

Nuclear pore localization and nucleocytoplasmic transport of eIF-5A: evidence for direct interaction with the export receptor CRM1

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SUMMARY

Eukaryotic initiation factor 5A (eIF-5A) is the only cellular protein known to contain the unusual amino acid hypusine. The exact *in vivo* function of eIF-5A, however, is to date unknown. The finding that eIF-5A is an essential cofactor of the human immunodeficiency virus type 1 (HIV-1) Rev RNA transport factor suggested that eIF-5A is part of a specific nuclear export pathway. In this study we used indirect immunofluorescence and immunogold electron microscopy to demonstrate that eIF-5A accumulates at nuclear pore-associated intranuclear filaments in

mammalian cells and *Xenopus* oocytes. We are able to show that eIF-5A interacts with the general nuclear export receptor, CRM1. Furthermore, microinjection studies in somatic cells revealed that eIF-5A is transported from the nucleus to the cytoplasm, and that this nuclear export is blocked by leptomycin B. Our data demonstrate that eIF-5A is a nucleocytoplasmic shuttle protein.

Key words: eIF-5A, CRM1, Nuclear pore complex, Nuclear export, HIV-1, Rev

INTRODUCTION

Human eukaryotic initiation factor 5A (eIF-5A) is a small acidic protein of 154 amino acids (aa) with a molecular mass of 16.7 kDa (Smit-McBride et al., 1989a). eIF-5A is unique, because it is the only cellular protein known to date to contain the unusual amino acid hypusine (reviewed by Park et al., 1993a). Although hypusine-modified eIF-5A appears to be essential for eukaryotic cell proliferation (reviewed by Park et al., 1993b), its precise function still remains elusive. Its designation as an 'initiation factor' originated from the initial finding that it stimulates the formation of the dipeptide analogue methionyl-puromycin in an *in vitro* assay that mimics the formation of the first peptide bond in protein synthesis (Park, 1989; Smit-McBride et al., 1989b). The activity of eIF-5A in this model assay, however, appears to be poor (Kang et al., 1993). More recent *in vivo* data has clearly indicated that eIF-5A is not an initiator of general protein translation. In fact, complete intracellular depletion of eIF-5A in the yeast *Saccharomyces cerevisiae* does not cause major changes in the overall rate of protein synthesis (Kang and Hershey, 1994). Thus, the precise *in vivo* function of eIF-5A in eukaryotic cells is as yet unknown.

Investigation of the posttranscriptional regulation of human immunodeficiency virus type 1 (HIV-1) gene expression indicated a potentially novel eIF-5A activity *in vivo*. HIV-1 requires the action of the viral Rev trans-activator protein in order to produce progeny viruses (for a detailed review see

Pollard and Malim, 1998). Rev constantly shuttles between the nucleus and cytoplasm of host cells and mediates the translocation of unspliced and incompletely-spliced viral mRNAs across the nuclear envelope. In particular, nuclear export of Rev is mediated by a distinct effector or activation domain, which is composed of four critically spaced leucine residues and also functions as a nuclear export signal (NES). Indeed, various proteins have been described that bind specifically to this domain and may therefore mediate Rev function. This includes the human nucleoporin-like protein hRIP/Rab (Bogerd et al., 1995; Fritz et al., 1995), the export factor CRM1 (Stade et al., 1997; Ossareh-Nazari et al., 1997; Fornerod et al., 1997a; Fukuda et al., 1997) and the hypusine-containing protein eIF-5A (Ruhl et al., 1993; Bevec et al., 1996). In the case of eIF-5A in particular, it has been possible to describe distinct eIF-5A mutant proteins that upon intracellular expression inhibit Rev trans-activation and thereby HIV-1 replication in human T-cells (Bevec et al., 1996; Junker et al., 1996). Furthermore, microinjection studies demonstrated that these eIF-5A mutant proteins or antibodies directed against eIF-5A block the nuclear export of Rev in somatic cells (Bevec et al., 1996; Schatz et al., 1998); this suggests that eIF-5A is part of a nucleocytoplasmic transport pathway.

In eukaryotic cells, the nuclear envelope creates two distinct cellular compartments that separate DNA replication and transcription from protein synthesis. Thus, a constant flow of macromolecules has to traverse the nuclear envelope in both

directions. This transport into and out of the nucleus is mediated by nuclear pore complexes (NPCs), which are supramolecular assemblies that are integral parts of the nuclear envelope (for recent reviews see Bastos et al., 1995; Davis, 1995; Pante and Aebi, 1996). The pore channel is flanked by two coplanar rings or annuli, one of which is attached to the cytoplasmic and the other to the nucleoplasmic pore margin (Akey and Radermacher, 1993; Jarnik and Aebi, 1991). The central plug or 'transporter assembly' is located within the pore channel through which active nucleocytoplasmic transport takes place (Akey and Goldfarb, 1989; Feldherr and Akin, 1997). In addition, short fibrils emanate from the cytoplasmic face of the NPCs (Franke and Scheer, 1974; Ris, 1991; Jarnik and Aebi, 1991). Significantly longer NPC-associated fibrils extend from the inner annulus into the nucleoplasm (Franke and Scheer, 1970, 1974; Jarnik and Aebi, 1991). It is conceivable that both of these fibrils provide docking sites in the receptor-mediated nucleocytoplasmic transport of proteins prior to their translocation through the NPC.

A large body of studies from different laboratories has shown that most proteins translocate through the NPC in an energy and signal-dependent manner (for details please refer to following reviews and references therein: Nigg, 1997; Corbett and Silver, 1997; Ohno et al., 1998; Görlich, 1998; Melchior and Gerace, 1998). As mentioned above, nuclear export is mediated by NES sequences. The prototypic NES was originally identified in the activation domain of the HIV-1 Rev protein (Fischer et al., 1995; Wen et al., 1995). Structurally and functionally equivalent signals have since been described in various proteins, including mitogen-activated protein kinase kinase (MAPKK) (Fukuda et al., 1996), I κ B (Arenzana Seisdedos et al., 1997; Fritz and Green, 1996), which is an inhibitor of the transcription factor NF- κ B, and cAMP-dependent protein kinase inhibitor (PKI) (Wen et al., 1995; Fridell et al., 1996a). For these examples, it has subsequently been shown that the importin- β -like factor CRM1 is essential for their nuclear export, suggesting that CRM1 is a general export receptor for leucine-rich NESs in eukaryotic cells (Stade et al., 1997; Ossareh-Nazari et al., 1997; Fornerod et al., 1997a; Fukuda et al., 1997). Furthermore, it has been shown that CRM1 interacts with leucine-rich NESs and Ran-GTP (Stade et al., 1997; Fornerod et al., 1997a). This finding suggests that translocation of the export substrate through the NPC is mediated by CRM1 and that Ran-GTP promotes CRM1/NES interaction. However, it remains to be seen whether additional adaptor molecules are required to provide a means of specific regulation.

In the present study we report the subcellular localization of eIF-5A in mammalian cells and *Xenopus* oocytes. We show, that eIF-5A accumulates at the nucleoplasmic face of the NPC and in particular at the NPC-associated filaments. Furthermore, eIF-5A interacts with CRM1 and is translocated from the nucleus to the cytoplasm. Thus, eIF-5A displays characteristics that are typical of a factor that constantly shuttles between the nucleus and cytoplasm of eukaryotic cells.

MATERIALS AND METHODS

Molecular clones

The construct pGEX-eIF-5A is a bacterial expression vector that

expresses human eIF-5A fused to the carboxy terminus of glutathione S-transferase (GST) (Bevec et al., 1996). The vector expressing eIF-5A/VSV-G fusion protein was constructed by cloning a cDNA copy of the eIF-5A coding sequence between the *Hind*III and *Xho*I sites of the vector pcDNA3 (Invitrogen, NV Leek, Netherlands). Subsequently, a double-stranded oligonucleotide, encoding the vesicular stomatitis virus G-protein (VSV-G)-derived sequence (NH₂-PGPTDIEMNRLGK-COOH) (Kreis, 1986) was ligated between the *Xho*I and *Xba*I sites of this plasmid, resulting in the expression vector p3eIF-5A/VSV-G. The plasmid p3CANc/VSV-G was prepared by straightforward exchange of the eIF-5A gene in p3eIF-5A/VSV-G for the gene sequence encoding the CAN carboxy terminus (CANc, aa 1864-2090) (von Lindern et al., 1992). For this, the CANc sequence was isolated from a HeLa cell-derived cDNA as a 0.7 kb *Hind*III-*Xho*I fragment by PCR technology, using the following primers to introduce terminal *Hind*III and *Xho*I sites, respectively: sense 5'-AAGCTT-ATGCAGCAATCATCCTCTCCAGTGG-3'; anti-sense 5'-CTCG-AGGCTTCGCCAGCCACCAAAACCC-3'. The PCR conditions included 35 cycles of denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute and primer extension at 72°C for 1 minute.

Biological materials, oocyte fractionation and preparation of oocyte extracts

Xenopus laevis were purchased from the South African Snake Farm (Fish Hoek, Cape Province, South Africa). Pieces of *Xenopus* ovary were freshly removed from animals and placed in modified Barth's medium (Gurdon and Wickens, 1983). Subsequently, oocytes from different stages (Dumont, 1972) were isolated by manual separation from the follicle cells. Nuclei, nucleoplasm and nuclear envelopes from stage IV, V and VI oocytes were isolated manually as described in detail previously (Krohne and Franke, 1983).

For preparation of total oocyte extracts, stage VI oocytes were manually separated as described above, homogenized in extract buffer (250 mM sucrose, 100 mM NaCl, 2.5 mM MgCl₂, 0.1 mM PMSF, 20mM Hepes, pH 7.2) and cleared twice by centrifugation at 13,000 g for 10 minutes as described previously (Yang et al., 1998).

Mammalian cells were cultured at 37°C and 5% CO₂. Vero and HeLa cells were maintained in minimum essential medium (MEM) containing 5% or 10% fetal calf serum (FCS), respectively (GibcoBRL, Eggenstein, Germany).

Antibodies

The rabbit polyclonal anti-eIF-5A-specific antibody (α -eIF-5A) used for the detection of mammalian or *Xenopus* eIF-5A has been described previously (Schatz et al., 1998). Monoclonal murine antibody RL1 raised against pore complexes (Snow et al., 1987; Holt et al., 1987) was a kind gift from Dr L. Gerace (The Scripps Research Institute). The murine monoclonal antibody P5D4 is directed against an epitope of the VSV-G protein (Kreis, 1986). The rabbit polyclonal anti-CRM1 antiserum (Kudo et al., 1997) was kindly provided by Dr M. Yoshida (The University of Tokyo). The rabbit polyclonal anti-*Xenopus* Tpr antiserum (xITpr-Pep3) (Cordes et al., 1997) and the monoclonal antibody PF790x7A8 directed against Nup153 (Cordes et al., 1993) were kind gifts from Dr W. Franke (German Research Cancer Center). Monoclonal antibodies specific for GST or bovine serum albumin (BSA) were obtained from Serotec (Oxford, England) or Sigma (Deisenhofen, Germany), respectively.

Purification of GST fusion protein

GST/eIF-5A fusion protein was expressed in *Escherichia coli* BL21 and purified from crude lysates by affinity chromatography with glutathione-Sepharose 4B according to the manufacturer's specifications (Pharmacia Biotech, Freiburg, Germany). Eluted eIF-5A-containing fractions were identified by western immunoblot analysis, pooled, concentrated by ultrafiltration with a PM10 filter device (Amicon Inc., Beverly, Massachusetts), and stored at -70°C.

Immunofluorescence microscopy

Indirect immunofluorescence studies were performed by fixing cultured cells with 3% paraformaldehyde for 30 minutes. Subsequently, cells were permeabilized using 0.1% Triton X-100 (Sigma, Deisenhofen, Germany) for 4 minutes and blocked with 1% BSA for 20 minutes at ambient temperature. Proteins were stained for 30 minutes with α -eIF-5A (1:300 dilution), mAb P5D4 (1:100 dilution), mAb RL1 (1:50 dilution), anti-CRM1 (1:200 dilution) or anti-GST (1:50 dilution) antibodies. Following extensive wash steps in PBS, cells were incubated with appropriate secondary antibodies coupled to Cy2 or Cy3 fluorophores (Biotrends, Köln, Germany) for 30 minutes. The samples were then washed in PBS, mounted in Mowiol (Calbiochem, La Jolla, California) and analyzed using a Zeiss Axiovert-135 microscope. Images were recorded with a cooled MicroMax CCD camera (Princeton Instruments, Stanford, California) and processed using the IPLab spectrum and Adobe Photoshop software package.

Small pieces of *Xenopus* ovary were shock frozen in isopentane cooled by liquid nitrogen. Frozen sections 5 μ m thick were air dried and incubated with 2% paraformaldehyde for 10 minutes. Subsequently, the sections were incubated with α -eIF-5A (at a 1:250 dilution) for 30 minutes followed, after several wash steps in PBS, by secondary Texas Red-conjugated anti-rabbit antibodies (Jackson Immunoresearch Laboratories, Baltimore, Maryland). Images were taken with a Zeiss Axiophot microscope equipped with epifluorescence optics and the appropriate filter sets.

Electron microscopy

Procedures used for EM immunocytochemistry of isolated nuclear envelopes from *Xenopus* oocytes have been described (Dabauvalle et al., 1988). The manually isolated and unfixed nuclear envelopes were incubated with anti-eIF-5A (α -eIF-5A; diluted 1:250) antibodies alone or in combination with anti-Nup153 antibodies (undiluted), followed by incubation for 3 hours with secondary antibodies coupled to 6 nm or 12 nm gold particles (Dianova, Hamburg, Germany) (diluted 1:10). In another set of experiments isolated nuclear envelopes were first incubated with GST/eIF-5A fusion protein (diluted 1:100 in PBS containing 100 μ M GTP) for 60 minutes. After several wash steps in PBS the nuclear envelopes were incubated with anti-GST antibodies alone (diluted 1:50) or in combination with anti-Tpr antiserum (diluted 1:200), followed by incubation as described above with secondary gold-coupled antibodies. Cryostat sections (5 μ m) of *Xenopus* ovaries were fixed with 2% paraformaldehyde in PBS for 10 minutes and incubated with α -eIF-5A for 1 hour. After thorough washing with PBS, anti-rabbit IgG coupled to 12 nm gold particles was added for 3 hours at room temperature. Specimens were then washed in PBS, postfixed, and processed for EM as described (Benavente et al., 1985).

Cultured Vero cells grown on coverslips were fixed with 2% paraformaldehyde in PBS for 5 minutes. After several wash steps in PBS, the cells were permeabilized by treatment with 0.2% Triton X-100 in PBS for 5 minutes and washed again. The specimens were then incubated for 60 minutes with α -eIF-5A followed by an overnight incubation with the secondary gold-coupled antibodies and processed for EM as described above for the cryostat sections.

Microinjection studies

Vero cells were microinjected in the nucleus with a CompiC INJECT computer-assisted injection system (Cellbiology Trading, Hamburg, Germany) in combination with an Eppendorf microinjector 5242 (Eppendorf Gerätebau, Hamburg, Germany). p3eIF-5A/VSV-G or p3CANc/VSV-G plasmid DNAs were injected at a concentration of 100 ng/ μ l. 22 hours after injection, cells were fixed and subsequently double-stained as described above, using anti-VSV-G (mAb P5D4) and anti-CRM1 or anti-eIF-5A (α -eIF-5A) antibodies.

The GST/eIF-5A or GST protein was injected at a concentration of 1.5 mg/ml, together with rabbit IgG (1.0 mg/ml). About 30 minutes

after injection, cells were fixed with 3% paraformaldehyde and analyzed as described before.

Protein gel electrophoresis and immunoblotting

Proteins were resolved by SDS-PAGE (Thomas and Kornberg, 1975) using 12% acrylamide. For immunoblots, polypeptides were electrophoretically transferred from gels to nitrocellulose. Membranes were blocked by overnight incubation with 10% nonfat dry milk in 150 mM NaCl, 10 mM Tris-HCl, 0.05% Tween-20, pH 8.0 (TBST) at 4°C, then incubated for 2 hours at 25°C with α -eIF-5A antibodies (diluted 1:1000). After several washes in TBST appropriate secondary antibodies coupled to peroxidase (Dianova, Hamburg, Germany) were added at a dilution of 1:10,000 in TBS containing 5% dry milk and incubated for 1 hour at room temperature. After several wash steps, bound antibodies were visualized using the enhanced chemical luminescence detection system (ECL) (Amersham Buchler, Braunschweig, Germany).

Overlay blot assays and pull-down experiments

Overlay blot assays were performed as described previously (Lounsbury et al., 1994) using GST or GST/eIF-5A fusion protein. Bound proteins were visualized by incubation with anti-GST (diluted 1:500) antibodies. For some experiments the nitrocellulose membrane was stripped of bound antibodies and reprobed as described in the manufacturer's protocol (Amersham Buchler, Braunschweig, Germany) with anti-CRM1 antiserum (diluted 1:250).

Pull-down experiments were essentially performed as described previously (Yang et al., 1998). In brief, GST or GST/eIF-5A fusion proteins were coupled to glutathione-Sepharose beads and blocked with 1% BSA in extract buffer for 45 minutes at ambient temperature. The beads were then incubated for an hour with total oocyte extract in extract buffer containing 1% BSA and subsequently washed three times using the same buffer. Finally, the binding proteins were resolved by SDS-PAGE followed by immunoblotting using anti-CRM1 antiserum (diluted 1:250). Unbound proteins were precipitated with acetone at -20°C, resuspended and analyzed identically.

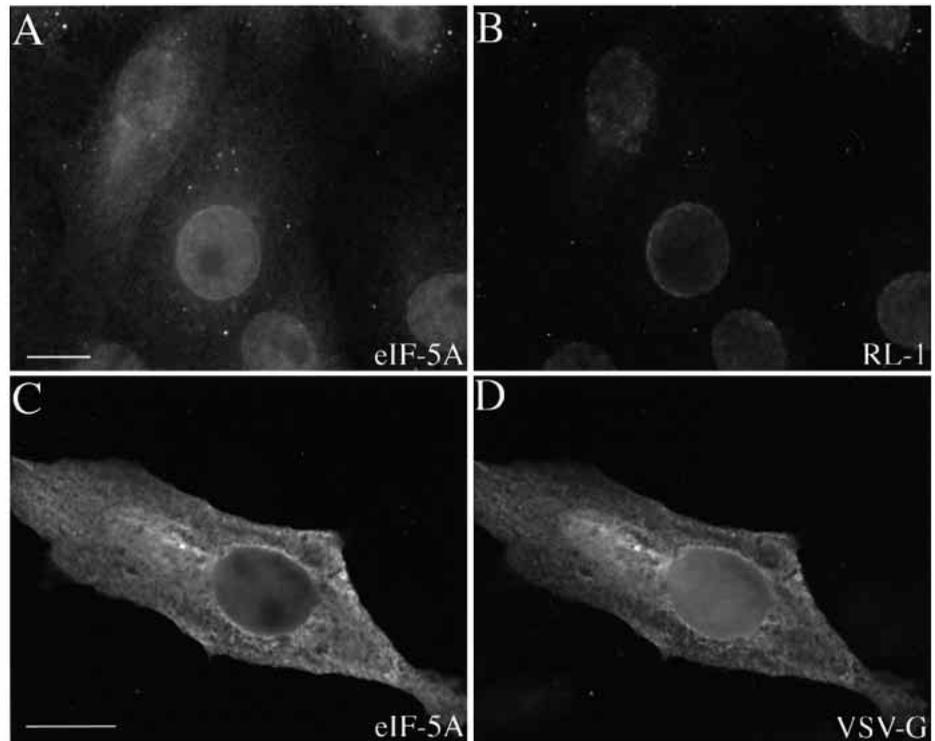
RESULTS

Localization of eIF-5A in mammalian cells

To characterize the subcellular localization of eIF-5A in detail, we investigated the intracellular distribution of eIF-5A in Vero cells by indirect double-label immunofluorescence microscopy. As shown in Fig. 1A, staining of endogenous eIF-5A with a specific rabbit polyclonal anti-eIF-5A antibody (α -eIF-5A) resulted in cytoplasmic and nucleoplasmic signals. The antibody also showed a moderate nuclear rim staining pattern that co-aligned with the signal obtained with mAb RL1 (Fig. 1B), an antibody directed against a group of O-linked glycoproteins of the NPC (Snow et al., 1987; Holt et al., 1987).

To control for undesired cross-reactions from the antibody used, we microinjected Vero cell nuclei with an expression plasmid that encodes epitope-tagged eIF-5A. In this expression vector, a heterologous sequence derived from the vesicular stomatitis virus G protein (VSV-G) is fused in-frame to the carboxyterminus of the human *eIF-5A* gene; this allows the intracellular detection of this eIF-5A/VSV-G fusion protein using a mouse monoclonal anti-VSV-G antibody (mAb P5D4) (Kreis, 1986). Clearly, overexpression of this fusion protein resulted in an increased cytoplasmic eIF-5A-specific signal. In agreement with the subcellular localization of endogenous eIF-5A, however, nucleoplasmic and nuclear rim staining patterns were also observed in these overexpressing cells (Fig. 1C,D).

Fig. 1. Localization of eIF-5A in cultured Vero cells by immunofluorescence microscopy. eIF-5A localizes in the cytoplasm and nucleus, particularly accumulating at the nuclear envelope. (A and B) Colocalization of eIF-5A with proteins of the nuclear pore complex. Double immunofluorescence of Vero cells with antibodies to eIF-5A (α -eIF-5A; A) and nucleoporins (mAb RL1; B). Bar, 20 μ m. (B and D) Detection of eIF-5A/VSV-G fusion protein. Vero cells were microinjected into the nucleus with p3eIF-5A/VSV-G expression plasmid DNA, subsequently cultured for 22 hours and analysed by double-label immunofluorescence microscopy using α -eIF-5A (C) and mAb P5D4 (α -VSV-G; D) antibodies. Bar, 20 μ m.



To investigate whether or not the nuclear rim accumulation of eIF-5A reflected binding to NPCs, we next employed preembedding immunogold electron microscopy. Strikingly, eIF-5A-specific gold clusters were enriched at the nucleoplasmic side of the NPC (Fig. 2A), in close association with fibrils that emanate from the nucleoplasmic surface of the NPC into the nuclear interior (Fig. 2B,C). This site of accumulation suggests that eIF-5A is bound to these NPC-attached nucleoplasmic filaments, which are thought to be the site within the NPC at which export substrates initially bind during nucleocytoplasmic translocation.

eIF-5A is present in *Xenopus* oocytes

Having localized eIF-5A in mammalian cells, we next examined whether eIF-5A is also detectable in *Xenopus*

oocytes. For this, total RNA from non-induced and hormone-induced oocytes of different stages (stage I, II, IV, V and VI) were isolated and subjected to *eIF-5A*-specific Northern blot analysis. As shown in Fig. 3A, no oocyte-derived *eIF-5A* mRNAs were apparent in this experiment (lane 1 to 6), irrespective of the sort of oocytes used, although the same hybridisation probe detected *eIF-5A* gene sequences in *Xenopus* DNA (Ruhl et al., 1993). In contrast, high levels of *eIF-5A* transcripts were present in total RNA isolated from HeLa cells (lane 7). These data are in agreement with previous reports (Ruhl et al., 1993; Bevec et al., 1994). Of note is the fact that we were also not able to detect *eIF-5A*-specific mRNA employing RT-PCR analysis (not shown).

Next we performed a western blot analysis of *Xenopus* oocyte proteins isolated from different oocyte stages. Using the

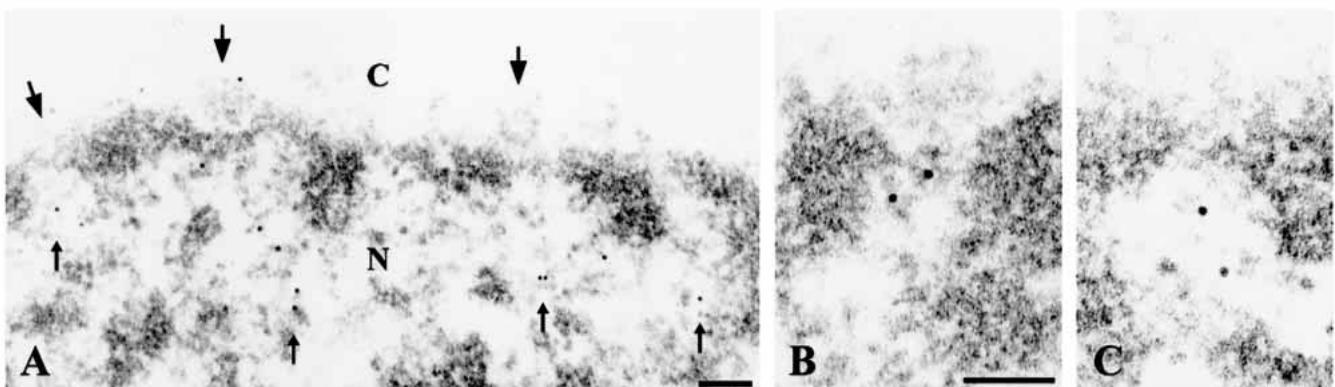


Fig. 2. Preembedding immunogold EM localization of eIF-5A. Vero cells were fixed with paraformaldehyde, Triton-permeabilized and incubated with α -eIF-5A followed by secondary gold-conjugated antibodies. (A) NPCs (indicated by large arrows) appear to be selectively labeled at the nucleoplasmic surface by eIF-5A-specific gold particles (small arrows). C, cytoplasm; N, nucleoplasm. Bar, 0.1 μ m. (B and C) eIF-5A appears to be associated with nucleoplasmic fibrils that emanate from the NPC into the nucleoplasm. Bar, 0.1 μ m.

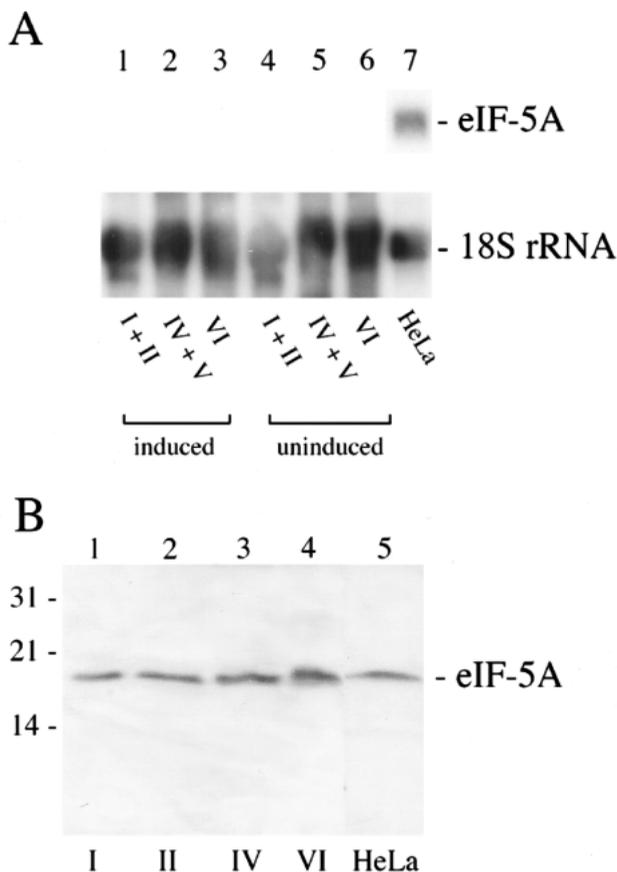


Fig. 3. Detection of eIF-5A in *Xenopus* oocytes. (A) Northern blot analysis using an eIF-5A-specific radiolabeled oligonucleotide probe and 15 μ g of total RNA from hormone-induced and uninduced oocytes of different stages (indicated by roman digits; lane 1 to 6) or from HeLa cells (lane 7). To control for loading of comparable quantities of RNA, the filters were stripped and rehybridized using a 18S ribosomal RNA probe. (B) Immunoblots using α -eIF-5A antibodies. Proteins from stage I, II, IV or VI oocytes (indicated at the bottom; lane 1 to 4) or from HeLa cell nuclei (lane 5) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with eIF-5A-specific antibodies. Molecular mass standards are indicated in kDa on the left.

α -eIF-5A antibody, an eIF-5A-specific signal was clearly detectable in all oocytes examined, as well as in the nuclei of HeLa cells (Fig. 3B). In agreement with previous reports (Cooper et al., 1982; Smit-McBride et al., 1989b), the eIF-5A protein was migrating at a relative molecular mass of ~18 kDa. Subsequent fractionation of oocytes revealed that eIF-5A is present in both the nucleus and cytoplasm (not shown), reflecting the subcellular distribution of eIF-5A seen in mammalian cells (Klier et al., 1995). Taken together the combined data suggest that the eIF-5A protein present in *Xenopus* oocytes may be of maternal origin.

To characterize further the nuclear envelope localization of eIF-5A in oocytes, frozen ovary sections (5 μ m) were investigated by immunofluorescence microscopy. As expected eIF-5A staining exhibited a pronounced localization at the nuclear envelope (compare Fig. 4A,B). In addition, some fluorescent dot-like structures were apparent in the cytoplasm of the oocytes (Fig. 4A, arrows), probably reflecting the

presence of annulate lamellae (Dabauvalle et al., 1991; Ewald et al., 1996), which are pore complexes that normally occur in the cytoplasm of a wide variety of cells and notably oocytes. In order to obtain a more detailed understanding of the nuclear localization of eIF-5A at the ultrastructural level, isolated nuclear envelopes (Fig. 4C to F) and frozen sections (Fig. 4G) were subjected to preembedding immunoelectron microscopy. The gold particles decorated the nucleoplasmic periphery of the nuclear envelope. Interestingly, at higher magnification the gold label was found to decorate the intranuclear NPC-attached filaments in particular (Fig. 4D to F). To confirm this observation, we performed the same analysis using oocyte cryosections. Again, eIF-5A-specific gold particles were found to localize at the nuclear pore-associated intranuclear filaments (Fig. 4G).

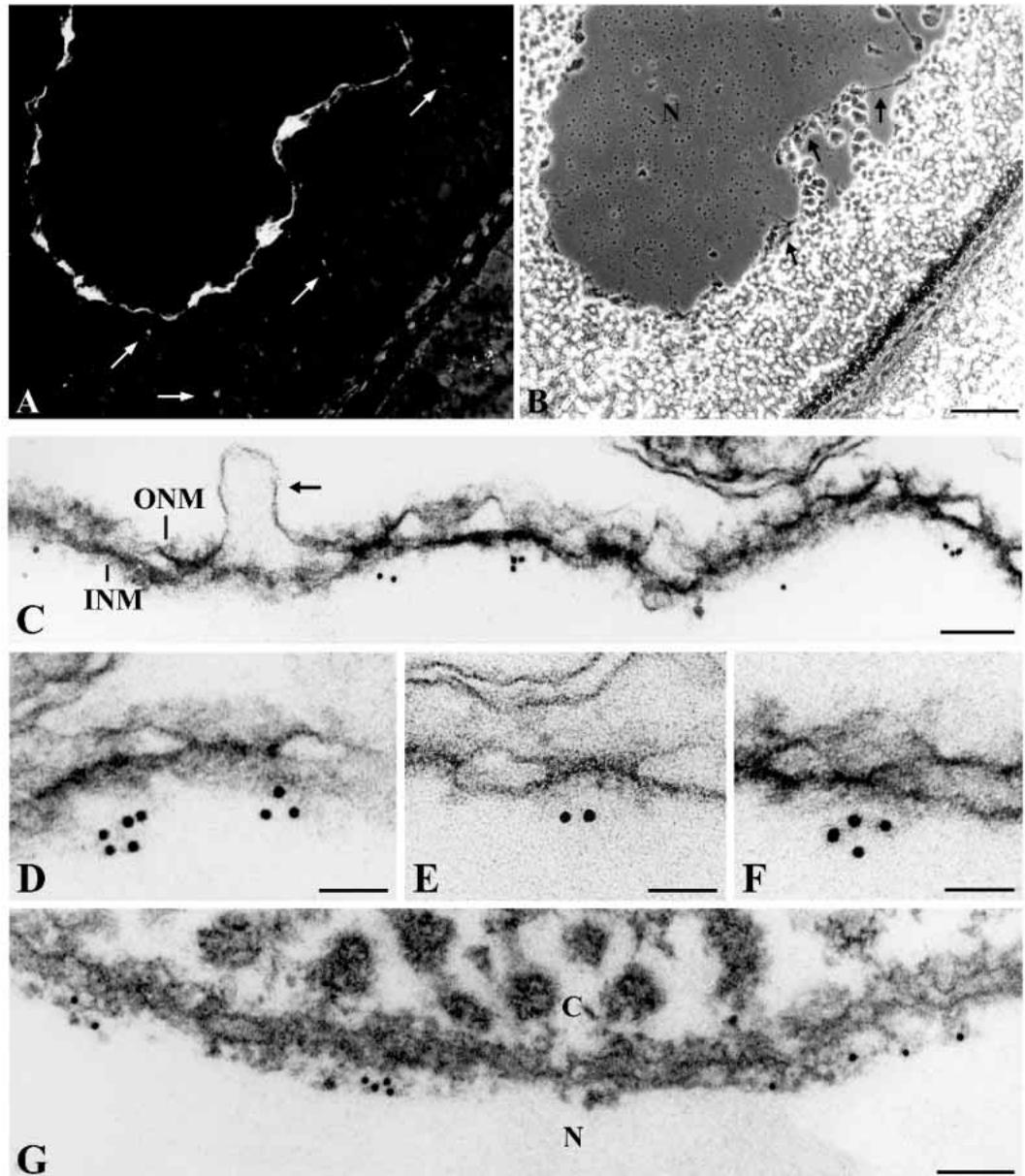
In another set of experiments, isolated nuclear envelopes were incubated with recombinant glutathione S-transferase (GST)/eIF-5A fusion protein and subsequently examined by immunogold electron microscopy, using a monoclonal antibody directed against GST. As shown (Fig. 5A to C), GST/eIF-5A fusion protein accumulated exclusively at the nucleoplasmic surface of NPCs, particularly at the intranuclear NPC-attached filaments, exactly replicating the localization pattern seen for endogenous eIF-5A. To unequivocally identify the topology of the nuclear envelopes, we next performed double immunogold labeling using anti-GST and anti-Tpr or anti-eIF-5A and anti-Nup153 antibodies, respectively. Both, the nucleoporin Nup153 and Tpr are components of the NPC-associated nucleoplasmic filaments (Bastos et al., 1996; Cordes et al., 1997). As expected, both proteins clearly co-localized with eIF-5A (Fig. 5D,E). Of note, double-labeling experiments using antibodies directed against Nup180, which is a constituent of the cytoplasmic NPC-associated fibrils (Wilken et al., 1995), verified that eIF-5A accumulates exclusively at the nucleoplasmic face of NPCs (data not shown).

The combined data demonstrated, that eIF-5A is present in *Xenopus* oocytes and specifically accumulates, as it does in mammalian cells, at NPC-attached nucleoplasmic filaments.

Interaction of eIF-5A with CRM1

The accumulation of eIF-5A at the nucleoplasmic periphery of the NPC indicated that eIF-5A might directly interact with components of the NPC. To observe NPC proteins that bind eIF-5A, *Xenopus* oocyte nuclear envelopes were isolated manually and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the separated proteins were transferred onto nitrocellulose filters and the filters were incubated with recombinant GST/eIF-5A or GST protein. Bound GST/eIF-5A or GST protein was visualized by standard western analysis using anti-GST antibodies. As shown in Fig. 6A, the GST/eIF-5A protein bound to several distinct proteins in this overlay blot assay (lane 1). Control experiments confirmed that GST alone does not bind (lane 2). Clearly, the most prominent binding signal in the GST/eIF-5A overlay blot assay originated from a ~110 kDa protein (denoted by a dot; Fig. 6A, lane 1). When this blot was stripped of the bound anti-GST antibodies and re-probed with anti-CRM1 antibodies (Kudo et al., 1997), the same ~110 kDa protein was labeled (Fig. 6A, lane 3), suggesting that eIF-5A binds to the general export receptor CRM1. In order to verify the interaction of eIF-5A with CRM1 in an independent

Fig. 4. Visualization of eIF-5A in *Xenopus* oocytes by light and electron microscopic immunocytochemistry using α -eIF-5A. (A) Nuclear envelope localization of eIF-5A as demonstrated on a (5 μ m) frozen oocyte section by indirect immunofluorescence analysis. Fluorescent dots seen in the cytoplasm most likely reflect the presence of annulate lamellae (indicated by arrows). (B) Corresponding phase contrast image of A. The nuclear envelope is indicated by arrows. N, nucleoplasm. Bar: 500 μ m. (C-F) Preembedding immunogold EM localization of eIF-5A on manually isolated nuclear envelopes from oocytes. The inner nuclear face of NPCs is selectively labeled as seen in transversely sectioned nuclear envelopes. The outer nuclear membrane (ONM) often forms vesicles (arrow in C; INM, inner nuclear membrane. Bar: 0.1 μ m. (D-F) eIF-5A-specific gold clusters accumulate at nucleoplasmic NPC-associated fibrils. Bar: 0.05 μ m. (G) Cryosection of manually isolated oocytes. C, cytoplasm; N, nucleoplasm. Bar: 0.1 μ m.



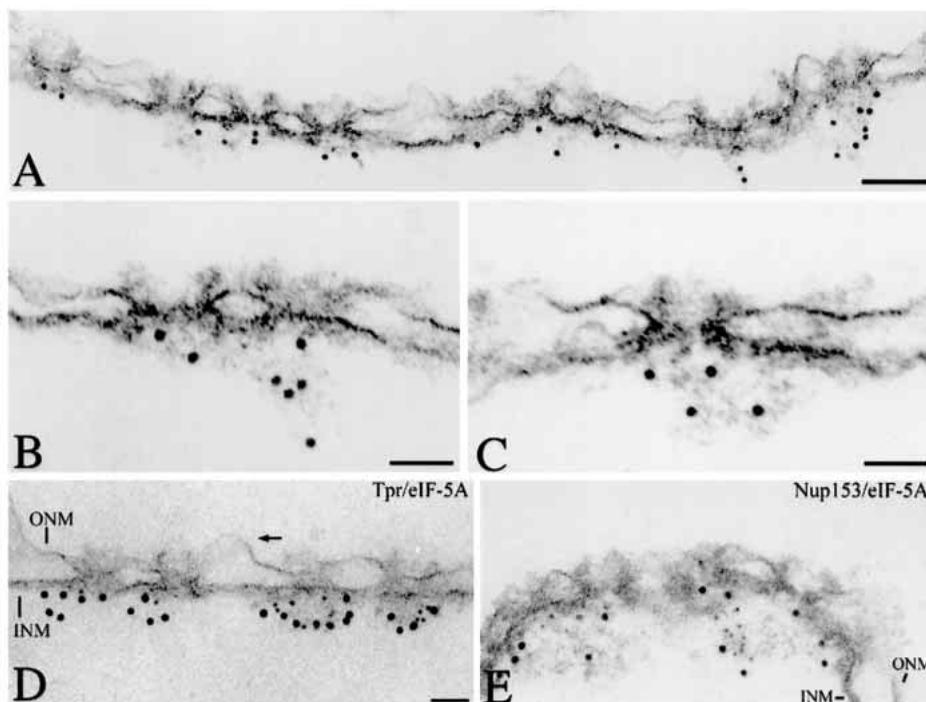
assay, we next performed binding studies in solution. GST/eIF-5A fusion protein was immobilized on glutathione-Sepharose beads and incubated with total extracts from stage VI oocytes. The beads were then pelleted by centrifugation and the bound and unbound material was analysed by western blot using the anti-CRM1 antiserum. As shown, CRM1 bound the GST/eIF-5A fusion protein (Fig. 6B, compare lane 1 and 1'), but not GST alone (Fig. 6B, compare lane 2 and 2') in this assay. In addition to the intact CRM1 protein, a ~66 kDa protein that cross-reacted with the anti-CRM1 antiserum was also always observed. This protein appears to be a specific CRM1 degradation product, since it is also the main product when CRM1 is expressed as a recombinant protein in *E. coli* (O. Rosorius, unpublished results).

CRM1 has been shown to localize in the nucleus as well as at both the nucleoplasmic and cytoplasmic surfaces of NPCs (Fornerod et al., 1997b). It has a significant homology to importin- β (Fornerod et al., 1997b; Stade et al., 1997; Görlich

et al., 1997) and associates in particular with the nucleoporin CAN (Fornerod et al., 1996). CAN by itself has been reported to accumulate at the cytoplasmic and also, in overexpressing cells, at the nucleoplasmic surface of NPCs (Kraemer et al., 1994; Boer et al., 1997). Furthermore, when expressed alone, the CAN carboxy terminus localizes to the nucleoplasm and, importantly, causes the disappearance of CRM1 from the nuclear envelope (Fornerod et al., 1995; Fornerod et al., 1997b). This can be explained by the fact that the carboxy-terminal half of CAN contains the nucleoporin-specific repeat region and the CRM1 binding site (Fornerod et al., 1996).

Since eIF-5A accumulates at the nucleoplasmic surface of the NPC (Figs 2, 4 and 5) and appears to bind CRM1 (Fig. 6), we presumed that expression of the CAN carboxy terminus might affect the *in vivo* localization of eIF-5A as well as CRM1. To test this hypothesis, we microinjected Vero cells with an expression vector that encodes the CAN carboxy terminus (CANc, aa 1864-2090) (von Lindern et al., 1992)

Fig. 5. Accumulation of eIF-5A at NPC-associated nucleoplasmic filaments. (A-D) Isolated nuclear envelopes were incubated with GST/eIF-5A fusion protein and subsequently analyzed using anti-GST antibodies alone (A-C) or in combination with anti-Tpr antibodies (D). (A) The nucleoplasmic surface is selectively labeled. Bar, 0.1 μ m. (B and C) The gold particles are specifically localized on the intranuclear filaments that are associated with the NPCs. Bars, 0.05 μ m. (D) eIF-5A (6 nm gold particles) localizes at Tpr-labeled (12 nm gold particles) intranuclear filaments. The outer nuclear membrane (ONM) forms vesicles (indicated by an arrow; INM, inner nuclear membrane; Bar, 0.05 μ m). (E) Double immunogold labeling of Nup153 (6 nm gold particles) and endogenous eIF-5A (12 nm gold particles).



fused to the VSV-G epitope-tag. Subsequent double-label immunofluorescence microscopy experiments using anti-VSV-G (mAb P5D4) and polyclonal anti-CRM1 antibodies revealed, as reported previously (Fornerod et al., 1997b), a marked effect on CRM1 subcellular localization, in cells overexpressing CANc (compare A and B, Fig. 7). A comparable effect was also seen using the α -eIF-5A antibody in these experiments (Fig. 7C,D). Control cells displayed the typical eIF-5A localization pattern in the cytoplasm, nucleoplasm and particularly at the nuclear envelope. In sharp contrast, overexpression of CANc abrogated the accumulation of eIF-5A at the nuclear envelope, resulting in equal distribution of the protein throughout the cell. Thus, overexpression of the carboxy terminus of CAN affects the subcellular localization of both, CRM1 and eIF-5A.

Nuclear export of eIF-5A

A hallmark of soluble nuclear export factors is their capacity to shuttle between the cellular compartments. Foremost is the fact that these factors are actively exported from the nucleus to the cytoplasm. The observation presented in this study, that eIF-5A localizes at the nucleoplasmic surface of NPCs, and previous data demonstrating that eIF-5A is a cofactor of HIV-1 Rev (Ruhl et al., 1993; Bevec et al., 1996), suggested that eIF-5A alone might be exported from the nucleus to the cytoplasm. To examine potential eIF-5A export activity we microinjected GST/eIF-5A fusion protein directly into the nucleus of Vero cells. In addition, rabbit IgG was coinjected in these experiments in order to establish the site of injection. After a period of ~30 minutes, cells were fixed and the injected proteins were visualized by double-label immunofluorescence microscopy. Clearly, a significant amount of the nuclear injected GST/eIF-5A protein was transported to the cytoplasm while, in contrast, the rabbit IgG injection control remained in the nucleus (see Fig. 8A,B). When the same experiment was carried out at low temperature, no nuclear export of eIF-5A

was detected (Fig. 8C,D), indicating the energy requirement for the nuclear export of eIF-5A. Importantly, nuclear export was also not observed in these experiments when the GST/eIF-5A protein was substituted by wild-type GST (Fig. 8G,H).

Leptomycin B (LMB) is a cytotoxin that has previously been shown to block the nucleocytoplasmic translocation of HIV-1 Rev (Wolff et al., 1997) by apparently preventing the formation of stable NES-CRM1 complexes (Ossareh-Nazari et al., 1997; Fornerod et al., 1997a; Fukuda et al., 1997). We therefore were also interested in testing the effect of LMB on eIF-5A trafficking. For this, the cell cultures were supplemented two hours prior to microinjection with LMB at a concentration of 5 nM. As shown, LMB totally abrogated the nuclear export of eIF-5A (Fig. 8E,F).

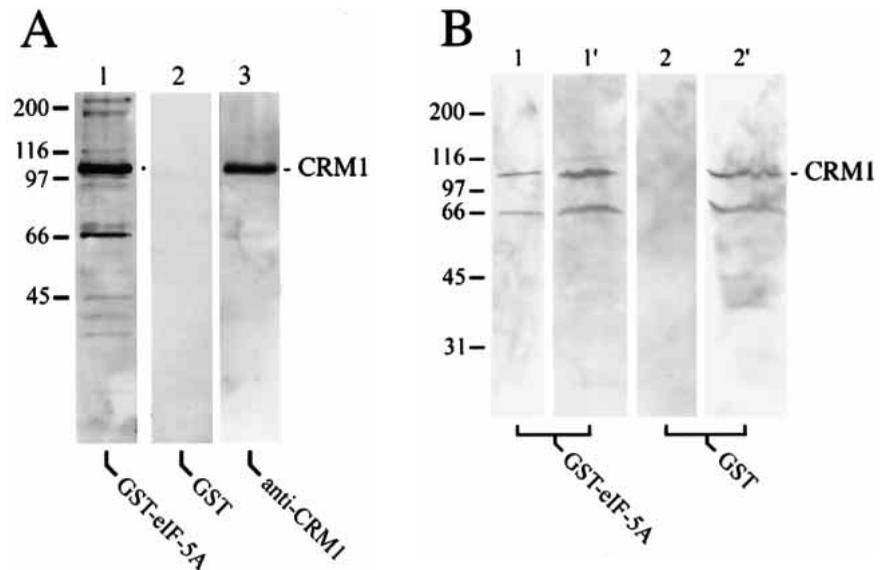
Taken together these data show that eIF-5A is exported from the nucleus to the cytoplasm in mammalian cells.

DISCUSSION

In the present study we have demonstrated that the hypusine-containing protein eIF-5A accumulates at the nucleoplasmic surface of NPCs. This localization and particularly the fact that eIF-5A has nuclear export capacity, indicate that this highly conserved protein might act in nucleocytoplasmic transport. This notion is in agreement with previous studies that identified eIF-5A as a cellular target of the activation domain of the HIV-1 Rev as well as of the human T-cell leukemia virus type I (HTLV-I) Rex trans-activator proteins (Ruhl et al., 1993; Katahira et al., 1995), both of which are retroviral regulatory factors required for the nuclear export of viral RNAs. Independent evidence in favour of this hypothesis has been provided by previous studies. Inhibitors of the hypusine-modification in eIF-5A cause the disappearance of specific mRNAs from polysomes (Hanuske-Abel et al., 1995), indicating that eIF-5A might be part of a nuclear export pathway involved in nucleocytoplasmic

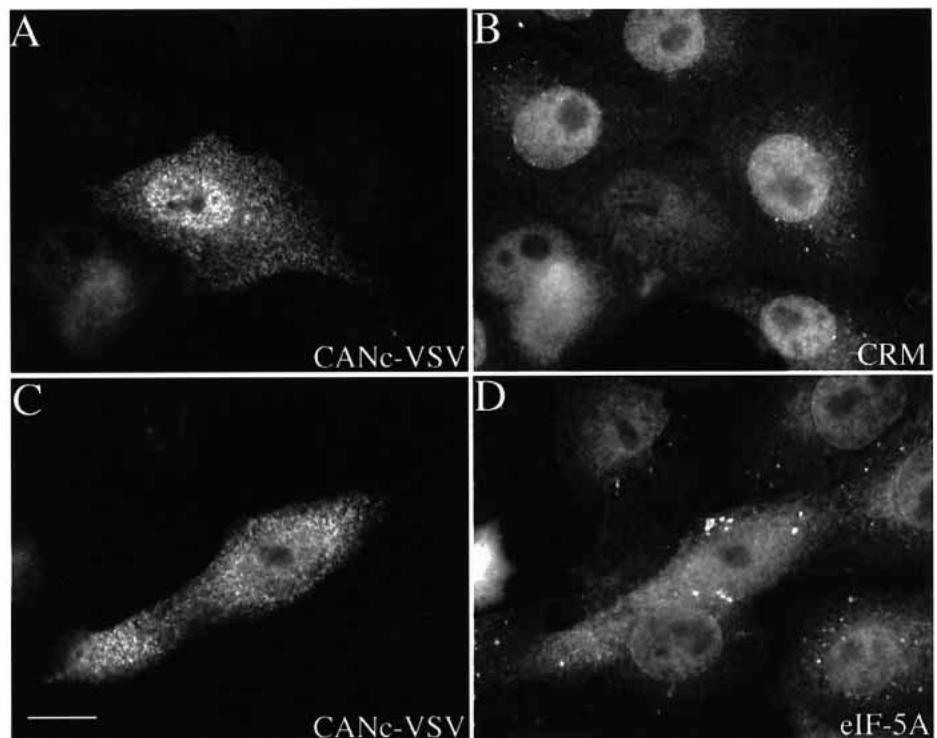
Fig. 6. Interaction of eIF-5A with CRM1.

(A) Interaction of GST/eIF-5A fusion protein (lane 1) or GST protein (lane 2) with nuclear envelope-associated proteins as determined by overlay blot assay. Membranes were stripped of bound anti-GST antibodies and reprobbed with anti-CRM1 antiserum (lane 3). Molecular mass standards are indicated in kDa on the left. The location of CRM1 is indicated by a dot and denoted at the right. (B) Solution binding of CRM1 to GST/eIF-5A fusion protein. Glutathione-Sepharose beads coupled to the indicated GST proteins were incubated with total extracts of stage VI oocytes, pelleted and processed for immunoblotting with anti-CRM1 antiserum. Bound proteins are shown in lane 1 and 2 and the unbound material is shown in lane 1' and 2', respectively. Molecular mass standards are indicated in kDa on the left and CRM1 is denoted at the right. The signal at ~66 kDa corresponds to a frequently observed CRM1 degradation product.



translocation of specific RNAs. This notion is supported by a more recent work in which conditionally expressed *eIF-5A* (*HYP2*) genes were investigated in yeast (Liu and Tartakoff, 1997). In this study, extensive intracellular depletion of eIF-5A resulted in a marked accumulation of specific mRNAs in the nuclear compartment, suggesting that eIF-5A functions in the export of a subset of mRNAs. In an independent study, temperature-sensitive yeast strains were investigated. These data revealed that eIF-5A affects the decay of specific yeast mRNAs, acting after the decapping process and before these transcripts are degraded by the exonuclease Xrn1p (Zuk and Jacobson, 1998). Interestingly, Xrn1p appears to be a cytoplasmic protein (Johnson, 1997), suggesting that eIF-5A may act at the level of nuclear export of these transcripts.

The subcellular localization of eIF-5A in mammalian cells and in *Xenopus* oocytes is identical; eIF-5A localizes in both the cytoplasmic and nuclear compartments and is concentrated at the nucleoplasmic face of NPCs. The presence of eIF-5A in oocytes is of particular interest because the previous reporting that the *eIF-5A* gene is transcriptionally inactive in oocytes (Ruhl et al., 1993) has subsequently been incorrectly interpreted as a complete absence of eIF-5A protein. The data presented here clearly demonstrate that this is not the case. This finding is also important with respect to investigating HIV-1 Rev function. Currently it is not clear why discrepancies with respect to Rev activity in oocytes exist, depending on the Rev assay applied. For example, standard Rev trans-activation assays which rely on the microinjection of expression plasmids

**Fig. 7.** Effect of the CAN carboxy terminus on subcellular distribution of CRM1 and eIF-5A. (A to D) Vero cells were microinjected with a vector expressing the CAN carboxyterminus (CANc; aa 1864–2090) fused to an epitope-tag derived from the VSV-G protein. Subsequent double-label immunofluorescence analyses using anti-VSV-G (A and C) and anti-CRM1 (B) or α -eIF-5A (D) antibodies revealed redistribution of CRM1 and eIF-5A in cells overexpressing CANc. Bar, 20 μ m.

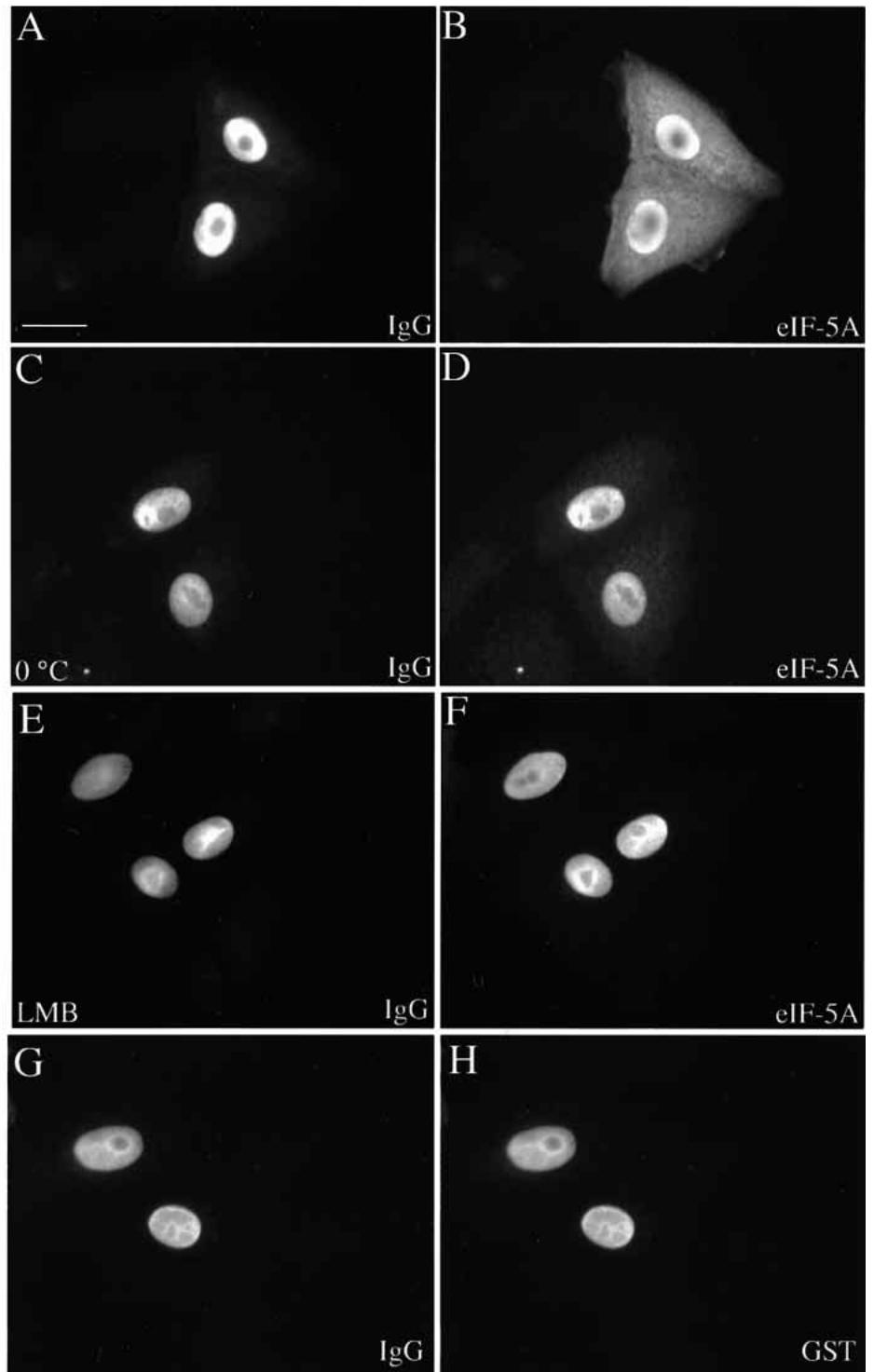


Fig. 8. Export of GST/eIF-5A from the nucleus to the cytoplasm in mammalian cells. (A to H) Vero cells were comicroinjected in the nucleus with GST/eIF-5A (eIF-5A) or GST and rabbit IgG (IgG, injection control). Cells were incubated ~30 minutes, fixed, and analyzed for GST-and rabbit IgG-specific localization by double-label indirect immunofluorescence microscopy. (A and B) Specific nuclear export of GST/eIF-5A. Bar, 20 μ m. Nuclear export of GST/eIF-5A is inhibited when microinjection and cell culture were carried out at 0°C (C and D) or in presence of 5 nM leptomycin B (LMB) (E and F). A control experiment demonstrated that wild-type GST protein is not exported from the nucleus to the cytoplasm (G and H).

encoding Rev and Rev-responsive reporter constructs fail to monitor Rev activity in oocytes (Ruhl et al., 1993). In contrast, assays dependent on the injection of (radiolabeled) Rev protein alone or in combination with radiolabeled RRE RNA demonstrate Rev export activity (Fischer et al., 1994, 1995; Stutz et al., 1996). Obviously, the simplest explanation for this discrepancy may be the sensitivity of the different assay systems employed. Detection of Rev activity using a standard trans-activation assay requires multiple activities at the level of

gene transcription, mRNA processing and translation and is significantly less sensitive than an assay that exclusively detects the nucleocytoplasmic translocation of a radiolabeled probe. Alternatively, injection of high levels of Rev protein into oocytes may mask the requirement of other essential factors, such as eIF-5A. This has been reported to be the case for the Ran- and p62-binding cytosolic import factor NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995). NTF2 is essential for cell viability in yeast (Nehrbass and Blobel, 1996;

Corbett and Silver, 1996). However *GSP1*, which encodes the yeast homologue of Ran/TC4, can function as a high copy number suppressor in a NTF2-deletion strain (Paschal et al., 1997). Furthermore, high levels of Ran/TC4 can relieve the requirement for NTF2 in a mammalian-permeabilized cell assay for nuclear protein import (Paschal et al., 1997).

Our finding that eIF-5A accumulates at NPC-associated intranuclear fibrils, the site where initial docking of export substrates to the NPC is conceived to take place and binds CRM1, provides further evidence that eIF-5A participates in nucleocytoplasmic transport processes. It is conceivable that eIF-5A may facilitate the interaction of the HIV-1 Rev NES and CRM1 at the nucleoplasmic site of the NPC. This model of eIF-5A function is in agreement with previously published *in vitro* binding studies, that investigated the co-operative interaction of the HIV-1 Rev NES with Ran-GTP and CRM1 (Fornerod et al., 1997a). These studies employed reticulocyte lysates, which are in fact a rich source of eIF-5A (Schatz et al., 1998). Interestingly, the Rev NES-CRM1 interaction appeared to be poor when purified proteins were used in a more recent study (Askjaer et al., 1998).

The concept that additional, possibly bridging molecules may be required to provide a higher degree of specificity to nucleocytoplasmic transport comes from considering the fact that CRM1 plays a central role in NES-mediated nuclear export (Stade et al., 1997; Ossareh-Nazari et al., 1997; Fornerod et al., 1997a; Fukuda et al., 1997). For example TFIIIA, which is involved in nucleocytoplasmic transport of 5S rRNA (Fridell et al., 1996b) and the yeast poly(A)⁺ RNA export factor Gle1p (Murphy and Went, 1996) are translocated in a NES-dependent manner from the nucleus to the cytoplasm. The same applies for proteins with no obvious activity in RNA export, such as the transcription factors p53 (Middeler et al., 1997), the nuclear factor of activated T-cells (NF-ATc) (Klemm et al., 1997), mdm-2 and IκB (the inhibitors of p53 and NF-κB, respectively) (Roth et al., 1998; Arenzana Seisdedos et al., 1997; Fritz and Green, 1996), and various other cellular proteins of different functions, including the aforementioned PKI and MAPKK (Wen et al., 1995; Fridell et al., 1996a; Fukuda et al., 1996). Although all of these export cargos contain Rev-type NESs that serve as potential CRM1 binding sites, the differing biological activities of these proteins necessitates that their nuclear export is differentially regulated. This hypothesis implies that inhibition of CRM1 activity should lead to a general block in NES-mediated nuclear export which, in turn, should then result in severe toxic side-effects on cellular metabolism. Indeed, this was reported in a recent study in which long-term toxicity of the CRM1 inhibitor leptomycin B had been observed in tissue culture (Wolff et al., 1997).

The presence of eIF-5A in *Xenopus* oocytes and particularly its accumulation at the nucleoplasmic face of NPCs will now allow the investigation of eIF-5A function in more detail. The use of isolated nuclear envelopes from oocytes will provide a new opportunity to identify the components of the NPC-associated import/export machinery with which eIF-5A interacts during its transit through the nuclear pore and determine particularly how these interactions might affect specific nuclear export pathways.

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