

Eukaryotic Initiation Factor 5A Is a Cellular Target of the Human Immunodeficiency Virus Type 1 Rev Activation Domain Mediating *Trans*-Activation

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Abstract. Expression of human immunodeficiency virus type 1 (HIV-1) structural proteins requires the presence of the viral *trans*-activator protein Rev. Rev is localized in the nucleus and binds specifically to the Rev response element (RRE) sequence in viral RNA. Furthermore, the interaction of the Rev activation domain with a cellular cofactor is essential for Rev function in vivo. Using cross-linking experiments and Biospecific Interaction Analysis (BIA) we identify eukaryotic initiation factor 5A (eIF-5A) as a cellular factor binding specifically to the HIV-1 Rev activation

domain. Indirect immunofluorescence studies demonstrate that a significant fraction of eIF-5A localizes to the nucleus. We also provide evidence that Rev *trans*-activation is functionally mediated by eIF-5A in *Xenopus* oocytes. Furthermore, we are able to block Rev function in mammalian cells by antisense inhibition of eIF-5A gene expression. Thus, regulation of HIV-1 gene expression by Rev involves the targeting of RRE-containing RNA to components of the cellular translation initiation complex.

HUMAN immunodeficiency virus type 1 (HIV-1)¹ encodes a *trans*-acting regulator of viral gene expression, called Rev, which is essential for virus replication (Feinberg et al., 1986; Sodroski et al., 1986; Sadaie et al., 1988; Terwilliger et al., 1988). It was shown that Rev function is required for the cytoplasmic expression of unspliced and singly spliced viral mRNAs encoding the viral structural proteins (Emerman et al., 1989; Hammarskjöld et al., 1989). Various modes of Rev action have been suggested including effects on the transport, stability, splicing, and translation of viral mRNA species (Chang and Sharp, 1989; Felber et al., 1989; Malim et al., 1989b; Arrigo and Chen, 1991; Kjems et al., 1991b; Lawrence et al., 1991; D'Agostino et al., 1992).

Previous studies demonstrated that Rev is a nuclear phosphoprotein which appears to accumulate in the nucleoli of expressing cells (Cullen et al., 1988; Hauber et al., 1988;

Cochrane et al., 1989; Malim et al., 1989a; Cochrane et al., 1990b). Rev *trans*-activates through a highly structured *cis*-acting RNA sequence, the Rev response element (RRE), which is encoded by sequences residing in the *env* gene (Rosen et al., 1988; Hadzopoulou-Cladaras et al., 1989; Malim et al., 1989b). In vitro studies demonstrated that the rev gene product binds directly and specifically to its RRE RNA target sequence (Daly et al., 1989; Zapp and Green, 1989; Cochrane et al., 1990a; Daefler et al., 1990; Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990b). Multiple Rev interaction sites within the RRE have been reported (Kjems et al., 1991a). However, a substructure of the RRE, termed Stem-Loop IIB, has been mapped as the high-affinity Rev binding site (Bartel et al., 1991; Cook et al., 1991; Heaphy et al., 1991; Kjems et al., 1992; Tiley et al., 1992). Recently, evidence was provided that multimerization of Rev monomers on the RRE is required for biological activity (Malim and Cullen, 1991). In addition, Rev multimerization before RRE RNA binding has also been reported (Olsen et al., 1990a; Zapp et al., 1991).

Mutational analysis of the *rev* gene revealed a modular structure similar to transcriptional activator proteins (Ptashne, 1988). A basic domain rich in arginine residues, which maps to amino acids (aa) 33 to 46 in the 116 aa Rev protein, serves as a nuclear localization signal and is required for the sequence specific binding of viral RRE RNA

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1. *Abbreviations used in this paper:* BIA, biospecific interaction analysis; CNBr, cyanogen bromide; HIV-1, human immunodeficiency virus type 1; RRE, Rev response element.

(Malim et al., 1989a; Hope et al., 1990; Olsen et al., 1990a; Böhnlein et al., 1991; Kjems et al., 1991b, 1992; Malim and Cullen, 1991; Zapp et al., 1991). Amino acid residues flanking this domain are reported to be involved in the oligomerization of the Rev molecule (Olsen et al., 1990a; Malim and Cullen, 1991; Zapp et al., 1991). In addition, a protein activation domain which is required to mediate Rev effector functions *in vivo* is located between aa 78 and aa 93 (Malim et al., 1989a, 1991; Mermer et al., 1990; Venkatesh and Chinnadurai, 1990; Hope et al., 1991; Weichselbraun et al., 1992). A peptide core motif rich in leucine residues (aa 81 to 84) has been identified as the functionally critical part of this domain (Hope et al., 1991; Malim et al., 1991). Mutations in this region resulted in Rev mutant proteins which display a *trans*-dominant (dominant-negative) phenotype. These data suggest that the Rev activation domain mediates Rev function *in vivo* by interacting with an unknown cellular host protein (Malim et al., 1989a, 1991; Mermer et al., 1990; Venkatesh and Chinnadurai, 1990; Hope et al., 1991). The identification of this cellular cofactor required for Rev function will provide insight into the molecular mechanism of Rev-mediated *trans*-activation in the course of HIV-1 infection.

We used biochemical methods to detect a cellular protein which localizes to the nucleus and interacts specifically with the activation domain of the HIV-1 Rev *trans*-activator protein. Subsequently, this cellular factor was identified by purification and amino acid sequencing. Finally, functional data are provided which demonstrate that this specific interaction mediates Rev *trans*-activation.

Materials and Methods

Cross-linking Experiments and Subcellular Fractionation

Total HeLa nuclei were prepared according to standard procedures and purified by centrifugation through a 60% sucrose cushion (Yamasaki et al., 1989).

Proteins from total nuclei were prepared by TCA precipitation (15% final concentration) and subsequently analyzed on 10–15% gradient SDS-PAGE followed by autoradiography. Nuclear envelopes were isolated by a combined nuclease digestion (DNase I: 45 U; RNase A: 0.75 U; Boehringer Mannheim, Mannheim, Germany) at 37°C for 30 min followed by high-salt extraction (1.6 M NaCl; 2 × 10 min at ambient temperature). The chromatin fraction (chromatin-associated proteins) was prepared by DNase I digestion as described above followed by salt extraction using 400 mM NaCl. The nucleoplasmic fraction was obtained by salt extraction using 300 mM NaCl. The cytoplasmic fraction was obtained by disrupting (Wheaton dounce tissue grinder, pestle "B"; 10 strokes) intact HeLa cells in hypotonic buffer (10 mM Hepes, pH 6.5; 10 mM NaCl; 4 mM MgCl₂; 1 mM EDTA; 2 μg/ml Aprotinin; 10 μg/ml Leupeptin) and low-speed centrifugation to remove the nuclei.

Peptide mimics of the HIV-1 Rev activation domain (Rev aa 75 to 93: NH₂-LPPLERLTDCNEDCGTSG-COOH) were coupled to radioiodinated N-[4-(p-azidosalicyl-amido)butyl]-3'-(2'-pyridyl-dithio)propionamide cross-linker molecules (APDP; Pierce, Rockford, IL). 5 × 10⁶ HeLa nuclei were equilibrated for 1 h at room temperature in the presence of 400 nM of ¹²⁵I-APDP-activation domain peptide (10⁶ cpm/nmol) in 2 mM Hepes, pH 7.0, 40 mM NaH₂PO₄, 50 mM NaCl, and 4 mM MgCl₂. The photoreactive moiety was activated at a distance of 10 cm at 366 nm for 10 min using a Hoefer Transilluminator with a bulb surface intensity of ~10,000 μW/cm². A Tat-derived peptide (Tat aa 73 to 86; NH₂-PTSQSRGNPTGPKQ-COOH) served as nonspecific competitor (Arya et al., 1985).

Protein Purification

500 g of HeLa cells were used to isolate 6 × 10¹⁰ nuclei which were

stored at –80°C in storage buffer (10% Sucrose, 10 mM Hepes, 10 mM NaCl, 4 mM MgCl₂, pH 6.5) as described above.

After thawing, 2 μg/ml Aprotinin and 20 μg/ml Leupeptin were added to the nuclei and the suspension was centrifuged at 2,800 g for 15 min at 4°C. The pellet was washed twice in 25 mM Hepes, 150 mM NaCl, 4 mM MgCl₂, 1 mM EGTA, 10% Glycerol, 0.6% *n*-Octylglucoside, 2 μg/ml Aprotinin, 20 μg/ml Leupeptin, pH 7.4 and resuspended in 250 ml of the same buffer. This solution was adjusted to a final concentration of 2% NP-40 and 2% *n*-Octylglucoside. Solubilization of the nuclei was finally achieved using 20 strokes of a motor driven Teflon homogenizer (1,200 rpm; 4°C). The obtained homogenate was cleared by centrifugation at 100,000 g for 60 min at 4°C and the supernatant subjected to ultrafiltration using an Amicon XM 300/76 device (Amicon Corp., Danvers, MA). The resulting filtrate was then concentrated using an Amicon YM 3/76 unit.

Size exclusion chromatography on Superose 75 (Pharmacia, Uppsala, Sweden) was carried out using a 16 × 1,200-mm column equilibrated with chromatography buffer (25 mM Hepes, 50 mM NaCl, 4 mM MgCl₂, 1 mM EGTA, 10% Glycerol, 0.6% *n*-Octylglucoside, 2 μg/ml Aprotinin, 20 μg/ml Leupeptin, pH 7.4). The protein homogenate was chromatographed at 1 ml/min and fractions were collected.

Fractions containing the 19-kD protein were pooled and loaded onto a Mono Q column (16 × 100 mm; Pharmacia) for anion exchange chromatography. Proteins were eluted by applying a salt gradient ranging from 50 mM to 1 M NaCl at a flow rate of 2 ml/min and fractions containing the desired protein were pooled. After this stage of the purification scheme, two alternative separation procedures were followed.

To prepare the Rev activation domain binding protein in its native state, the technique of chromatofocusing was applied. A Mono P column (high resolution [HR]; 10 × 300 mm; Pharmacia) was equilibrated with chromatography buffer and the protein sample was loaded using a flow rate of 1 ml/min. A self-forming pH gradient was created by titrating the sample-containing Mono P matrix at pH 7.4 with elution buffer (Pharmacia-Polybuffer 74 diluted 1:20 with chromatography buffer lacking Hepes). The obtained native protein was subjected to two-dimensional (2D) gel electrophoresis for determination of purity and identity confirmation.

For sequencing, the Rev binding protein was prepared by reversed phase chromatography using a Vydac C4 column (4.6 × 150 mm). Separation was achieved by applying a gradient of water/0.1% Trifluoroacetic Acid (TFA) to acetonitrile/0.1% TFA.

Amino Acid Analysis and Sequence Determination

Approximately two pmol of the purified Rev binding protein were gas-phase hydrolyzed with 6 N HCL containing 1% phenol at 110°C for 20 h on a pico tag workstation (Waters Associates, Milford, MA) and subsequently dried. Amino acid derivatives were formed by automated pre-column reaction with *o*-Phthalaldehyde and FMOc. The amino acid derivatives were analyzed by reversed phase HPLC on a model HP 1090 M liquid chromatograph (Hewlett-Packard Co., Palo Alto, CA) using a micro-bore column (2 × 150 mm) and fluorescence detection. Elution was carried out according to the manufacturer's recommendations.

For CNBr cleavage ~50 pmol of the isolated protein were dissolved in 100 μl of 70% formic acid containing 3 mg/ml cyanogen bromide and incubated at 20°C for 16 h in the dark. Subsequently the sample was dried and 10% of the material was used to verify the cleavage reaction by SDS-PAGE. The remainder was redissolved in TFA/acetonitrile/water (1:500:500), spotted on a polybrene coated glass filter disk and subjected directly to sequence determination.

Automated amino acid sequence analysis was performed on a model 470A sequencer. Amino acid derivatives were analyzed on-line by reversed phase HPLC with a model 120A PTH amino acid analyzer (Applied Biosystems, Inc., Foster City, CA) using a C-18 micro-bore column (2.1 × 220 mm). Instrument control, data collection, and analysis was performed using a model 900A controller (Applied Biosystems, Inc.).

Antisera

Chemically synthesized HIV-1 Rev activation domain peptide and eIF-5A protein, which was purified as described previously (Park et al., 1986), were conjugated separately to cationized BSA (SuperCarrierTM; Pierce) according to the manufacturer's protocol.

Female BALB/c mice received 50 μg conjugated Rev activation domain peptide (NH₂-LPPLERLTDCNEDCGTSG-COOH) in Freund's complete adjuvant intraperitoneally (ip) and subcutaneously (sc) on day 0. The same amount was administered in Freund's incomplete adjuvant on day 21. Two animals received intraperitoneally 20 μg conjugated eIF-5A protein in saline on day 36.

IgG and IgM antibodies were detected in the sera by an ELISA system as previously described (Bahr et al., 1985).

To raise polyclonal anti-eIF-5A antibodies, glutathione-S-transferase-eIF-5A fusion protein was prepared as previously described (Smith and Johnson, 1988) and conjugated to cationized BSA. A rabbit was immunized on day 0 with 200 μ g of this material after mixing with Freund's complete adjuvant. The rabbit was boosted by additional injections on day 8, 15, and 22 and the responses rapidly demonstrated a high level of specific anti-eIF-5A antibody as measured by immunoprecipitation of eIF-5A protein.

Biospecific Interaction Analysis (BIA)

Biospecific Interaction Analysis (BIA) was performed on a BIAcore™ processing unit (Pharmacia) according to the manufacturer's system manual.

The eIF-5A protein used in these studies was purified as described previously (Park et al., 1986). The immunoaffinity purified anti-Rev activation domain antibodies were prepared as described previously (Kristiansen, 1978) by passing 1.5 ml of high titered pooled mouse antiserum diluted with PBS (1:3) through a Rev-Sepharose column (5 \times 20 mm). Bound mouse anti-Rev antibody was eluted at pH 2.8 with 0.1 M Glycin/HCL buffer and neutralized immediately after desorption. eIF-5A and the immunoaffinity purified anti-Rev antibody were immobilized on the BIAcore chip surface at a surface concentration of 15 ng/mm². Antiserum from one mouse designated P3 was diluted 1:20 with PBS and subsequently 5–20 μ l were used for serum sample analysis. Preincubation experiments were carried out by exposing the mouse antiserum (P3) to 20 μ g/ml of eIF-5A or BSA for 30 min at ambient temperature before BIA.

Plasmid Constructions

The pcRev, pBC12/CMV, pBC12/CMV/II-2, and pDM128/CMV constructs and the plasmids encoding the Rev mutants M10, M20, and M32 have been described in detail previously (Cullen, 1986a; Malim et al., 1988, 1989b, 1991). The RRE-deficient plasmid pDM128/CMV Δ RRE was constructed by cloning the 1.7-kb XbaI-BglII fragment from the Rev-specific CAT reporter construct pDM128 (Hope et al., 1990) between the Sall and HindIII site of pgTat (Malim et al., 1988). Substitution of the II-2 specific sequences in pBC12/CMV/II-2 (Cullen, 1986a) by the 3.7-kb HindIII-BamHI fragment of pSV- β -Galactosidase (Promega, Madison, WI) resulted in the construct pBC12/CMV/ β Gal. The gene encoding eIF-5A (Smit-McBride et al., 1989a) was isolated from a human cDNA as a 0.5-kb HindIII-BamHI fragment by PCR technology, using the following primers to introduce terminal HindIII and BamHI sites, respectively: sense 5'-AAG-CTTGCGCGAGTTGGAATCGAAGCCTCTT-3'; anti-sense 5'-GGATCC-CCTGGAGCCAGTTATTTGCC-3'. The PCR conditions included 30 cycles of denaturation at 95°C for 1 min, primer annealing at 60°C for 2 min, and primer extension at 73°C for 3 min. The reaction product was digested by HindIII and BamHI and subsequently inserted in the expression vector pBC12/CMV to generate peIF-5A. Plasmid peIF-5Aas expresses the antisense strand of the full-length human eIF-5A cDNA.

Immunofluorescence

Indirect immunofluorescence studies were performed as previously described (Malim et al., 1989a) to localize the eIF-5A protein within transfected and wild-type COS cells. The primary rabbit anti-eIF-5A antibody was used at a 1:1000 dilution. The second antibody, rhodamine-conjugated goat anti-rabbit IgG, was used at a 1:50 dilution.

For confocal laser scanning microscopy COS cells were fixed using 3.7% formaldehyde/2% sucrose in PBS and permeabilized with 0.5% Triton X-100 in PBS. The cells were incubated with anti-eIF-5A antiserum at a 1:500 dilution in PBS containing 1% BSA. To label the nuclear envelope, a mouse mAb (CHON 211) that specifically binds to components of the nuclear pore complex (Park et al., 1987) was included in the primary antibody incubations. CHON 211 ascites was used at a 1:2000 dilution. The secondary antibodies were FITC-conjugated goat anti-mouse IgM and Texas red-conjugated goat anti-rabbit IgG (diluted 1:100 in PBS/BSA). All antibody incubations were carried out for 1 h at ambient temperature.

Two color images were obtained in the dual channel mode with a BioRad MRC 600 confocal laser scanning system (Bio Rad Laboratories, Cambridge, MA) mounted on a Zeiss IM 10 inverted microscope (Carl Zeiss Inc., Thornwood, NY). Spill-over of fluorescein into the Texas red channel was corrected for by applying the available "bleed" software. The two images were merged using the "nmerge" command.

Transfections

For indirect immunofluorescence and studies using antisense constructs,

COS cells were transfected using DEAE-Dextran as previously described (Cullen, 1986b).

CAT enzyme levels in protein extracts of the transfected COS cells were determined as previously described (Neumann et al., 1987).

Microinjection Experiments

Microinjection was performed into nuclei of stage VI *Xenopus* oocytes (Dumont, 1972). Typically, 15 ng pDM128/CMV, 15 ng pcRev and 7.5 ng pBC12/CMV/ β Gal DNA together with 0.05 ng eIF-5A protein or 15 ng peIF-5A DNA in 80 mM NaCl, 10 mM Hepes, pH 6.8, were co-microinjected in oocytes using 15 oocytes per experiment. In some experiments the pDM128/CMV reporter construct was substituted by pDM128/CMV Δ RRE. In all of the experiments the DNA concentration was kept constant by addition of pBC12/CMV DNA.

Incubation of the injected oocytes was carried out at 21°C for 24 h in modified Barth's medium (Gurdon and Wickens, 1983). Protein extracts were prepared by disrupting the oocytes with a pipette tip in 200 μ l of 250 mM Tris-HCl, pH 7.6, and cleared by centrifugation.

CAT assays were normalized to the expression of β -galactosidase activity expressed from the internal control plasmid pBC12/CMV/ β Gal (Gorman et al., 1982).

RNA Isolation and SI Nuclease Protection Analysis

Nuclear and cytoplasmic RNAs were isolated from 100 mm cell cultures (2.5×10^6 COS cells) 60 h after transfection as described previously (Malim et al., 1989b).

SI nuclease protection analysis was carried out as published previously (Malim et al., 1988). The probe used was isolated as a 629-bp Sall-EcoRI fragment from pDM128/CMV (Malim et al., 1991), fused to heterologous non-HIV-1 sequences and radioactive labeled at the Sall site using Klenow enzyme. The probe spans the HIV-1-derived splice donor site (Hope et al., 1990) and allows the input probe to be distinguished from the protected fragments corresponding to unspliced and spliced pDM128/CMV-derived RNAs.

Results

Detection of the Cellular Rev Activation Domain Binding Factor

To detect an interaction between the HIV-1 Rev activation domain and host cell proteins, we used a synthetic peptide to mimic the Rev activation domain in cross-linking experiments. The radioactive labeled cross-linker APDP (N-[4-(p-azidosalicyl-amido)butyl]-3'-(2'-pyridyl-dithio)propionamide) was coupled chemically to the Rev activation domain peptide and the resulting conjugate was incubated with total nuclei or cellular extracts from HeLa cells (Fig. 1). Cellular proteins interacting with the conjugated Rev activation domain were identified through activation of the cross-linker by UV-light, followed by a reductive cleavage of the cross-

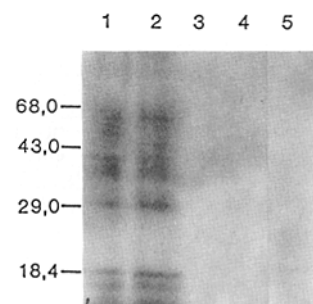


Figure 1. Intracellular distribution of HIV-1 Rev activation domain sequence binding proteins. Intact nuclei from HeLa cells were incubated with Rev activation domain peptide coupled to radioiodinated N-[4-(p-azido-salicylamido)butyl]-3'-(2'-pyridyl-dithio)propionamide (APDP;Pierce) and subsequently cross-linked. Subfractions of labeled nuclei were

subjected to SDS-PAGE and the cross-linked proteins were visualized by autoradiography. Lane 1, total nuclei; lane 2, nuclear envelope; lane 3, chromatin; lane 4, nucleoplasm; lane 5, cytoplasm. Molecular mass standards (in kilodaltons) are indicated on the left.

linked peptide-protein complexes. This protocol resulted in the transfer of the radioactive label from the cross-linker molecule onto the cellular target proteins which were subsequently subjected to gel electrophoresis and visualized by autoradiography. A typical result is shown in Fig. 1. Predominant signals migrating at a relative molecular mass of 65, 41, 39, 34, 30, 19, and 15 kD were detectable when the Rev activation domain peptide was incubated with total nuclei from HeLa cells (Fig. 1, lane 1). Fractionation of the nuclei resulted in an identical cross-linking pattern for the nuclear envelope fraction (Fig. 1, lane 2 versus lane 1). No signals were detected in the HeLa cell chromatin, nucleoplasmic or cytoplasmic fractions (Fig. 1, lanes 3 to 5). To demonstrate binding specificity, competition experiments were performed using unlabeled peptide. A 6–10-fold molar excess of the Rev activation domain peptide completely abrogated the cross-linking to the 19-kD protein from the envelope fraction of HeLa nuclei (Fig. 2 A, lanes 4 and 5 versus lane 1). Densitometric analysis of this data revealed that the 19-kD signal is reduced by maximal 82% when compared with the nonspecific 15-kD band (not shown). In contrast, the use of a nonspecific competitor peptide derived from the HIV-1 Tat carboxyterminus (Tat aa 73 to 86) did not abolish the interaction of the HIV-1 Rev activation domain peptide with the 19-kD host protein (Fig. 2 B, lanes 2 and 3 versus lanes 4 and 5). Therefore, the band of 19 kD exhibited the highest degree of specific competition and was selected for further analysis in this study.

Purification and Identification of the Rev Binding Factor

A radiotracer isolation procedure was used to isolate the 19-kD Rev binding host protein. This approach was chosen since the detected binding factor lacks Rev binding activity

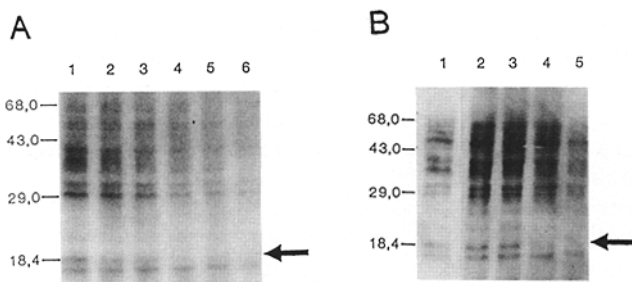


Figure 2. Binding specificity of nuclear proteins recognizing the HIV-1 Rev activation domain. Autoradiographs of SDS-PAGE gels are shown. The molecular mass standards (in kilodaltons) are indicated on the left. An arrow indicates the position of the 19-kD nuclear protein. (A) Cross-linking experiments using an unlabeled specific competitor (Rev activation domain peptide). The molar excess of the specific competitor used was as follows: lane 1, no competitor; lane 2, onefold; lane 3, threefold; lane 4, sixfold; lane 5, 10-fold; lane 6, 50-fold. (B) Comparison of specific with nonspecific competition. A Tat-derived peptide (Tat aa 73 to 86; NH₂-PTSQSRGNPTGPKQ-COOH) served as nonspecific competitor. The molar excess of the competitors used were as follows: lane 1, no competitor; lanes 2 and 3, 5- and 10-fold molar excess of nonspecific competitor, respectively; lanes 4 and 5, 5- and 10-fold molar excess of specific competitor, respectively.

when solubilized from its native nuclear context (data not shown). For this the HeLa cell nuclei were cross-linked to the Rev activation domain peptide as described above and the 19-kD target protein was purified on an analytical scale using various extraction and column purification methods (not shown). In addition, the isoelectric point was determined to be pI 5.1 by 2D gel electrophoresis using the identical material. This information was subsequently used to adjust the scheme to large-scale purification (detailed in

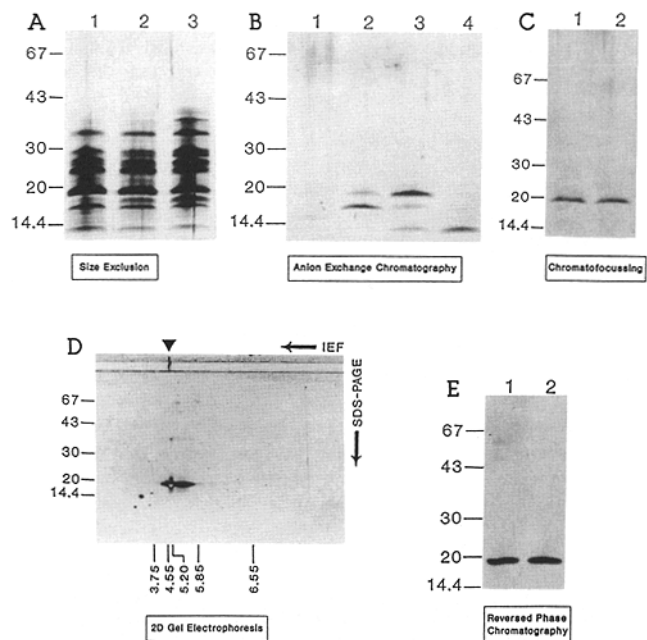


Figure 3. Purification of the Rev activation domain binding factor. 10 μ g of Rev activation domain binding factor were purified from 500 g of HeLa cells. Size fractions which contained the desired protein and which were collected for further purification are shown on silver stained SDS-PAGE gels. (A) Size exclusion Chromatography. Solubilized nuclear proteins were fractionated according to size on a Superose 75 (Pharmacia) gel-filtration column. SDS-PAGE analysis of the fractions collected is shown. Molecular mass standards (in kilodaltons) are indicated on the left. (B) Anion Exchange Chromatography. The charge differences at physiological pH were exploited to separate the acidic Rev activation domain binding factor from the neutral and basic nuclear protein fractions using a Mono Q resin (Pharmacia). The 19-kD Rev cofactor eluted at a salt concentration of \sim 400 mM NaCl which was monitored by SDS-PAGE (lane 3). (C) Separation according to isoelectric properties was achieved using Chromatofocusing on a Mono P matrix (Pharmacia). This procedure ensured the recovery of the desired protein in a native state. SDS-PAGE analysis revealed the purity of the fractions collected which eluted at a pH of 5.5 (lanes 1 and 2). (D) 2D gel electrophoresis was used to demonstrate purity and identity of the protein prepared under native conditions. pI standards for isoelectric focussing (IEF) as the first dimension are indicated on the bottom. The purified Rev activation domain binding factor is indicated by a triangle. Molecular mass standards for SDS-PAGE as the second dimension are shown on the left. (E) To prepare the protein in a solvent mixture suitable for amino acid sequence determination, reversed phase chromatography on a Vydac C4 support was performed. The 19-kD protein desorbed at an acetonitril concentration of 55%. Again, the purity of the protein was confirmed by SDS-PAGE (lanes 1 and 2).

1	MADDLDFETGDAGASATFPM	20
CNBr peptide #1		
21	QCSALRKNGFVVLKGRPCKIVEM	43
CNBr peptide #2		
44	STSKTGKKGHAKVHLVGIDIFTG	79
	KKYEDICPSTHNM	
CNBr peptide #3		
80	DVPNIKRNDFQLIGIQDGYLSLLQ	141
	DSGEVREDLRLPEGDLGKEIEQK	
	YDCGEEILITVLSAM	
CNBr peptide #4		
142	TEEAVAIKAM	152
CNBr peptide #5		
153	AK	154

Figure 4. Amino acid sequence determination after cyanogen bromide (CNBr) cleavage of the Rev binding factor. The amino acid sequence of the 154-amino acid (aa) eIF-5A protein is shown. The sequence of the indicated CNBr-derived peptides (boxes) gave an exact match with the published human eIF-5A sequence (Smit-McBride et al., 1989a). No sequence could be determined for aa 1 to 20 due to an amino-terminal modification in eIF-5A (Wolff et al., 1992).

Materials and Methods) to isolate quantitative amounts. Since Rev binding activity is lost after solubilization, the protein purification could not be directly followed by a binding assay. However, the chromatographic behavior derived from the abovementioned small-scale procedure allowed the path of the 19-kD protein to be followed. 2D gel electrophoresis was used to identify fractions containing this protein during anion exchange chromatography and chromatofocusing (Fig. 3 D). The isolated protein displayed identical extraction and elution behavior to the photoaffinity-labeled protein. The subsequent purification steps and the protein

fractions which were processed to determine a partial amino acid sequence of the Rev binding host cell factor are shown in Fig. 3.

50 pmol of the purified protein was subjected to NH₂-terminal sequence analysis (for technical details see Materials and Methods). However, this analysis revealed NH₂-terminal blocking of the purified Rev binding protein. Therefore the protein was subjected to amino acid analysis and subsequently cleaved by cyanogen bromide (CNBr) to generate a peptide mixture now accessible to sequence determination.

The sequences obtained were used in a subsequent search in the NIH sequence database using the FASTA program (Devereux et al., 1984). A perfect match with the amino acid sequence of eukaryotic initiation factor 5A (eIF-5A; formerly named eIF-4D) was detected (Smit-McBride et al., 1989a). In agreement with our results obtained with the uncleaved protein, the sequence of the NH₂-terminal peptide was also blocked (see Fig. 4). Taken together, these data strongly suggested that the isolated Rev binding protein was identical with eIF-5A.

eIF-5A Recognizes the HIV-1 Rev Activation Domain

To raise mAbs specific for the Rev activation domain, mice were immunized with the activation domain peptide (Rev aa 75 to 93: NH₂-LPPLERLTLDCNEDCGTSG-COOH) conjugated to BSA. Interestingly, serum from one mouse designated P3, taken 7 d after a boost with the peptide-BSA conjugate (bleed day 28; Table I) displayed not only IgG antibodies to the complete Rev protein but also IgM antibodies to purified eIF-5A. This IgM response to eIF-5A was induced to switch to an IgG response after injection of the mouse on day 36 with eIF-5A protein (bleed day 47; Table I). Sera from control mice immunized with the carrier alone totally lacked antibodies with specificity against either Rev or eIF-5A proteins. In addition, all preimmune sera did not react with Rev-derived peptides, Rev or eIF-5A (data not shown).

Biospecific interaction analysis was performed using a BIAcore™ processing unit (Pharmacia) to investigate the binding pattern of the P3 antiserum. As expected from the antibody levels detected, eIF-5A protein immobilized on the BIAcore support-chip retained effectively P3 serum compo-

Table I. Antibody Levels of Immunized BALB/c Mice

Animal	Immunization		Bleed day	Antibody levels* against			
	Peptide‡	eIF-5A‡		Rev		eIF-5A	
				IgG§	IgM	IgG	IgM
P1	+	-	28	1.01	0.10	0.03	0.06
P2	+	-	28	1.40	0.11	0.03	0.08
P3	+	-	28	0.41	0.08	0.07	0.36
P3	+	+	47	ND	ND	0.20	0.31
P4	+	-	28	0.84	0.04	0.00	0.09
P5	-	-	28	0.04	0.02	0.02	0.07
P5	-	+	47	ND	ND	0.10	0.28

Animals immunized with BSA and the preimmune sera of the animals presented showed no response to the Rev-derived peptide, Rev or eIF-5A (not shown).

* Absorbance values read at 492 nm.

‡ Animals were immunized using a Rev-derived peptide (Rev activation domain; Rev aa 75 to 93) on day 0 and day 21 or using eIF-5A protein on day 36. Both antigens were conjugated to cationized BSA (for details see Materials and Methods).

§|| The presented absorbance values were obtained at a 1:200§ or 1:50|| serum dilution.

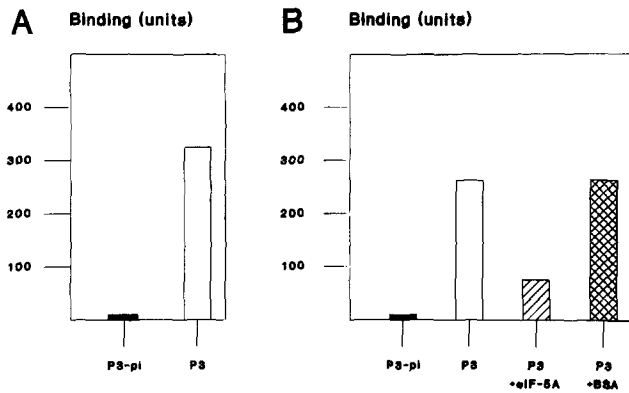


Figure 5. Biospecific interaction analysis (BIA). (A) Interaction of immobilized eIF-5A with the preimmune- (P3-pi; ■) and the immune-serum (P3; □) from a BALB/c mouse immunized with Rev activation domain peptide (Rev aa 75 to 93). Binding values represent the relative levels and have been corrected for background activity. (B) Interaction of the P3-derived serum with immobilized immunoaffinity-purified anti-Rev activation domain antibodies. P3 immune-serum preincubated with 20 μ g/ml eIF-5A for 30 min at ambient temperature before BIA (P3+eIF-5A; ▨); P3 immune-serum preincubated with 20 μ g/ml BSA for 30 min at ambient temperature before BIA (P3-BSA; ▩).

nents compared to P3 preimmune serum (see Fig. 5 A). Immobilization of immuno-purified anti-Rev activation domain peptide antibodies on the BIAcore support-chip revealed an almost identical reactivity with the P3 immune serum. Under identical conditions P3 preimmune serum failed to react (Fig. 5 B). However, preincubation of the P3 immune serum with eIF-5A reduced the observed reactivity by 70%. In contrast, preincubation with BSA had no detectable effect on the reactivity of the P3 serum with the anti-Rev activation domain antibody (Fig. 5 B).

Taken together, these data suggest that the reactivity of the P3 antiserum to eIF-5A is an anti-idiotypic response to the initial serum antibodies generated against the Rev activation domain. Thus, the activation domain is the interaction site of the HIV-1 Rev *trans*-activator with eIF-5A.

Nuclear Localization of eIF-5A

To demonstrate directly that a significant fraction of eIF-5A localizes to the nucleus, we performed indirect immunofluorescence studies.

An anti-eIF-5A polyclonal antiserum was raised in rabbits by immunization of the animals with bacterially expressed glutathione S-transferase-eIF-5A fusion protein (see Materials and Methods). The specificity of the antiserum ob-

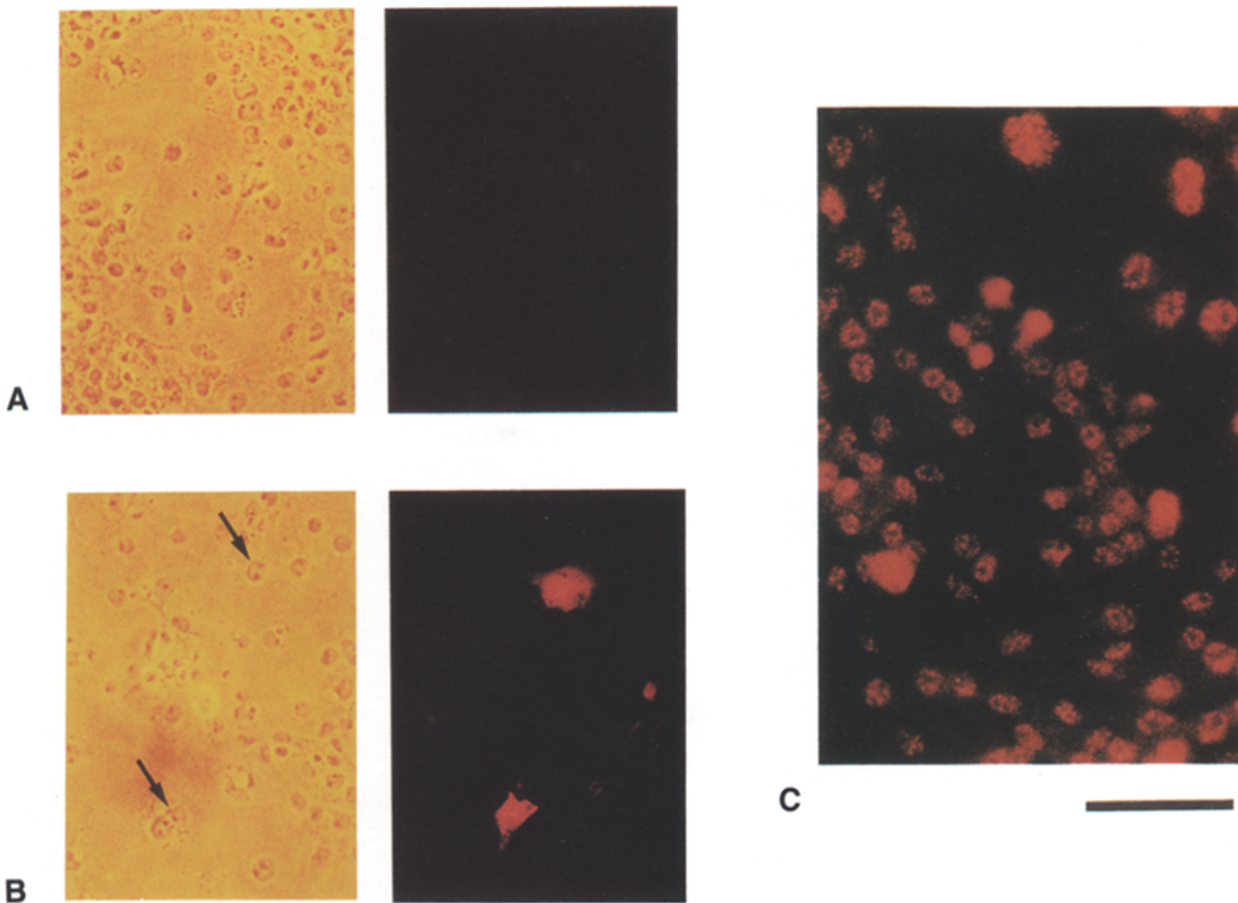


Figure 6. Subcellular localization on eIF-5A by indirect immunofluorescence. (A) Phase-contrast (*left*) and corresponding immunofluorescence photograph (*right*) of fixed COS cells transfected with 0.5 μ g of peIF-5A. Cells in this experiment were treated with rabbit pre-immune serum. (B) Phase-contrast and corresponding immunofluorescence photograph of peIF-5A-transfected COS cells incubated with polyclonal rabbit anti-eIF-5A antiserum. (C) Fluorescence photograph of nontransfected COS cells stained with anti-eIF-5A antiserum. Bar: (A and B) 130 μ m; (C) 85 μ m.

tained was verified by immunoprecipitation analysis of eIF-5A protein (data not shown).

We next transfected COS cell cultures with the peIF-5A expression plasmid and subjected the fixed cells to eIF-5A specific indirect immunofluorescence microscopy. No eIF-5A protein was detected upon incubation with pre-immune serum (Fig. 6 A). Using the anti-eIF-5A antiserum, strong cytoplasmic and nuclear fluorescence was easily detectable in transfected cells (Fig. 6 B). In this experiment the exposure time was very short to visualize eIF-5A in overexpressing cells. However, a weak fluorescence originating from endogenous eIF-5A protein could be detected in all cells using a longer exposure time combined with a higher magnification (Fig. 6 C). Clearly, the cytoplasmic fluorescence was faint compared with the more intense signals detectable in the cell nuclei.

To demonstrate the nuclear localization of eIF-5A more clearly, confocal laser scanning microscopy was performed on nontransfected wild-type COS cells (Fig. 7). The fixed cells were incubated together with anti-eIF-5A antiserum and a mouse mAb (CHON 211) that specifically recognizes components of the nuclear pore complex (Park et al., 1987). The immunostaining of the COS cell nuclear envelope by CHON 211 is shown in Fig. 7 A and the eIF-5A specific signal is shown in Fig. 7 B. Combination of both images clearly demonstrated that a significant fraction of eIF-5A is localized to the nucleus (see Fig. 7 C). Similar results were also obtained using HeLa cells (not shown).

Reconstitution of Rev Function in *Xenopus* Oocytes by eIF-5A

Having identified eIF-5A as a nuclear binding factor of the HIV-1 Rev activation domain, we next examined whether eIF-5A is also the cellular co-factor required for Rev func-

tion. A Northern blot analysis of eIF-5A encoding mRNAs revealed that, in contrast to the cell lines examined (HeLa, CEM, Jurkat, U937), no eIF-5A gene expression was detectable in *Xenopus* oocytes (see Fig. 8 A). Therefore, the *Xenopus* oocyte system was chosen to investigate Rev function. An additional Southern blot analysis of the corresponding genomic DNAs using the same eIF-5A-specific probe demonstrated probe specificity (Fig. 8 B). Multiple signals were detected in EcoRI-digested genomic DNA of mammalian cells (Fig. 8 B, lanes 1 to 4). This hybridization pattern might indicate the existence of multiple eIF-5A genes. In contrast, only a single signal was detected in *Xenopus* DNA (Fig. 8 B, lane 5).

Various Rev expressing plasmids and eIF-5A protein were microinjected into the nuclei of *Xenopus* oocytes. Rev activity was monitored by coinjection of the Rev-responsive chloramphenicol acetyltransferase (CAT) expression vector pDM128/CMV (Malim et al., 1991). All experiments were internally controlled by addition of the pBC12/CMV/ β Gal construct. β Gal expression was used to equalize the amount of protein extract used in the CAT assay. Before microinjection, the identity and purity of eIF-5A, which was purified as described previously (Park et al., 1986), was confirmed by 2D gel electrophoresis and amino acid sequence analysis.

In these assays Rev failed to induce CAT activity from the Rev-responsive reporter construct pDM128/CMV, indicating the lack of cellular components required for Rev trans-activation in oocytes (Fig. 9, lane 3 versus lane 4). However, addition of eIF-5A clearly reconstituted Rev function in the oocytes while eIF-5A alone had no influence on the reporter activity (Fig. 9, lane 5 versus lane 6). In previous studies, mutagenesis of the *rev* gene combined with functional analysis in mammalian cells was used to generate Rev activation domain mutants with defined phenotypes in vivo. The Rev mutants M10 and M32, both of which have alterations in the

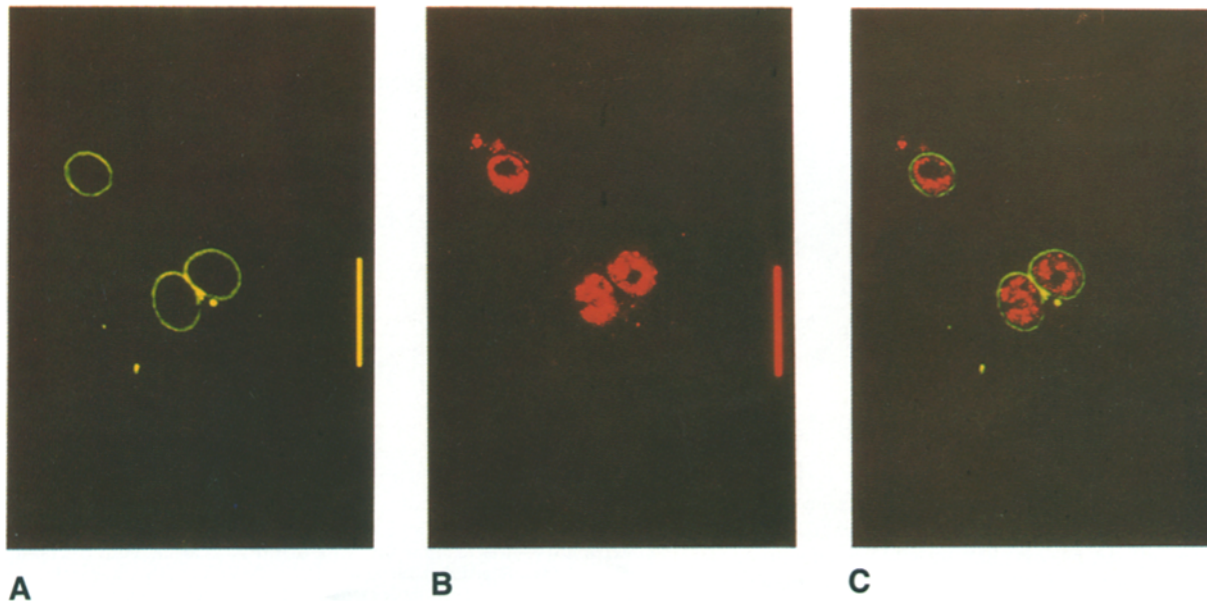


Figure 7. Nuclear localization of eIF-5A by confocal laser scanning microscopy. COS cells were viewed using a Bio Rad MRC 600 confocal imaging system. Corresponding images are shown. (A) Staining of the nuclear envelope using CHON 211 antibody and FITC-conjugated goat anti-mouse secondary antibody. (B) Detection of eIF-5A in the nucleus using anti-eIF-5A antiserum and Texas red-conjugated goat anti-rabbit secondary antibody. (C) The images shown in A and B were merged using the available software to obtain a single image combining both signals. Bars, 25 μ m.

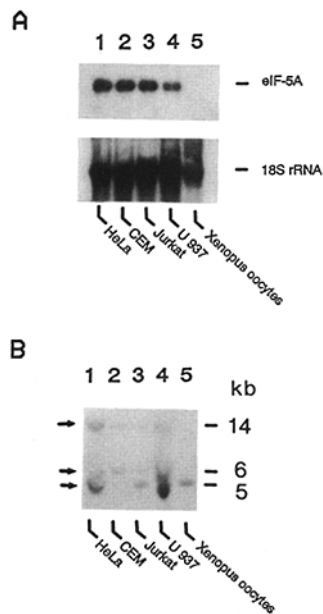


Figure 8. Detection of eIF-5A mRNA and gene sequences. (A) 10 μ g of total RNA from the indicated cell lines and *Xenopus* oocytes were subjected to Northern blot analysis as described previously (Valent et al., 1991). eIF-5A-specific transcripts of 1.4 kb were detected using a radiolabeled synthetic oligonucleotide probe (gene position, 300–329; Smit-McBride et al., 1989a). The same result was obtained using the full-length eIF-5A cDNA (not shown). To control for comparable RNA amounts the filters were stripped and rehybridized using a 18 S ribosomal RNA probe. (B) Southern blot analysis of 10 μ g of EcoRI digested genomic DNA from the indicated cell

lines and *Xenopus* oocytes was performed as previously described (Weiss et al., 1989). The filter used in this experiment was hybridized with the oligonucleotide probe described in A. eIF-5A-specific signals are indicated by arrows on the left. The hybridization pattern obtained might indicate the existence of multiple eIF-5A genes in mammalian cells. Size standards (in kilobase pairs) are shown on the right.

leucine-rich core motif of the Rev activation domain, are nonfunctional proteins with *trans*-dominant phenotypes (Malim et al., 1989a, 1991). In agreement with these *in vivo* observations, RevM32 and RevM10 failed to *trans*-activate in the presence of eIF-5A in *Xenopus* oocytes (Fig. 9, lanes

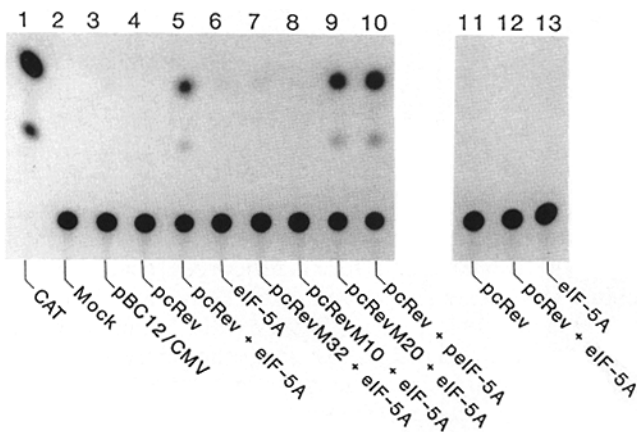


Figure 9. Reconstitution of Rev function in *Xenopus* oocytes by eIF-5A. Oocytes were microinjected with the indicated expression plasmids and purified eIF-5A protein. Rev activity was monitored by injection of the CAT expressing Rev-responsive reporter construct pDM128/CMV (lanes 3–10) or the RRE-deficient construct pDM128/CMV Δ RRE (lanes 11–13). All experiments were internally controlled by constitutive coexpression of β -Galactosidase from the plasmid pBC12/CMV/ β Gal. Lane 1 (CAT), CAT enzyme (assay control); lane 2 (Mock), noninjected oocytes (negative control).

7 and 8). Furthermore, a mutation in the activation domain (M20) which did not abolish biological Rev activity (Malim et al., 1991) was functional in our assay (Fig. 9, lane 9). Substitution of the microinjected protein by a cDNA encoding eIF-5A resulted in comparable Rev *trans*-activation in our assay system (Fig. 9, lane 10). Finally, no *trans*-activation could be detected in control experiments when the reporter pDM128/CMV was substituted by the RRE-deficient construct pDM128/CMV Δ RRE (Fig. 9, lanes 11–13).

These results directly demonstrate that eIF-5A is a cellular cofactor mediating Rev *trans*-activation. In addition, they provide evidence that the Rev leucine-motif is required for functional Rev-eIF-5A interaction and that *trans*-activation is dependent on the presence of the RRE target sequence.

eIF-5A Gene Expression Is Required for Rev *Trans*-Activation in Mammalian Cells

Finally we tested the effect of eIF-5A on Rev function in mammalian cells. For this, we transiently blocked *eIF-5A* gene expression in COS cells with an eIF-5A antisense construct (peIF-5Aas) and monitored Rev function by co-transfection of the Rev-responsive reporter construct pDM128/CMV. Again, these experiments were internally controlled by inclusion of the pBC12/CMV/ β Gal expression plasmid in order to rule out an effect of peIF-5Aas on general translation initiation. As summarized in Table II, the expression of eIF-5A antisense sequences resulted in a significant inhibition (83%) of Rev function as measured by Rev-dependent CAT activity, indicating the necessity of *eIF-5A* gene expression for Rev function.

Previous studies demonstrated that Rev function directly affects the intracellular distribution of incompletely spliced viral mRNAs in mammalian cells, including COS (Malim et al., 1988, 1989b; Felber et al., 1989). It was shown, that HIV-1-derived RRE-containing RNAs are retained in the nucleus and only appear in the cytoplasm when Rev is present. Therefore, we also investigated the effect of peIF-5Aas on the cytoplasmic accumulation of these RNAs. Transfected COS cell cultures were subjected to subcellular fractionation and

Table II. Effect of eIF-5A on HIV-1 Rev Activity in Transfected COS Cells

Transfection*	Level of CAT expression (cpm) [‡]	Percent inhibition of Rev function
pDM128/CMV	60	—
pDM128/CMV + pcRev	15,066	—
pDM128/CMV + pcRev + peIF-5Aas	2,576	83
pDM128/CMV + peIF-5Aas	222	—

* 2.5 \times 10⁵ COS cells were co-transfected together with 100 ng of pDM128/CMV, 50 ng of pcRev and 590 ng of peIF-5Aas as previously described (Cullen, 1986b). In addition, 60 ng of pBC12/CMV/ β Gal DNA was included in every transfection. Total input DNA was kept constant by inclusion of the parental expression vector pBC12/CMV as a negative control. Expression of β Gal served as internal control in order to normalize the CAT assays.

[‡] Protein extracts were prepared 60 h after transfection and assayed for CAT activity as described previously (Neumann et al., 1987). Values represent the relative levels of CAT expression measured when the change in transacetylation was linear with respect to time and have been corrected for background (mock) activity.

nuclear and cytoplasmic RNAs were subsequently analyzed by S1 nuclease protection (see Fig. 10). The probe used in this assay was designed to detect both spliced and unspliced (RRE-containing) RNA species expressed from the Rev-responsive reporter construct pDM128/CMV (Hope et al., 1990; Malim et al., 1991). Attachment of heterologous sequences also allowed us to distinguish the full-length input probe from the fragments rescued by the unspliced and spliced transcripts. As shown in Fig. 10, Rev function resulted in the cytoplasmic accumulation of unspliced RRE-containing RNA (lanes 1 and 2 versus lanes 3 and 4), which is in agreement with previous studies (Malim et al., 1988, 1989b). However, the Rev phenotype, indicated by these cytoplasmic RRE-containing RNAs, was almost completely abrogated by inclusion of the peIF-5A antisense construct (peIF-5Aas) to the transfection (see Fig. 10, lanes 5 and 6). Expression of peIF-5Aas in absence of Rev had no effect on pDM128/CMV expression in a control experiment (Fig. 10, lanes 7 and 8).

The data presented demonstrate that eIF-5A gene expression is necessary for Rev function in mammalian cells. eIF-5A is critically required for the Rev-mediated cytoplasmic accumulation and expression of incompletely spliced RRE-containing RNAs.

Discussion

The Rev *trans*-activator protein of HIV-1 plays a key role in the complex regulation of viral gene expression. Rev function results in the expression of the viral structural proteins

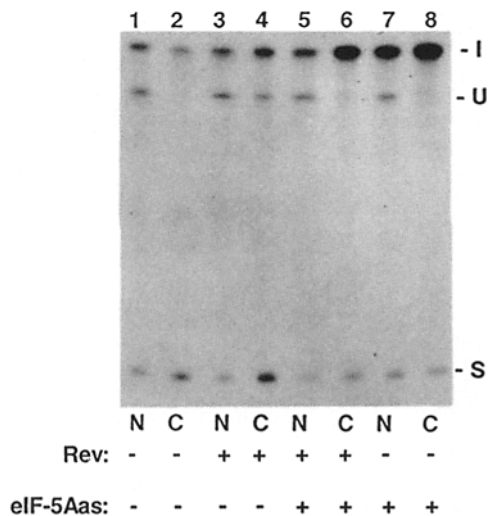


Figure 10. Effect of eIF-5A gene expression on the intracellular distribution of RRE-containing RNAs. COS cell cultures (2.5×10^6 cells) were transfected with 0.8 μ g of the Rev-responsive reporter construct pDM128/CMV and the expression plasmids pcRev (0.4 μ g) and peIF-5Aas (4.72 μ g) as indicated at the bottom. Total input DNA was maintained constant in every experiment by inclusion of the parental expression vector pBC12/CMV as a negative control. Nuclear (N) and cytoplasmic (C) RNAs were isolated at 60 h after transfection as described previously (Malim et al., 1989b). 4.0- μ g aliquots of total RNA were subjected to S1 nuclease analysis using an input probe (I) which was designed to detect both, unspliced (U) and spliced (S) pDM128/CMV-derived mRNA in the transfected COS cultures.

and hence, in the generation of infectious particles (Terwilliger et al., 1988; Emerman et al., 1989; Hammarskjöld et al., 1989). Experimental data suggested that interaction of Rev with an unknown cellular component is required for Rev function (Malim et al., 1989a, 1991; Mermer et al., 1990; Venkatesh and Chinnadurai, 1990). In this study we demonstrate that eukaryotic initiation factor 5A (eIF-5A, previously called eIF-4D) is a host co-factor required for Rev *trans*-activation.

Human eIF-5A is a small 154-aa protein with a molecular mass of 16.7 kD (Smit-McBride et al., 1989a). The isoelectric point of the acidic eIF-5A was determined previously with a pI of ~ 5.1 , which is in agreement with our results (Park et al., 1986; Smit-McBride et al., 1989b).

eIF-5A is the only known cellular protein that contains a hypusine residue, which is formed by posttranslational modification of the lysine residue at position 50 within eIF-5A (Fig. 4) (Park et al., 1986). The hypusinated protein stimulates the formation of the dipeptide analogue methionyl-puromycin in an in vitro assay which mimics the formation of the first peptide bond during protein synthesis (Park, 1989; Smit-McBride et al., 1989b; Hershey et al., 1990; Park et al., 1991). Thus, eIF-5A appears to function on the level of protein synthesis initiation. However, the precise function of eIF-5A in vivo is still unknown. eIF-5A might have activities in addition to, or instead of, translation initiation (for a recent review see Park et al., 1993).

Implications for Rev Function

The direct interaction of Rev with eIF-5A now provides the basis to significantly improve our understanding of Rev-specific *trans*-activation at the molecular level. It seems reasonable to assume that the targeting of RRE-containing mRNAs to eIF-5A could result in an improved utilization of these viral mRNAs by the translational machinery. Recently, D'Agostino and co-workers provided evidence that the presence of Rev affected not only transport, but also translation of viral mRNAs encoding the structural proteins, by promoting polysomal loading (D'Agostino et al., 1992).

The modification or inactivation of translation initiation factors to repress host and enhance viral mRNA translation is a strategy which is used by various viruses. Adenovirus infection involves viral-mediated dephosphorylation of a component of the cap-binding protein complex eIF-4F, while poliovirus inactivates eIF-4F by proteolytic degradation of an eIF-4F subunit (for a recent review see Thach, 1992). Influenza virus, vesicular stomatitis virus and adenovirus are able to control the efficiency with which different mRNAs are translated by blocking phosphorylation of a subunit of the translation initiation factor eIF-2.

The identification of eIF-5A as a cellular factor interacting with the Rev activation domain allows the model of Rev function to be refined. Rev activity depends on the presence of *cis*-acting repressive sequences (CRS) which are located in the HIV-1 structural genes and act to retain the viral mRNAs in the nucleus (Rosen et al., 1988; Cochrane et al., 1991; Maldarelli et al., 1991; Schwartz et al., 1992). Inefficient splice-sites present on the viral mRNAs may delay an otherwise very rapid in vivo splicing reaction (Chang and Sharp, 1989). This delay would allow the specific recognition of the RRE RNA sequence by Rev, followed by Rev-specific mul-

timer formation, which in turn results in the observed dissociation of spliceosomes on the viral mRNAs; a reaction which occurs independent of the Rev activation domain (Kjems et al., 1991b). Finally, the subsequent interaction of the RRE-Rev complex with eIF-5A may not only induce nucleo/cytoplasmic RNA translocation but may also result in the preferential translation of RRE-containing mRNAs. In this model, although Rev initially acts at a nuclear location by binding the viral RNA, it also provides cytoplasmic functions directly affecting translation via eIF-5A.

A Nuclear Role for eIF-5A

eIF-5A was originally described as an abundant cytoplasmic protein which is transiently attached to ribosomes in the course of initiation of eukaryotic cellular protein synthesis. The major evidence for its function comes from a nonphysiological *in vitro* reaction; the synthesis of methionyl-puromycin (Park, 1989; Smit-McBride et al., 1989b; Hershey et al., 1990; Park et al., 1991). However, the results presented in this study reveal that successful cross-linking of eIF-5A to the Rev activation domain is only achieved with eIF-5A residing in the nuclear compartment (Fig. 1). In agreement with this finding, our eIF-5A-specific immunofluorescence studies clearly demonstrated that a substantial fraction of this protein exists inside the nucleus. It is conceivable that this material was trapped in the nuclear envelope fraction of our initial fractionation experiment (Fig. 1). Nevertheless, this nuclear eIF-5A may possibly extend activities associated with translation initiation. Intriguingly, a similar nuclear localization characterized by speckles (see Fig. 7) was also recently demonstrated by Lejbkowitz and co-workers for a fraction of eukaryotic initiation factor 4E (eIF-4E), which is the mRNA 5' cap-binding protein (Lejbkowitz et al., 1992). Thus it is evident that the nuclear localization of some initiation factors is a common principle in eukaryotic cells, which may serve functions with respect to targeted nucleo-cytoplasmic mRNA transport and/or interference with the nuclear mRNA splicing machinery.

In recent studies the idea of variant eIF-5A activities, including functions other than protein synthesis, have been suggested. In CHO cells as well as in chick embryo fibroblasts, isoforms of eIF-5A could be detected (Park, 1989; Wolff et al., 1992). In the yeast *Saccharomyces cerevisiae*, two highly similar genes encoding eIF-5A are expressed differentially in response to aerobic growth conditions (Schnier et al., 1991). Two slightly different genes, termed *NeIF-5A1* and *NeIF-5A2*, were also described in tobacco (Chamot and Kuhlemeier, 1992). While *NeIF-5A2* appears to be a housekeeping protein involved in general translation initiation, *NeIF-5A1* seems to regulate the light-dependent translation of specific transcripts. Taken together, these data indicate that multiple forms of eIF-5A exist which may serve different functions during posttranscriptional regulation of eukaryotic gene expression.

It is tempting to speculate that the subcellular localization of Rev-binding eIF-5A is of functional significance for the selective translocation of specific viral mRNAs across the nuclear envelope. Indeed, the translocation of poly(A)-rich mRNA requires a protein carrier which is part of a complex system located in the nuclear envelope (Gerace and Burke, 1988). It is conceivable that eIF-5A is part of a RNP which is involved in the translocation of nuclear RNA to the

cytoplasm. Intriguingly, nuclear RNPs are concentrated in discrete nuclear domains, serving as interconnecting channels that extend through the nuclear interior to the nuclear envelope (Gerace and Burke, 1988). Also, the interaction of eIF-5A with the small preribosomal subunit already in the nuclear compartment may result in co-translocation of the viral mRNA. After transit through the nuclear pores and assembly of translation-competent ribosomes, the RRE-containing RNA would be preferentially used in the process of protein synthesis (Arrigo et al., 1991; Lawrence et al., 1991; D'Agostino et al., 1992).

The finding that eIF-5A is a cellular Rev activation domain binding factor required for Rev *trans*-activation has several consequences. It should now be possible to dissect the complete pathway of Rev function step by step, starting with the specific RRE binding and finally resulting in the preferential translation of these viral mRNAs. It will also be of interest to examine whether other complex retroviruses exploit the same pathway to regulate their gene expression at a posttranscriptional level. Therapeutic interference with Rev-eIF-5A interaction may provide a new opportunity to block viral replication by inhibiting the Rev-regulated expression of the structural proteins.

Clearly, our findings also demonstrate a new biological activity for eIF-5A. Studies aimed to identify the cellular interaction partners of eIF-5A in the nuclear compartment will generate new insights into mechanisms that are applied in eukaryotic cells in order to translate specific transcripts preferentially.

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References

- Arrigo, S. J., and I. S. Y. Chen. 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 *vif*, *vpr*, and *env/vpu* 2 RNAs. *Genes & Dev.* 5:808-819.
- Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal. 1985. *Trans*-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science (Wash. DC)*. 229:69-73.
- Bahr, G. M., D. S. Tello, and L. A. Chedid. 1985. Marked enhancement *in vivo* of adjuvant activity of muramyl dipeptide to protein antigens and to synthetic weak immunogens with monoclonal anti-muramyl dipeptide antibodies. *Infect. Immun.* 49:312-319.
- Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak. 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell*. 67:529-536.
- Böhnlein, E., J. Berger, and J. Hauber. 1991. Functional mapping of the human immunodeficiency virus type 1 Rev RNA binding domain: new insights into the domain structure of Rev and Rex. *J. Virol.* 65:7051-7055.
- Borer, R. A., C. F. Lehner, H. M. Eppenberger, and E. A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell*. 56:379-390.
- Chamot, D., and C. Kuhlemeier. 1992. Differential expression of genes encoding the hypusine-containing translation initiation factor, eIF-5A, in tobacco. *Nucleic Acids Res.* 20:665-669.
- Chang, D. D., and P. A. Sharp. 1989. Regulation by HIV Rev depends upon recognition of splice sites. *Cell*. 59:789-795.
- Cochrane, A., R. Kramer, S. Ruben, J. Levine, and C. A. Rosen. 1989. The human immunodeficiency virus *rev* protein is a nuclear phosphoprotein. *Virology*. 171:264-266.
- Cochrane, A. W., C.-H. Chen, and C. A. Rosen. 1990a. Specific interaction

- of the human immunodeficiency virus Rev protein with a structured region in the env mRNA. *Proc. Natl. Acad. Sci. USA.* 87:1198-1202.
- Cochrane, A. W., A. Perkins, and C. A. Rosen. 1990b. Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. *J. Virol.* 64: 881-885.
- Cochrane, A. W., K. S. Jones, S. Beidas, P. J. Dillon, A. M. Skalka, and C. A. Rosen. 1991. Identification and characterization of intragenic sequences which repress HIV structural gene expression. *J. Virol.* 65:5305-5313.
- Cook, K. S., G. J. Fisk, J. Hauber, N. Usman, T. J. Daly, and J. R. Rusche. 1991. Characterization of HIV-1 REV protein: binding stoichiometry and minimal RNA substrate. *Nucleic Acids Res.* 19:1577-1583.
- Cullen, B. R. 1986a. Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. *Cell.* 46:973-982.
- Cullen, B. R. 1986b. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* 152:684-704.
- Cullen, B. R., J. Hauber, C. Campbell, J. G. Sodroski, W. A. Haseltine, and C. A. Rosen. 1988. Subcellular localization of the human immunodeficiency virus trans-acting *art* gene product. *J. Virol.* 62:2498-2501.
- D'Agostino, D. M., B. K. Felber, J. E. Harrison, and G. N. Pavlakis. 1992. The Rev protein of human immunodeficiency virus type 1 promotes polyosomal association and translation of *gag/pol* and *vp1/env* mRNAs. *Mol. Cell. Biol.* 12:1375-1386.
- Daefler, S., M. E. Klotman, and F. Wong-Staal. 1990. Trans-activating rev protein of the human immunodeficiency virus 1 interacts directly and specifically with its target RNA. *Proc. Natl. Acad. Sci. USA.* 87:4571-4575.
- Daly, T. J., K. S. Cook, G. S. Gray, T. E. Maione, and J. R. Rusche. 1989. Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element *in vitro*. *Nature (Lond.)*. 343:816-819.
- Devereux, J., P. Haeblerli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Dumont, J. N. 1972. Oogenesis in *Xenopus leavis* (Daudin). *J. Morphol.* 136:153-180.
- Emerman, M., R. Vazeux, and K. Peden. 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell.* 57:1155-1165.
- Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal. 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell.* 46:807-817.
- Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis. 1989. Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc. Natl. Acad. Sci. USA.* 86:1495-1499.
- Gerace, L., and B. Burke. 1988. Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* 4:335-374.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
- Gurdon, J. P., and M. P. Wickens. 1983. The use of *Xenopus* oocytes for the expression of cloned genes. *Methods Enzymol.* 101:370-386.
- Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis. 1989. The rev (*trslart*) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. *J. Virol.* 63:1265-1274.
- Hammarskjöld, M.-L., J. Heimer, B. Hammarskjöld, I. Sangwan, L. Albert, and D. Rekosh. 1989. Regulation of human immunodeficiency virus env expression by the rev gene product. *J. Virol.* 63:1959-1966.
- Hauber, J., M. Bouvier, M. H. Malim, and B. R. Cullen. 1988. Phosphorylation of the rev gene product of human immunodeficiency virus type 1. *J. Virol.* 62:4801-4808.
- Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. M. Green, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner. 1990. HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. *Cell.* 60:685-693.
- Heaphy, S., J. T. Finch, M. J. Gait, J. Karn, and M. Singh. 1991. Human immunodeficiency virus type 1 regulator of virion expression, rev, forms nucleoprotein filaments after binding to a purine-rich "bubble" located within the rev-responsive region of viral mRNAs. *Proc. Natl. Acad. Sci. USA.* 88:7366-7370.
- Hershey, J. W. B., Z. Smit-McBride, and J. Schnier. 1990. The role of mammalian initiation factor eIF-4D and its hypusine modification in translation. *Biochim. Biophys. Acta.* 1050:160-162.
- Hope, T. J., X. Huang, D. McDonald, and T. G. Parslow. 1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. *Proc. Natl. Acad. Sci. USA.* 87:7787-7791.
- Hope, T. J., B. L. Bond, D. McDonald, N. P. Klein, and T. G. Parslow. 1991. Effector domains of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex are functionally interchangeable and share an essential peptide motif. *J. Virol.* 65:6001-6007.
- Kjems, J., M. Brown, D. D. Chang, and P. A. Sharp. 1991a. Structural analysis of the interaction between the HIV-1 Rev protein and the Rev response element. *Proc. Natl. Acad. Sci. USA.* 88:683-687.
- Kjems, J., A. D. Frankel, and P. A. Sharp. 1991b. Specific regulation of mRNA splicing *in vitro* by a peptide from HIV-1 Rev. *Cell.* 67:169-178.
- Kjems, J., B. J. Calnan, A. D. Frankel, and P. A. Sharp. 1992. Specific binding of a basic peptide from HIV-1 Rev. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1119-1129.
- Kristiansen, T. 1978. Matrix-bound antigens and antibodies. In *Affinity Chromatography*. O. Hoffman-Ostenhof, editor. Pergamon Press, Oxford. 191-206.
- Lawrence, J. B., A. W. Cochrane, C. V. Johnson, A. Perkins, and C. A. Rosen. 1991. The HIV-1 Rev protein: a model system for coupled RNA transport and translation. *New Biol.* 3:1220-1232.
- Lejbkowitz, F., C. Goyer, A. Darveau, S. Neron, R. Lemieux, and N. Sonnenberg. 1992. A fraction of the mRNA 5' cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. *Proc. Natl. Acad. Sci. USA.* 89:9612-9616.
- Maldarelli, F., M. A. Martin, and K. Strebel. 1991. Identification of post-transcriptionally active inhibitory sequences in HIV-1 RNA; novel level of gene regulation. *J. Virol.* 65:5732-5743.
- Malim, M. H., and B. R. Cullen. 1991. HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. *Cell.* 65:241-248.
- Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen. 1988. Immunodeficiency virus trans-activator modulates the expression of the viral regulatory genes. *Nature (Lond.)*. 335:181-183.
- Malim, M. H., S. Böhnlein, J. Hauber, and B. R. Cullen. 1989a. Functional dissection of the HIV-1 Rev trans-activator - derivation of a trans-dominant repressor of Rev function. *Cell.* 58:205-214.
- Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen. 1989b. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (Lond.)*. 338: 254-257.
- Malim, M. H., L. S. Tiley, D. F. McCarn, J. R. Rusche, J. Hauber, and B. R. Cullen. 1990. HIV-1 structural gene expression requires binding of the Rev trans-activator to its RNA target sequence. *Cell.* 60:675-683.
- Malim, M. H., D. F. McCarn, L. S. Tiley, and B. R. Cullen. 1991. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. *J. Virol.* 65:4248-4254.
- Mermer, B., B. K. Felber, M. Campbell, and G. N. Pavlakis. 1990. Identification of trans-dominant HIV-1 rev protein mutants by direct transfer of bacterially produced proteins into human cells. *Nucleic Acids Res.* 18: 2037-2044.
- Neumann, J. R., C. A. Morency, and K. O. Russian. 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques.* 5: 444-447.
- Olsen, H. S., A. W. Cochrane, P. J. Dillon, C. M. Nalin, and C. A. Rosen. 1990a. Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in env mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. *Genes & Dev.* 4: 1357-1364.
- Olsen, H. S., P. Nelböck, A. W. Cochrane, and C. A. Rosen. 1990b. Secondary structure is the major determinant for interaction of HIV rev protein with RNA. *Science (Wash. DC).* 247:845-848.
- Park, M. H. 1989. The essential role of hypusine in eukaryotic translation initiation factor 4D (eIF-4D). *J. Biol. Chem.* 264:18531-18535.
- Park, M. H., T.-Y. Liu, S. H. Neece, and W. J. Swiggard. 1986. Eukaryotic initiation factor 4D. *J. Biol. Chem.* 261:14515-14519.
- Park, M. K., M. d'Onofrio, M. C. Willingham, and J. A. Hanover. 1987. A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked N-acetylglucosamine is part of the immunodeterminant. *Proc. Natl. Acad. Sci. USA.* 84:6462-6466.
- Park, M. H., E. C. Wolff, Z. Smit-McBride, J. W. B. Hershey, and J. E. Folk. 1991. Comparison of the activities of variant forms of eIF-4D. *J. Biol. Chem.* 266:7988-7994.
- Park, M. H., E. C. Wolff, and J. E. Folk. 1993. Hypusine: its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. *BioFactors* 4:95-104.
- Ptashne, M. 1988. How eukaryotic transcriptional activators work. *Nature (Lond.)*. 335:683-689.
- Rosen, C. A., E. Terwilliger, A. Dayton, J. G. Sodroski, and W. A. Haseltine. 1988. Intragenic cis-acting *art* gene-responsive sequences of the human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA.* 85:2071-2075.
- Sadaie, M. R., T. Benter, and F. Wong-Staal. 1988. Site-directed mutagenesis of two trans-regulatory genes (*tat-III*, *trsl*) of HIV-1. *Science (Wash. DC).* 239:910-914.
- Schnier, J., H. G. Schwelberger, Z. Smit-McBride, H. A. Kang, and J. W. B. Hershey. 1991. Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 11:3105-3114.
- Schwartz, S., B. K. Felber, and G. N. Pavlakis. 1992. Distinct RNA sequences in the gag region of HIV-1 decrease RNA stability and inhibit expression in the absence of the Rev protein. *J. Virol.* 66:150-159.
- Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene (Amst.)*. 67:31-40.
- Smit-McBride, Z., T. E. Dever, J. W. B. Hershey, and W. C. Merrick. 1989a. Sequence determination and cDNA cloning of eukaryotic initiation factor 4D, the hypusine-containing protein. *J. Biol. Chem.* 264:1578-1583.

- Smit-McBride, Z., J. Schnier, R. J. Kaufman, and J. W. B. Hershey. 1989b. Protein synthesis initiation factor eIF-4D. *J. Biol. Chem.* 264:18527-18530.
- Sodroski, J., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. Haseltine. 1986. A second post-transcriptional *trans*-activator gene required for HTLV-III replication. *Nature (Lond.)*. 321:421-417.
- Terwilliger, E., R. Burghoff, R. Sia, J. Sodroski, W. Haseltine, and C. Rosen. 1988. The *art* gene product of human immunodeficiency virus is required for replication. *J. Virol.* 62:655-658.
- Thach, R. E. 1992. Cap recap: the involvement of eIF-4F in regulating gene expression. *Cell.* 68:177-180.
- Tiley, L. S., M. H. Malim, H. K. Tewary, P. G. Stockley, and B. R. Cullen. 1992. Identification of a high-affinity RNA-binding site for the human immunodeficiency virus type 1 Rev protein. *Proc. Natl. Acad. Sci. USA.* 89:758-762.
- Valent, P., D. Bevec, D. Maurer, J. Besemer, F. Di Padova, J. H. Butterfield, W. Speiser, O. Majdic, K. Lechner, and P. Bettelheim. 1991. Interleukin 4 promotes expression of mast cell ICAM-1 antigen. *Proc. Natl. Acad. Sci. USA.* 88:3339-3342.
- Venkatesh, L. K., and G. Chinnadurai. 1990. Mutants in a conserved region near the carboxy-terminus of HIV-1 Rev identify functionally important residues and exhibit a dominant negative phenotype. *Virology.* 178:327-330.
- Weichselbraun, I., G. K. Farrington, J. R. Rusche, E. Böhnlein, and J. Hauber. 1992. Definition of the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex protein activation domain by functional exchange. *J. Virol.* 66:2583-2587.
- Weiss, E. H., D. Bevec, G. Messer, S. Schwemmle, C. Großhaus, M. Steinmetz, and W. Schmidt. 1989. Organization of the AKR Qa region: structure of a divergent class I sequence, Q5^t. *J. Immunogenet. (Oxford)*. 16:283-290.
- Wolff, E., T. G. Kinzy, W. C. Merrick, and M. H. Park. 1992. Two isoforms of eIF-5A in chick embryo. *J. Biol. Chem.* 267:6107-6113.
- Yamasaki, L., P. Kanda, and R. E. Lanford. 1989. Identification of four nuclear transport signal-binding proteins that interact with diverse transport signals. *Mol. Cell. Biol.* 9:3028-3036.
- Zapp, M. L., and M. R. Green. 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. *Nature (Lond.)*. 342:714-716.
- Zapp, M. L., T. J. Hope, T. G. Parslow, and M. R. Green. 1991. Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. *Proc. Natl. Acad. Sci. USA.* 88:7734-7738.