - már előző kísérleteinkben érdekessé vált - fehérje nagy fokú feldúsítását értük el (6. ábra A). A I. közleményben ismertetett eredményeink alapján megállapítottuk, hogy a 36-kDa-os AUBP aktivitásért legalább két fehérje felelős. A tripszin-szenzitiv komponenst azonosítottuk, mint heteronukleáris RNS-kötő fehérjeként

ismert hnRNPA1-gyet, a másik, tripszin rezisztens összetevő "kilétére" az AUBP-k tisztítását követően derült fény. Heparin Sepharose, majd poli(U) Sepharose-on végzett affinitás kromatográfia után a 36-kDa-os fehérjét homogenitásig tisztítottuk (7. ábra B és C). A poli(U) oszlopról 250 mM NaCl-dal eluált frakció fehérjéit nitrocellulóz membránra transzferáltuk, festéssel a fehérjéket láthatóvá tettük, és a 36-kDa molekulatömegűt kivágtuk. N-terminális aminosavszekvencia-analízis egy jól ismert glikolítikus enzimet, a gliceraldehid-3-foszfát dehidrogenázt azonosította, mint a 36-kDa AUBP-t. A GAPDH-ról ismert, hogy relatíve rezisztens tripszin emésztésre, ami megerősíti, hogy a limfociták citoplazmatikus lizátumában detektált 36-kDa-os aktivitás tripszin-rezisztens összetevője nagy valószínűséggel a GAPDH.

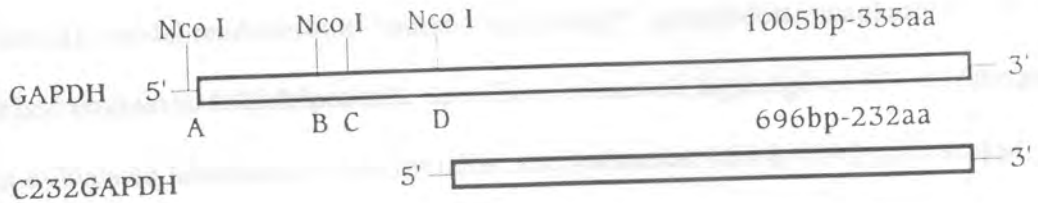


7. ábra AU-gazdag RNS-kötő fehérjék tisztításának lépései (A) Frakcionális ammónium szulfát kicsapás (B) Heparin Sepharose affinitás kromatográfia és (C) Poly(U) Sepharose affinitás kromatográfia során nyert frakciók ^{32}P-UTP jelölt IFN-γ 3'UTR RNS-kötő fehérjéi

II. A Rossmann hajlat, mint RNS-kötő domén vizsgálata: Delécios mutáns GAPDH fehérjék létrehozása, RNS-kötésük vizsgálata

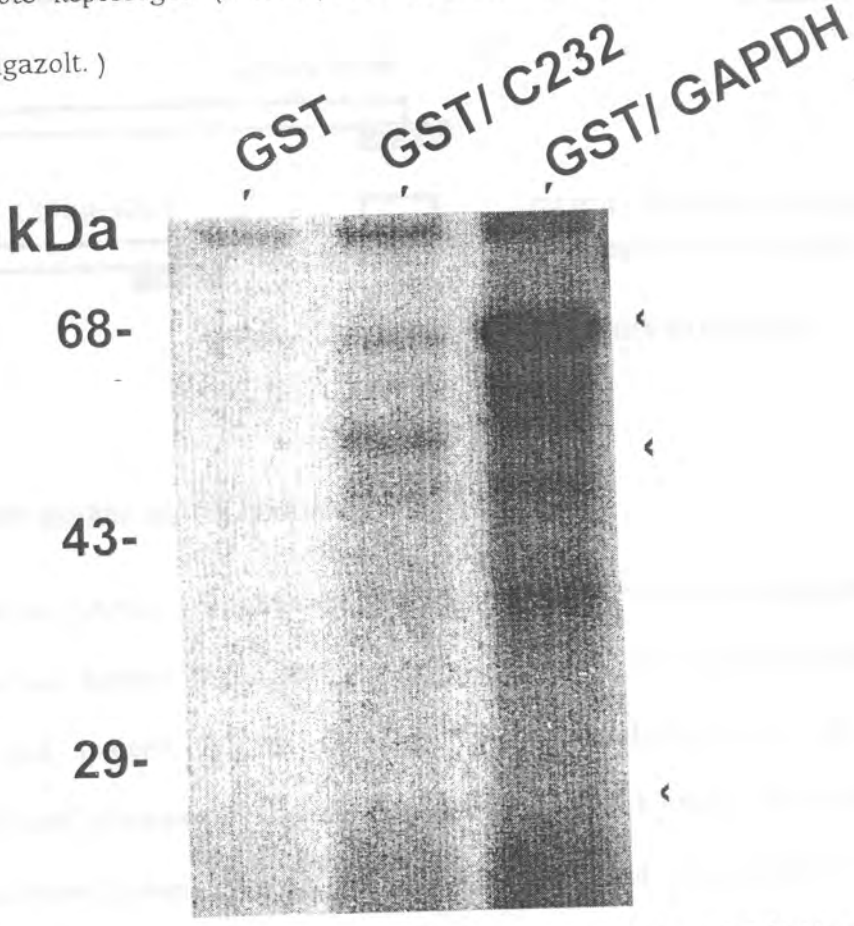
A nukleinsav-kötő domének felismerése, ezen belül az RNS-kötő régiók meghatározása nagyban elősegítette az RNS/fehérje interakció vizsgálatát, újabb RNS-kötő fehérjék azonosítását. A GAPDH esetében különösen indokolt az RBD-t (RNA-binding domain) meghatározni. Két indirekt és egy direkt kísérletes eredmény indokolja, hogy a Rossmann hajlatot, a NAD^+-kötő régiót gyanusítsuk. i., Több irodalmi adat is utal arra, hogy a NAD^+ kompetitíve gátolja a GAPDH nukleinsav kötését (78, 79, 82, IV. Közlemény). Ez azért nem fogadható el direkt bizonyítéknak, mert alloszterikus hatás sem zárható ki. ii., Számos metabolikus enzimről mutattak ki RNS-kötő aktivitást, a legtöbbjük dehidrogenáz, méghozzá NAD^+-függő. Szerkezetükben az egyedüli közös a NAD^+-kötő régió. iii., Az egyedüli direkt bizonyítékot a mi kísérleteink szolgáltatták, amelyben ténylegesen lokalizáltuk az RNS-kötést az N-terminális ~60 aminosavat tartalmazó peptidre. További kísérleteinkben azt akartuk meghatározni, hogy a Rossmann hajlat két mononukleotid-kötő régiója közül melyik ill. hány feltétlenül szükséges az RNS-kötéshez, ill. mi az a minimális fehérjeszekvencia, ami RNS-kötővé tehet egy egyébként RNS-t nem kötő proteint.

E célból fúziós fehérjéket hoztunk létre, melyekben az RNS-t nem kötő fúziós partner a glutathion-S-transzferáz. E fehérje génjéhez kapcsoltuk a GAPDH-ét, ill. annak különböző hosszúságú szakaszait reprezentáló DNS-szakaszokat. A hibrid fehérjéket bakteriális expressziós vektorok segítségével állítottuk elő, a Megközelítések és módszerek c. fejezetben részletezett módon. Elsőként a teljes hosszúságú GAPDH-t hordozó fúziós fehérjét (GST/GAPDH) állítottuk elő, restrikciós enzimmel plazmid DNS-ből kivágott GAPDH inzert segítségével. A GST/GAPDH hibridet kódoló plazmid további restrikciós enzimmel (Nco I) való csonkítása révén jutottunk a Rossmann hajlatot nem tartalmazó GAPDH-hoz, amely a C-terminális 2/3-nak felel meg (7. ábra).



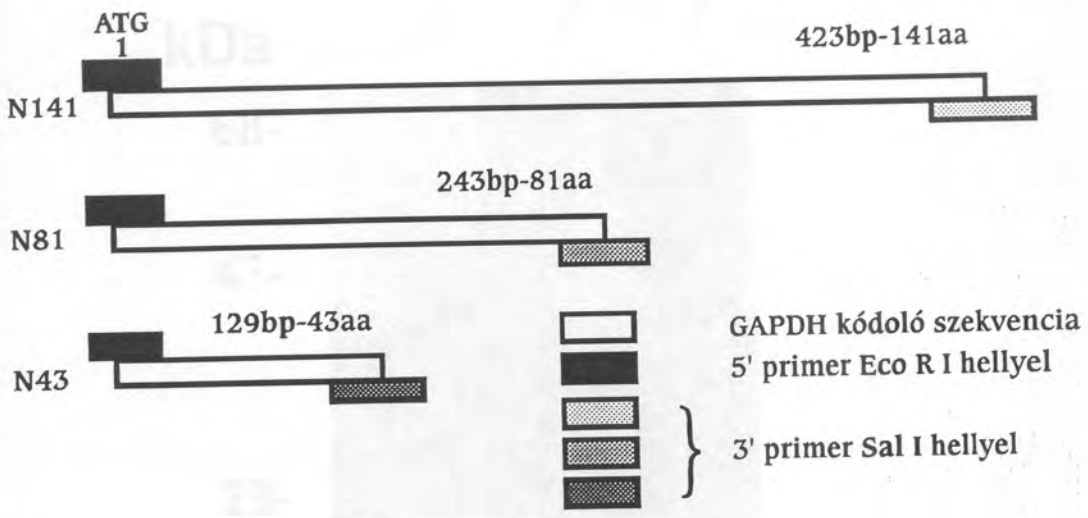
8. ábra Deléciós mutáns GAPDH fehérjék I.

A fehérjék glutation affinitás-kromatográfiával való tisztítását követően elvégeztük RNS-kötő vizsgálatukat. A 8. ábrán látható autoradiográfia arról tanúskodik, hogy az általunk előállított ~63-kDa-os GST/GAPDH a várt módon képes RNS-t kötni, míg a NAD^+-kötő régiójától megfosztott ~50-kDa molekulatömegű fehérje nem. A GST önmagában nem hordoz RNS-kötő képességet. (A fehérjék azonos mennyiségben való jelenléte a gélben CBB festéssel igazolt.)



8. ábra A GST fúziós GAPDH, és a Rossmann hajlatot nem tartalmazó deléciós mutáns RNS-kötése Az RNS-kötést label transzfer módszerrel, ^{32}P-UTP jelölt IFN-γ 3'UTR RNS próbával történt. A fúziós fehérjék festéssel meghatározott pozícióját nyilak mutatják.

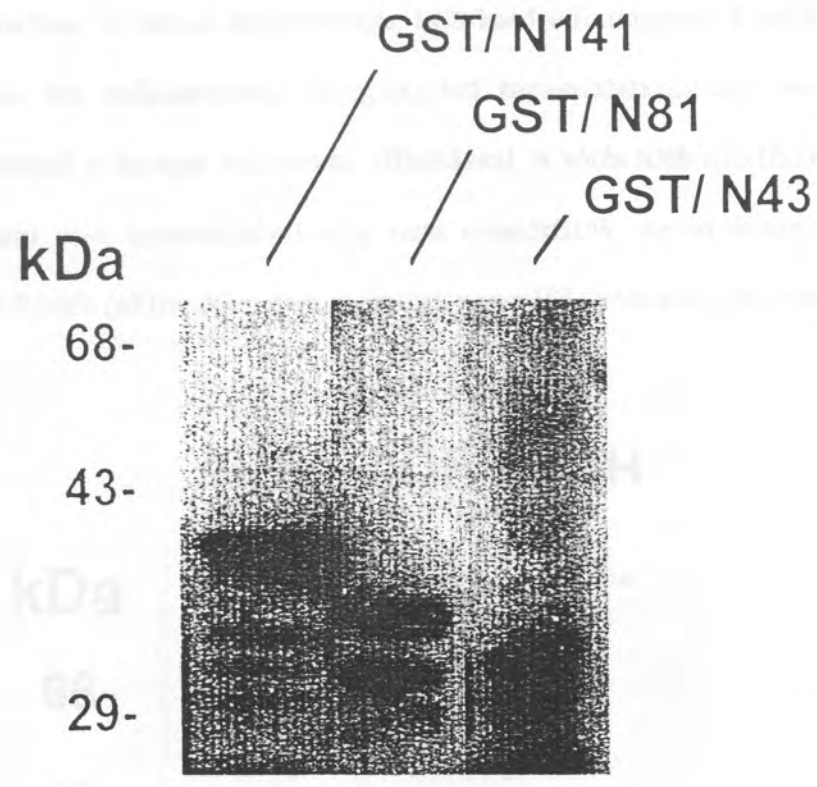
Miután modellrendszerünk "müködőképessége" igazolódott, megkezdtük az RNS-kötő régió pontosabb feltérképezését. Specifikus primer-ek segítségével PCR-rel felszaporítottuk a különböző hosszúságú N-terminális fehérjerészeket kódoló DNS-szakaszokat, és azokat szintén a GST génhez fúzionáltattuk. Így jutottunk az GAPDH N-terminális 141, 81, és 43 aminosavait hordozó hibridfehérjékhez (9. ábra). Az N141 a teljes koenzim-kötő régiót, tehát mindkét mononukleotid-kötő régiót hordozza. Az N43 csak az első βαβ hajlatot, vagyis βA-αB-βB és az αC felét, az N81 pedig az N43 aminosavain kívül az αC másik felét, és βC-t is (lásd 4. ábra).



9. ábra Deléciós mutáns GAPDH fehérjék II.

Mivel a csonkított GAPDH-t tartalmazó hibrid fehérjék térbeli alakja a baktériumot, amely termeli semmilyen korrekt foldingra nem emlékezteti, ezért heat shock proteinjei (HSP) "missfolded"-nak ismerik fel, és kirekesztik a sejtmetabolizmusból, zárványtestbe szekvesztrálódnak, ahonnan csak erős denaturánssal lehet kinyerni. E fehérjéket ezért SDS-PAGE mintapufferben oldottuk fel, szeparáltuk, majd nitrocellulóz membránra transzferáltuk, renaturáltuk, és Northwestern analízissel vizsgáltuk. Az, hogy ez egy járható út, és a GAPDH ill. a ~6-kDa-os RNS-kötő N-terminális peptid nem veszíti el RNS-

felismerő képességét, már előzőleg igazoltuk (IV. Közlemény). A deléciós mutáns hibridfehérjék Northwestern analízise azt mutatja (10. ábra), hogy még a legkisebb, 43 aminosavat hordozó peptid is jelölődik radioaktív RNS-sel, vagyis a kötés, vagy annak egy része biztosan az első mononukleotid régióra tehető, annak is a βαβ hajlat területére.

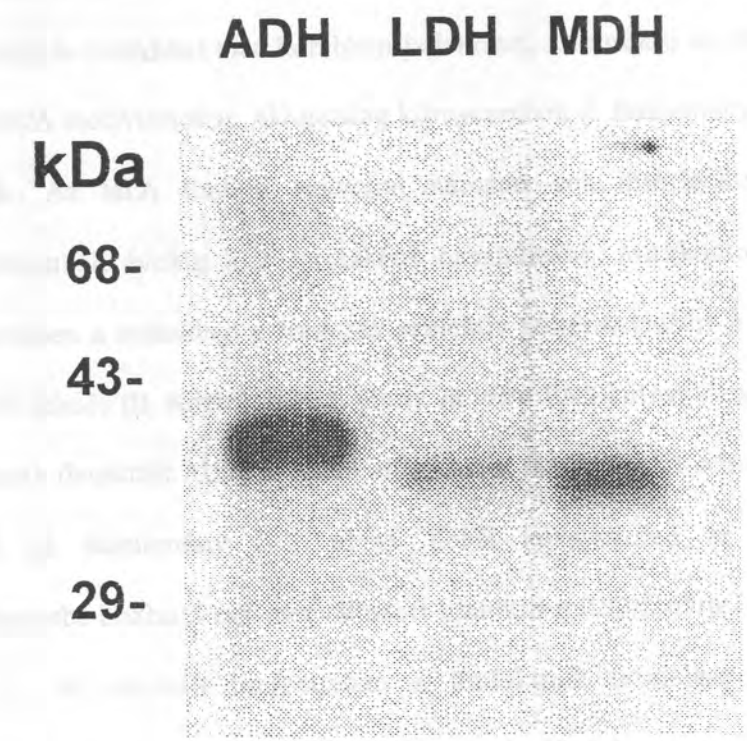


10. ábra Deléciós mutáns GAPDH fúziós fehérjék RNS-kötése Northwestern analízissel
Azonos mennyiségű fúziós fehérjét analizáltunk ^{32}P-UTP jelölt IFN-γ 3'UTR RNS-sel.

III. Egyéb NAD$^+$-függő dehidrogenázok AU-gazdag RNS-kötő aktivitásának vizsgálata

Miután számos dehidrogenázról kiderült, hogy *in vitro* RNS-kötésre képes, kíváncsiak voltunk azokra a dehidrogenázokra is, amelyekről még nem jelentettek RNS-kötő tulajdonságot. Az NAD$^+$-dependens izocitrát dehidrogenáz a GAPDH-hoz hasonlóan AU-

gazdag szekvenciákat ismer fel (68), ezért indokoltnak láttuk az AU-gazdag RNS próbáinkat használni az RNS-kötéshez. Gyári, tisztított ADH-t, LDH-t és MDH-t vizsgáltunk a molekulatömeg alapján számított azonos moláris mennyiségben, azonos pufferben való dialízist követően. A 11. ábrán látható autoradiogramról leolvasható, hogy mindhárom dehidrogenáz köt AU-gazdag RNS-t *in vitro*, de különböző erősséggel. Az ADH feltűnően erősebb affinitása nagyon konzekvens volt a háromszor megismételt kísérletsorozatban. A három dehidrogenáz RNS-kötő affinitásának korrekt elvégzését is tervbe vettük, bár előkísérleteink és gyakorlati tapasztalataink azt mutatják, hogy a radioaktív szignál erősségge arányos az affinitással. A kötés RNS-specificitását nem jelölt RNS-próbákkal való kompetícióval még nem ellenőriztük, de az bizonyos, hogy nem NAD^+-kötő fehérjék (aktin, BSA) nem mutattak semmiféle RNS-kötő aktivitást.



11. ábra NAD^+-függő dehidrogenázok AU-gazdag RNS-kötése. Azonos moláris mennyiségű (10 pmole) fehérjék (monomerre számítva) ^{32}P-UTP jelölt IFN-γ 3'UTR RNS-kötése

ÖSSZEFOGLALÁS

A dolgozatban bemutatott eredményeinket és a vázolt tudományterülethez való hozzájárulásunkat a kérdésfelvetésben követett logika szerint foglalom össze.

1. Szekvencia-specifikus RNS-kötő fehérjék azonosítása aktivált T limfocitákban, ezek résztvétele a limfokin termelés poszt-transzkripcionális szabályozásában.

Három különböző modellben folytattuk párhuzamosan vizsgálatainkat.

a., Az aktivált T sejt modellben a laboratóriumban végzett előkísérletek tanúsága szerint a limfokin termelés szabályozása a megfelelő gének transzkripciós aktiválásán túl mRNS féléletidő változáson keresztül történik (62). Olyan RNS-kötő fehérjéket azonosítottunk, melyek aktivitása megnő *in vitro* fitohemagglutininnel (PHA) stimulált limfocitákban (I. Közlemény, 1. ábra), és amelyek szekvencia-specifikus módon felismerik az instabilitás elemként már korábban jellemzett, a limfokin és citokin mRNS 3' UTR-ban lévő AUUUA motívumokat, AU-gazdag környezetben (I. Közlemény, 2. ábra).

b., Az MLA limfoid sejtvonal kórosan sok interleukin 2-t termel, ezért az immunológusok évekig IL-2 forrásként használtánk. Munkacsoportunk megállapította, hogy ezekben a sejtekben e limfokin szelektív túltermeléséért a kórosan megnövekedett féléletidő felelős (II. Közlemény, 2. ábra). *In vitro* RNS-kötési vizsgálatok tanúsága szerint az általunk detektált AUBP-k eltérő affinitással ismerik fel a retrovirálisan modifikált IL-2 3'UTR-t (II. Közlemény, 3-5. ábra). Ebből következően az RNS-stabilitás változás összefüggésbe hozható egy megváltozott limfokin mRNS/fehérje interakcióval.

c., Az aktivált limfocitában a limfokinek termelése különböző kezelésekkel (hormonok, egyéb immunsejtek által termelt limfokinek, stb.) módosítható (55-62). Munkacsoportunk igazolta, hogy a "natural killer" sejt stimuláló faktor (NKSF, IL-12), mely ismert módon az IFN-γ termelést stimulálja, szelektív módon megnöveli az IFN-γ mRNS stabilitását, míg az IL-2 mRNS-t nem érinti (III. Közlemény, 2-3. ábra). Az IFN-γ ill.

IL-2 mRNS 3' nem átíródó régióit, mint RNS-próbákat használva azonban nem tudtunk a Northern analízissel felfedett mRNS stabilitás-beli különbségnek megfelelő RNS-kötésbeli eltéréseket kimutatni (III. Közlemény, 5-6. ábra). Két magyarázat adódott. Az első szerint az mRNS molekulák egyéb részei közvetítenék a stabilitás szelektív befolyásolását. Miután azonban tejles IFN-γ ill. IL-2 mRNS molekulákat, mint próbákat használva sem tudtunk IFN-γ kódoló vagy 5' UTR-specifikus RNS-kötő fehérjéket detektálni (nem közölt adat), így a második feltételezésünket tartottuk valószínűnek, vagyis azt, hogy a sejtek feltárása, a fehérjék izolálása során kritikus *in vivo* molekuláris asszociációkat, és így információkat veszítünk, melyek nem rekonstruálhatók *in vitro*. E feltételezésünk a Bevezetőben ismertetett irodalmi hivatkozások alapján teljesen reálisnak is tűnik.

2. AU-gazdag RNS-kötő fehérjék jellemzése

a., Elsőként a szekvencia-specificitás kérdését elemeztük részletesen. A különböző limfokin, citokin és proto-onkogén mRNS-ek 3' nem átíródó szakaszait analizálva megállapítottuk, hogy az általunk detektált AUBP-k (25-, 36-, 43-, 50-, 70- és 90-kDa) némi affinitásbeli különbséggel ugyan, de felismerik az összes általunk használt ARE RNS-t, nem AU-gazdag szekvenciákat viszont nem, vagy nagyon alacsony affinitással (I. Közlemény, 1-2. ábra).

b., Az RNS-metabolizmust drámaian befolyásoló RNS polimeráz-gátlók ismerten megnövelik citoplazmatikus RNS-kötő fehérjék, RNP-k mennyiségét ill. affinitását, sőt bizonyos mRNS-ek stabilitást is befolyásolják (117). Ezért érdekesnek tűnt ezt a mi esetünkben is megvizsgálni, hogy megerősítsük: *bona fide* RNS-kötő fehérjékkel van dolgunk. Különösen egy 36-kDa molekulatömegű AUBP mutatott Actinomycin D ill. DRB-függő aktivitás növekedést (I. Közlemény, 3. ábra).

c., Részleges tripszin-emésztéssel megállapítottuk, hogy a 36-kDa-os AUBP aktivitás legalább két fehérjétől származik. Egy tripszin-rezisztens összetevőből, és egy

~25-kDa (és valószínűleg egy, a gélen nem detektálható ~11-kDa) molekulatömegű fragmentet eredményező tripszinre érzékenyből (I. Közlemény, 6. ábra, felső panel jobb oldala).

d., Az RNS-kötő fehérjék irodalmát tanulmányozva szereztünk tudomást a 36-kDa molekulatömegű heteronukleáris RNS-kötő A1 fehérje (hnRNPA1) citoplazmatikus jelenlétéről (118, 119). A hnRNPA1 nukleocitoplazmatikus mozgását RNS polimeráz-gátlók segítségével ismerték fel immunhisztokémiai és sejtfrakcionálási módszerekkel. Actinomycin D ill. DRB e fehérje citoplazmában való akkumulációját okozza. Részleges tripszin emésztése egy 25-kDa, UP-1-nek nevezett fehérjét eredményez. Mindezek alapján gyanunk támadt, hogy a 36-kDa-os AUBP tripszin-szenzitiv komponense a hnRNPA1-gyel lehet azonos. Továbbí biokémiai és immunológiai vizsgálatok megerősítették a feltételezésünket, sőt a 41-43-kDa molekulatömegű AUBP-t is identifikálni tudtuk, mint hnRNPC1 fehérjét (I. Közlemény, 4-6. ábra). Ez utóbbiról ismeretes volt, hogy poli(U) homopolimerhez nagy affinitással kötődik (120). Későbbi irodalmi adatok azt is megerősítették, hogy a hnRNPA1 a pre-mRNS-eket kikíséri a magból egészen a riboszómákig (121).

e., Az AU-gazdag RNS-kötő fehérjék jobb megismerése, funkcionális jellemzése érdekében humán limfocitákból ill. lépből AUBP-eket tisztítottunk, melynek végeredményeképpen egy nagy tisztaságú frakcióban egy 36-kDa molekulatömegű fehérjét izoláltunk (6. ábra). Aminosavszekvencia-analízis alapján azonosság merült fel a glikolítikus enzimként ismert gliceraldehid-3-foszfát dehidrogenázzal.

<u>3. A gliceraldehid-3-foszfát dehidrogenáz RNS-kötésének jellemzése.</u>

a., Meglepő eredményünket megerősítendő, kiegészítő vizsgálatokban igazoltuk, hogy a 36-kDa-os, lépből tisztított AUBP-t specifikus anti-GAPDH monoklonális ellenanyag Western-blot analizísben felismeri, oldatban immunoprecipitálja, valamint

tisztított "gyári" GAPDH (Sigma, nyúl vázizomból) is rendelkezik AU-gazdag RNS-kötő képességgel (IV. Közlemény, 1. ábra).

b., További kísérletekben demonstráltuk, hogy az általunk tesztelt összes AUUUA-tartalmú nem jelölt RNS próba (IFN-γ, c-myc, GM-CSF és IL-2 3'UTR) effektíven kompetált a radioaktívan jelzett IFN-γ 3'UTR RNS-sel, míg a nem specifikus, AUUUA-t nem tartalmazó, nem AU-gazdag próba nem mutatott kompetíciót. Hasonló kísérletekben igazoltuk, hogy poli(U) hathatósan megakadályozza a GAPDH/IFN-γ 3'UTR interakciót, míg poli(C) RNS nem mutat ilyen hatást, poli(A) pedig intermedier módon kompetál, tovább erősítve az AUUUA-specificitást (IV. Közlemény, 2-3. ábra). A korábban ismertetett tRNS-kötő képességgel (82) összehasonlítva az ARE-hez való affinitás erősebbnek mutatkozott (IV. Közlemény, 4. ábra).

c., További kísérleteinkben azt a hipotézisünket teszteltük, hogy a NAD^+-kötő régió, a Rossmann hajlat RNS-kötő doménként is funkcionálhat, mégpedig koenzim-függő módon. Ezen elképzelés alapján a glikolítikus funkció és az RNS-kötő képesség egymást egy időben kizáró módon, reciprok szabályozás révén valósulna meg. Ezt a mechanizmust feltételezve a Rossmann hajlatban specifikusan kötődő molekulák kompetitíve gátolják a GAPDH/AU-gazdag RNS interakciót. Eredményeink azt mutatják, hogy NAD^+, NADH és ATP koncentráció-függő módon, a μM-os tartományban gátolni képes a GAPDH/IFN-γ 3'UTR interakciót (IV. Közlemény, 6. ábra). A fél-maximális kompetíciót eredményező koncentrációk nagyon közel vannak az e anyagokra mért K_m értékekhez, még a NAD^+ és NADH közötti affinitás-különbséget is tükrözik kompetíciós kísérleteink. Negativ kontrollként az UTP használtuk, amelynek affinitása a Rossmann hajlathoz kb. 20-szor alacsonyabb, mint az ATP-é. Ennek megfelelően kompetíciós modellünkben az UTP-re ~1mM, az ATP-re ~50 μM K_m értéket mértünk.

Munkahipotézisünk helyességének kezdeti megerősítését követően direktebb módszertani megközelítéssel folytattuk vizsgálatainkat. Specifikus endoproteáz-kezeléssel peptid fragmentekre hasítottuk a GAPDH-t majd ezt követően végeztük el az AU-gazdag RNS-kötés detektálását. Ennek eredményeként megállapítottuk, hogy hosszantartó, magas enzim koncentrációval végzett emésztés után egy nagyon rezisztens, SDS-gélen kb. 6-kDa molekulatömegűnek mutatkozó peptidre lokalizálható a GAPDH RNS-kötő helye (IV. Közlemény, 7. ábra). E peptid mérete és az aminosavszekvencián alapuló fragment-térkép alapján ez a régió a GAPDH N-terminális végén lévő 6.8-kDa számított molekulatömegű peptidnek felel meg, mely magába foglalja a Rossmann hajlat egy részét. Ezt követően elvégeztük ezen RNS-kötő peptid aminosavszekvencia-analízisét, mely egyértelműen megerősítette ezt a következtetésünket (IV. Közlemény, 8. ábra). A dolgozatban ismertetett deléciós mutáns GAPDH fúziós fehérjék megerősítik, hogy a Rossmann hajlat első mononukleotid-kötő régiója elégséges az RNS-kötéshez, és mint egy RNS-kötő domén viselkedik: képes RNS-kötő képességet átvinni nem RNS-kötő fehérjére.

d., A GAPDH más nukleinsav-kötő fehérjékhez hasonlóan érzékenyen reagál redox státuszának változtatására, -SH csoportjainak oxidálására. A reverzibilis oxidálószer diamid hatására az RNS-kötés drámaian lecsökkent, 2-merkaptoetanol hozzáadásával az RNS-kötő aktivitás visszahozható volt (IV. Közlemény, 5. ábra)

e., *In vitro* kísérleteink után a GAPDH, mint RNS-kötő fehérje - kérdéskör funkcionális vizsgálatát kezdtük meg, vagyis arra kerestünk bizonyítékot, hogy a GAPDH-hoz e szerep fiziológiásan, *in vivo* is hozzárendelhető-e. Ennek megválaszolását az RNS-metabolizmus befolyásolását követő, GAPDH-t érintő változások detektálásával kezdtük. Az irodalomból jól ismert, hogy a nukleáris RNS-szintézis megbénításával (Actinomycin D, DRB) a citoplazmában az RNS metabolizmussal kapcsolatban álló molekulák, RNS-kötő fehérjék asszociációja megváltozik. Az RNS-metabolizmus - ezen belül is az RNS-stabilitás és transzlálhatóság - citoplazmatikus szabályozásának legfontosabb lépései a

poliszómákra lokalizálható. Ezen okokból követtük a GAPDH mennyiségi változásait humán limfociták poliszóma frakcióiban RNS-szintézis gátlás hiányában és azt követően. Western blot analízis segítségével megállapítottuk, hogy RNS-szintézis gátlás eredményeként a poliszómákra lokalizált GAPDH mennyisége megnő (IV. Közlemény, 9. ábra). Ami ennél is érdekesebb, egy addig csak nagyon alacsony koncentrációban jelenlévő, eltérő elektroforetikus mobilitású GAPDH izoform szaporodik fel szignifikánsan.

E kísérleti eredmények nem csak az *in vitro* RNS-kötés NAD^+-függése alapján feltételezett fiziológiás szerepet támogatják, hanem kiegészítik e komplex szabályozást a kompartmentalizáció felvetésével is. E kibővített modellben a GAPDH a citoplazmában, ahol a NAD^+ és NADH koncentrációja magas főleg glikolítikus funkciót látna el, míg a poliszómákon, ahol alacsony NAD^+, NADH, és magas AU-gazdag RNS-koncentráció feltételezhető, RNS-kötő fehérjeként lenne involválva az RNS-metabolizmus szabályozásában. Ehhez sokban hasonlatos a már említett IRE-BP esete, mely a citoplazmában RNS-kötő fehérje, *alter ego*-ja, az akonitáz pedig a mitokondriális kompartmentben citrát-köri enzim (64). E fehérje poliszómákon igazolt jelenlétét, valamint az RNS-polimerizáció gátlására bekövetkező poliszómális GAPDH mennyiségi változását indirekt bizonyítéknak tekintjük, hogy a GAPDH/RNS interakció valóban létezik *in vivo*.

Egy új módszer kidolgozásával *in vivo* képződött RNS/fehérje, azon belül is ARE/AUBP interakciókat vizsgáltunk (V. Közlemény). Az élő sejtek UV-fénnyel való besugárzásával az RNS/fehérje komplexeket kovalensen fixáljuk, így azok nem esnek szét a sejtek frakcionálása során, és ezt követően végezzük el az *in vitro* RNS-kötést radioaktív AU-gazdag próbákkal. E metódussal sikerült különbséget kimutatnunk normál és malignusan transzformált limfociták citoplazmatikus AUBP/ARE-komplex képződésében. A módszert

egy másik munkacsoport is átvette és sikerrel alkalmazta nem ARE-specifikus RNS-kötő fehérjék *in vivo* asszociációjának vizsgálatára (122). A módszer révén lehetőségünk nyílik a GAPDH *in vivo* RNS-partnereinek kimutatására.

Legújabb kísérleteinkben kimutattuk, hogy az aktivált limfociták citokalazin kezelése a limfokin mRNS-ek megváltozott stabilitását eredményezi, vagyis az aktin citoszkeleton átrendeződése befolyásolja ezen mRNS szubpopuláció metabolizmusát. A molekuláris hatásmechanizmus felderítésének kezdeti lépéseiben AUBP fehérjék aktinhoz való asszociációját mutattuk ki. A citoplazmatikus frakciójában detektált 36-kDa AUBP aktin ellenesen antitesttel az aktinnal együtt immunprecipitálható, mennyisége ill. aktivitása citokalazin kezelésre befolyásolható (VI. Kézirat).

Egyre több közlemény támogatja azt az elképzelést, hogy az RNS metabolizmus részfolyamatai - a magból a citoplazmába jutás, a féléletidő és a transzláció szabályozása - szorosan kapcsoltak. Erre támaszkodva tehát feltételezhető olyan RNS-kötő fehérjék létezése, melyek mindhárom esemény során az RNS-hez kötve maradnak, és protein-protein, protein-RNS interakciók révén közvetítik regulatórikus szerepüket az általunk felismert RNS-ek metabolizmusában (RNS-chaperonok).

Eredményeink alapján az általunk felismert GAPDH-RNS interakciónak igen fontos szerepe lehet az eukarióta sejtek aktivációjában, külső stimulusra adott válaszaiban a rapidan indukálható fehérjék expressziójának szabályozása révén. Ez egyben arra is utal, hogy nagy mennyiségben jelenlévő, eddig enzimekként ismert és emlegetett fehérjék fontos szerepet tölthetnek be az RNS-metabolizmus szabályozásában. E multifunkcionális molekulák valószínűleg egy igen komplex, összehangolt reguláció központi szabályozói.

FOLYAMATBAN LÉVŐ MUNKA, TÁVLATI KÍSÉRLETI TERVEK

Most van folyamatban a fúziós fehérjék termelésének optimizálása (induktor koncentrációja, hőmérséklet, indukció ideje, oxigenáltság), hogy minél több fehérje elkerülje a zárványtestbe kerülést, ill. az odakerülők urea-szolubilizálás utáni renaturációjával elegendő mennyiségű rekombináns fehérjéhez jussunk az RNS-kötés oldatban, a fehérje natív állapotában való elvégezhetőségéhez. Ugyanis az RNS-kötő minimális peptidszekvencia RNS szekvencia-specificitását, a GAPDH RNS-felismeréshez szükséges egyéb részeinek meghatározását is szeretnénk elvégezni. A rekombináns fúziós expressziós vektorok azt is lehetővé teszik, hogy "site-directed" mutagenezis segítségével meghatározhassuk az RNS-sel létrejövő kontaktusban kritikus aminosavakat.

A fentiekben bemutatott fúziós fehérjék, mint antigének segítségével monoklonális antitestek gyártását is megkezdtük, melyek elengedhetetlenek további funkcionális és *in vivo* vizsgálatokhoz. A minimális RNS-felismerő, N-terminális 43 aminosav ellenes antitestek közül leginkább az RNS-kötést megakadályozókat (neutralizálókat) szeretnénk használni és hatásukat *in vitro* mRNS lebomlási modellben (*in vitro* mRNA decay system) és *in vitro* transzlációs rendszerben (nyúl retikulocita lizátum) vizsgálni. Az *in vitro* meghatározott RNS-ligand alapján az AUUUA pentamereket, AU-gazdag szekvenciákat hordozó, gyors turnover-ű növekedési faktor, limfokin, citokin mRNS-ek stabilitásának és/vagy transzlálhatóságának befolyásolása valószínűsíthető. Mivel a GAPDH jelenléte mindkét rendszerben bizonyított, amennyiben az RNS-kötést neutralizáló anti-GAPDH antitest ill. NAD^+ hozzáadása változást okoz AU-gazdag szekvenciákat hordozó mRNS-ek (IFN-γ, IL-2) *in vitro* stabilitásában ill. transzlálhatóságában, és ez a változás GAPDH hozzáadásával visszafordítható, úgy kritikus információkhoz jutunk a fiziológiás szerepet illetően.

Jelenleg a GAPDH NAD^+-mentesítését, autofoszforilációját ill. foszfatáz kezelését követő izoform megoszlás analízisét végezzük két-dimenziós elektroforézis technikával. Ezt követően megvizsgáljuk a különböző izoformok RNS-kötését, hogy meghatározhassuk, miben rejlik az RNS-kötő képesség-beli különbség. Olyan antitest termelését is megkíséreljük, mely specifikusan felismeri az RNS-kötő formát, mint antigént, de a többit nem. Ily módon olyan értékes immunológiai reagenshez jutunk, mely hisztológiai módszerekkel kombinálva lokalizálni képes a GAPDH RNS-kötő szubpopulációját a sejten belül.

A kompartmentalizáció kérdését szintén anti-GAPDH antitestekkel kívánjuk vizsgálni immunfluoreszcenciás ill. immunogold ELMI technikák felhasználásával. A sejtmetabolizmus különböző befolyásolása (glikolízis bénítás, RNS-transzkripció gátlás, fehérjszintézis-gátlás) hatására bekövetkező GAPDH szubcelluláris megoszlási változását szeretnénk közelebbről megvizsgálni. Figyelmünket a nukleocitoplazmatikus megoszlásra ill. a poliszómális lokalizáció köré összpontosítjuk.

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Act D	Actynomycin D
ALA-	δ-aminolevulinsav-
ARE	adenin-uracil - rich element
AU-	adenin-uracil -
AUBP	adenin-uracil - binding protein
BSA	bovine serum albumin
CAPS	3-[Cyclohexylamino]-1-propane sulfonic acid
CBB	Coomassie Brillant Blue
DRB	5,6-dichloro-1-b-ribouranosylbenzimidazole
DTT	dithiothreitol
ELMI	elektronmikroszkópia
FBS	fetal bovine serum
GAPDH	gliceraldehid-3-foszfát dehidrogenáz
GDH	glutamát dehidrogenáz
GM-CSF	granulocyta-macrophage colony stimulating factor
GST	gluthation-S-transferáz
hnRNP	heterogenous nuclear ribonucleoprotein
IL-2	interleukin 2
IFN-γ	gamma interferon
IRE-BP	iron-response element binding protein
LDH	laktát dehidrogenáz
MDH	malát dehidrogenáz
mRNS	messenger ribonukleinsav
NAD^+	nicotinamid dinukleotid, oxidált
NADH	nicotinamid dinukleotid, redukált
NTP	nukleotid trifoszfát
PHA	phytohaemagglutinin
PMSF	phenylmethylsulfonyl fluoride
PBMC	peripheral blood mononuclear cell
RBD	RNA-binding domain
SDS-PAGE	sodium dodecyl sulphate poliacrylamide gel electrophoresis
ssDNS	single strand dezoxiribonukleinsav, egyes szálú DNS
TBS	Tris-buffered saline
TfR	transzferrin receptor
UDG	uracil-DNS glikoziláz
UTP	uridin trifoszfát
3'UTR	3' untranslated region, 3' nem átíródó régió

PUBLIKÁCIÓS LISTA

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KÖSZÖNETNYILVÁNÍTÁS

Legelsősorban szüleimnek köszönöm, hogy humán érdeklődésem ellenére gyengéd erőszakkal a tudományos pálya felé irányítottak, és az egyetemi évek során is tudományos diákköri munkára biztattak.

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A POTE Klinikai Kémiai Intézete összes dolgozóját köszönet illeti, amiért mindenben és mindenkor készségesen segítségemre voltak. Külön hálás vagyok Dr. Eckert Máriának és Pappné Bácskai Saroltának, akik a kísérletekben közreműködtek. Dr. Ludány Andrea, Dr. Bogner Péter hasznos tanácsai, Pappné Bácskai Sarolta, Fábián Jánosné és Dr. Borbély Csaba nagyszerű és sokrétű segítsége nagyban hozzájárult a PhD disszertáció elkészítéséhez.

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