A Leptomycin B-sensitive Homologue of Human CRM1 Promotes Nuclear Export of Nuclear Export Sequence-containing Proteins in Drosophila Cells*

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The Rev protein of human immunodeficiency virus is a nuclear shuttling protein that promotes nuclear export of mRNAs that encode the viral structural proteins Gag, Pol, and Env. Rev binds to a highly structured RNA motif, the Rev-responsive element (RRE), that is present in all Rev-responsive viral transcripts and facilitates their entry into a nuclear export pathway by recruiting cellular export factors. In mammalian and yeast cells, the principal export receptor engaged by Rev has been identified as the importin/transportin family member CRM1/exportin 1. CRM1 binds directly to a leucine-rich nuclear export sequence (NES) present in Rev, and similar motifs have been identified in a variety of cellular nuclear shuttling proteins. We and our colleagues previously demonstrated that, in transfected Drosophila cells, HIV-1 Rev is fully functional and promotes expression of the viral envelope glycoprotein. We now demonstrate that the fundamental mechanism of Rev action in insect cells is identical to that observed in the mammalian systems. In particular, we show that Drosophila cells express a leptomycin B-sensitive homologue of human CRM1 that supports Rev-dependent gene expression and is required for nuclear export of NES-containing proteins in insect cells.

In eukaryotic cells diverse physiological processes such as mRNA accumulation (1–5), transcription (4, 5), cell cycle progression (6–8), cellular differentiation (9–10), and the cells response to genotoxic stress (8, 11, 12) are controlled by differentially regulating the nucleocytoplasmic transport of effector molecules. At the appropriate time transcription factors, RNA-binding proteins and components of the DNA replication machinery must be imported into the nucleus, whereas many nuclear proteins, tRNA, rRNA, and mRNA-protein complexes are subject to nuclear export. The cellular factors and pathways that orchestrate these nuclear transport reactions are the subject of intense experimental investigation, and the molecular details of nuclear import, though far from complete, are now well understood (13–15). Nuclear protein import requires a nuclear localization sequence (NLS)* within the import substrate (16) and can be reconstituted in vitro with permeabilized cell nuclei (17) and four soluble cytosolic factors, importin α, importin β, Ran, and p10, a small Ran-binding protein (18, 19). An experimentally accredited model for nuclear import suggests that NLS-containing substrates bind to importin α, which recruits importin β to form a trimeric transport complex within the cytoplasm (18, 19). In turn, the NLS-importin-α/β complex binds directly to components of the nuclear pore. Initial binding of the transport complex to the nuclear pore is stabilized by Ran-binding protein 1 (RanBP1) (20) and is likely stimulated by the interaction of Ran-GDP with pore components. Pore-mediated binding events promote transport through the pore and entry into the nucleus by an as yet poorly defined mechanism. Following entry into the nucleoplasm, the trimeric transport complex binds Ran-GTP, which dissociates the complex and releases the transported substrate (21, 22). The importin α and β subunits are subsequently recycled back to the cytoplasm (23). The fundamental mechanism by which substrates are imported into nuclei is highly conserved, and components required for import have been identified in yeast (24), plant (25), and animal (26) cells. In contrast to nuclear import, in vitro nuclear export assays have been difficult to develop, and consequently the nuclear export reactions are only beginning to be unraveled. In particular, the cellular mRNA processing pathways that promote nuclear export of mRNA are poorly understood. However, the process of regulated nuclear export of mRNA has been extensively studied for human immunodeficiency virus type-1 (HIV-1).

HIV-1 gene expression is regulated in a temporal manner by a post-transcriptional process that involves regulated nuclear export of incompletely spliced mRNAs (27, 28). Early in the viral infection cycle, short multiple-spliced transcripts that encode the viral regulatory proteins Tat, Rev, and Nef locate to and accumulate within the cytoplasm of infected cells, leading to expression of these nonstructural regulatory proteins. At this early time point, the unspliced and single-spliced transcripts that encode the structural genes, gag/pol and env, are produced but are strictly retained within the nucleus of infected cells; consequently, the structural proteins are not ex-

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pressed. In the late stages of infection, a pronounced change in the pattern of viral mRNA accumulation occurs. At this time, the full-length and single-spliced transcripts are released from the nucleus, facilitating expression of the structural proteins. This dramatic switch in the pattern of viral mRNA accumulation and protein expression is absolutely dependent upon the activity of the viral rev gene product.

Rev is a 116-amino acid nuclear shuttling protein (29) that demonstrates sequence-specific RNA binding activity (30–31). Rev binds to a highly structured RNA target sequence, known as the Rev-Responsive Element (RRE), which is located within the env coding region and is present in all Rev-responsive mRNAs. Rev promotes the nuclear export and cytoplasmic accumulation of RRE-containing transcripts by accessing a nuclear protein-export pathway (32, 33). Definitive genetic and biochemical studies of Rev structure and function (34, 35) and converging developments in the field of cell biology and nuclear transport (13–15) have provided significant insight into the molecular events that underlie Rev-mediated gene expression. In addition to an arginine-rich RNA-binding domain, Rev also contains an effector domain rich in leucine and hydrophobic amino acids (34–36). The leucine-rich region is absolutely required for Rev function as mutations within this region block Rev-dependent gene expression, exhibit a transdominant negative phenotype, and prevent nuclear export of Rev in nuclear shuttling assays (34). It is now clear that the Rev leucine-rich effector domain is a prototypic example of a group of transferable nuclear export sequences (NES) (34, 36). Fusion of these NES elements to heterologous proteins can promote nuclear export of the chimeric proteins following microinjection into cell nuclei (32, 36). Importantly, a cellular cofactor required for NES-directed nuclear export has been identified in both yeast and human cells.

The cellular NES-receptor protein CRM1/exportin is a member of the importin/transportin family of nucleocytoplasmic transport receptors, which require the small GTPase Ran and nuclear pore components for translocation into or of the nucleus (37–41). CRM1 binds directly to Rev-NES, but not to NES-mutants, in a RanGTP-dependent manner in vitro, and overexpression of CRM1 enhances Rev-NES nuclear export (37, 38). Moreover, CRM1 binds the Streptomyces metabolite leptomycin B, a compound that specifically blocks nuclear export of Rev in cell-based assays (42, 43). A model for Rev-mediated gene expression suggests that Rev bound to viral RNA, encoding the RRE, associates with CRM1 and RanGTP and is rapidly exported from the nucleus by CRM1-orchestrated interactions with the nucleoporins and nuclear pore complex (37, 38, 41). Upon entry into the cytoplasm, RanGTP hydrolysis promotes dissociation of the CRM1/Rev/RNA complex, thereby facilitating translation and expression of the viral structural proteins and recycling of CRM1.

We and our colleagues have previously demonstrated Revresponsiveness and Rev-function in a model system based upon stably transfected Drosophila melanogaster S2 cells (44, 45). Using this system, we have been able to recapitulate the Rev-dependent expression of the HIV-1 envelope glycoprotein that is normally observed in both transfected heterologous mammalian cells and infected human CD4+ T-cells (27). As observed in the more conventional mammalian systems, no envelope protein is expressed in the absence of Rev, and the block to expression can be overcome by supplying Rev in trans (44, 45). Significantly, analysis of these cell lines indicates that, in the absence of Rev, the RRE-containing env transcripts are retained within the nuclear compartment of transfected cells, and only when Rev is co-expressed do the env transcripts locate to, and accumulate within, the cytoplasm resulting in expression of envelope proteins (45).

Based upon data obtained from the Drosophila system, we have suggested that the fundamental mechanism and cellular factors underlying both the nuclear retention of HIV-1 transcripts and their trans-activation by Rev are highly conserved (44–46). Moreover, the robust Rev-dependent effects on envelope expression obtained in Drosophila cells provide us with a versatile reporter system for dissection of the fundamental molecular processes that underlie regulated nuclear export in insect cells. We have now used this reporter system to examine the cellular processes that facilitate Rev-dependent gene expression and nuclear export of Rev in the Drosophila system in more detail. Here we confirm and extend upon our previous observations and demonstrate that, in Drosophila cells, Rev functions as a mechanism that is functionally indistinguishable from that observed in mammalian cell systems. Moreover, we now demonstrate that a leptomycin B-sensitive Drosophila homologue of hCRM1 promotes nuclear export of NES-containing proteins in Drosophila cells.

** MATERIALS AND METHODS 

**Generation of Cell Lines—**Stably transfected D. melanogaster S2 cell lines were generated by transfection of 3 × 10^6 S2 cells with 19 μg of DNA and 1 μg of pCOHygro (unless otherwise indicated), using a calcium phosphate precipitation method (47), and selected for 4–6 weeks in selection medium (Shields and Sang M3 Insect Medium, Sigma); 10% fetal bovine serum (Life Technologies, Inc.); 1% penicillin/streptomycin; 300 μg/ml hygromycin B (Calbiochem), at 23 °C (44, 45). Cells were washed and replated in fresh medium every 4 days over the selection period. Stably transfected cell were induced at 5 × 10^6 cells/ml with 0.5 mM copper sulfate for heterologous protein expression, and cells were harvested after 3–5 days.

**Inhibition of Envelope Glycoprotein Expression by Leptomycin B—**Drosophila cells co-transfected with pMt120352 or pMt160352 (44, 45) in the presence or absence of pMtRev were induced by addition of 0.5 mM copper sulfate to the culture medium. Cells were incubated for 8 h and then washed with fresh medium. The cells were resuspended in fresh medium containing 0.5 mM CuSO4 plus or minus leptomycin B (0–10 nm). Cells were incubated overnight and subsequently assayed for envelope glycoprotein expression by Western blot analysis of culture medium and cell lysates using an anti-gp120 rabbit primary antibody.

**Western Blot Analysis—**Proteins were resolved on SDS-polyacrylamide (10% or 12%) gels and electrophoretically transferred overnight to nitrocellulose filters (Schleicher & Schuell) in transfer buffer (1 × Tris-glycine, 20% methanol) at 150 mA (48). Filters were blocked in blocking buffer (5% dried non-fat milk, 0.25% Triton X-100, PBS) for 1 h, washed twice for 10 min in wash buffer (0.5% dried non-fat milk, 0.25% Triton X-100, PBS), probed with primary antibody 1:6000 rabbit anti-gp120 (44, 45), 1:3000 rabbit anti-cCRM1 (44, 45), mouse anti-hCRM1 (S678), or 1:3000 rabbit anti-CRM1 (R278) at room temperature for 1 h, washed twice for 10 min, probed with horseradish peroxidase-conjugated secondary antibody (1:3000), and washed four times in wash buffer. Bound antibodies were detected using an enhanced chemiluminescence technique (ECL) and exposed to film, as directed by the manufacturer (Amersham Pharmacia Biotech).

**cDNA Cloning—**To isolate the Drosophila CRM1 homologue, the GenEMBL data bases were searched using a conserved CRM1 peptide GVQDMACDFIKI as TFasta query sequence. Primers dEx1545 (5′-CGGTATCCAGTTAAG-3′) and dEx3181 (5′-TGGACCAGAATCATG-3′) were designed based on a conserved CRM1 peptide sequence in GenEMBL data bases and amplified with total RNA isolated from mixed stage Drosophila embryos. First strand cDNA was synthesized using the 5′-TGGACCAGAATCATG-3′ and dCRM1-specific primer 5′-TGGACCAGAATCATG-3′ using Taq PCR.
polymerase (Sigma). Multiple 5’-RACE products from independent reactions were cloned into pCR-BLUNT (Invitrogen) and sequenced. Full-length dCRM1 was constructed by digesting the dCRM1 partial cDNA with SstI and KpnI and subcloning it into similarly digested pCR-BLUNT-dCRM1. Full-length dCRM1 was sequenced on both strands (GenBank™ accession number AF190557) by another accredited laboratory. Unpublished results were performed on an ABI Prism 377 Automated DNA Sequencer (Perkin-Elmer).

RNA Isolation and Analysis—Total RNA fractions were prepared using TRIReagent as directed by the manufacturer (Sigma). Subsequently, poly(A)+ mRNA was selected using an oligo(dT)-cellulose batch procedure as directed by the manufacturer (microFastTrack, Invitrogen). Poly(A)+ RNA samples (2 μg) were resolved on 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes (9). To detect crm1, mRNA blots were dried, UV cross-linked using a Stratalinker (Stratagene, CA), blocked, and probed (49) with a 32P-labeled random primed crm1 cDNA fragment. Blots were washed, dried, and exposed to x-ray film.

Plasmid Construction—Vectors pM160Δ32, pMt120I32, and pMTRev have been described (44, 45). The prototype Rev M10 and Rev M5 mutants are identical to those described by Malim et al. (34) and were generated by site-directed mutagenesis of a Rev cDNA in pBlue-script-KS. Subsequently, the coding regions for the Rev mutants were PCR-amplified incorporating an SpeI site 5’ of the Rev coding region and an NcoI site 3’ of the Rev stop codon. The amplified regions were digested with SpeI and StuI and cloned into the reciprocal sites within the polyclinker of the previously described Drosophila expression vector pMta (44) to generate pMTRev-M10 and pMTRev-M5. This places each of the mutant Rev open reading frames into a transcription unit that is driven from the Drosophila metallothionein promoter and terminated by the SV40 early poly(A) site. pMTRev-Tg is identical to pMTRev but includes the 8-amino acid Flag epitope (45) fused in frame to the carboxyl terminus of the Rev open reading frame. Plasmid MtRev-Tg was constructed by PCR amplification of the Rex open reading frame from cDNA generated from HeLa cells expressing HTLV-1 antigens. The primers incorporated SpeI and StuI sites 5’ and 3’ of the Rex coding region, respectively, and introduced an 8-amino acid FLAG epitope tag fused in frame to the carboxyl terminus of Rex. The Rex-FLAG encoding PCR product was subsequently cloned into the Drosophila expression vector pMta as described above. The fidelity of all sequences were confirmed by dideoxy-chain termination sequence analysis.

pGE1X3-hCRM1 (aa 805–1071) was constructed by excision of hCRM1 (aa 805–1071) from pBAS2-hCRM12 with MscI and BamHI and insertion into pGE1X3 (Amersham Pharmacia Biotech), cut with BamHI and PstI. pBAS-cCRM1 was constructed by digesting dCRM1 from pCR-BLUNT (Invitrogen) with ApaI, blunt-ending ApaI ends with T4 DNA polymerase (Roche Molecular Biochemicals GmbH), and cloning into Smal-digested pBAS2. pBAS-cCRM1-Flag was generated by amplifying a carboxyl-terminal dCRM1-FLAG PCR product with primers dE2130 (5’TGGGATCTGCTGAATG) and dE2-CTERM (5’TATA-GAGCTCTACTGTCATCGTCCTGTCGTTAATCTCCTGCTGATCAT- AATC), digesting the product with StyI and SacI, and using the digested product to replace the corresponding fragment in StyI-SacI-digested pBAS-cCRM1. Standard cloning procedures were performed as described (49). Restriction enzymes were obtained from New England Biolabs Inc. Manual sequencing was performed using the dideoxy-chain termination method and the T7 Sequenase Version 2.0 sequencing kit (Amersham Pharmacia Biotech). Automated sequencing was performed using an ABI Prism 377 Automated DNA Sequencer (Perkin-Elmer).

Recombinant Proteins and Antibodies—100 ml of pGE1X3-hCRM1 (aa 805–1071) cultures were induced with 0.1 mM IPTG (Sigma) for 3 h. GST-hCRM1 (aa 805–1071) inclusion bodies in the cell pellet were washed once with 500 ml of 0.2× Triton X-100/PBS and twice with 0.5× Triton X-100/PBS. Washed GST-hCRM1 (aa 805–1071) crude protein (1.5 mg) was loaded on a 10% preparative SDS-PAGE gel and electrophoresed, and the protein was excised using 1 M KCl precipitation to locate the GST-hCRM1 band. GST-hCRM1 (aa 805–1071) was electroeluted from the gel slice into 5 ml of 1× Tris-glycine, 0.1% SDS in dialysis tubing (Life Technologies, Inc.) at 100 V for 5 h. Electro-eluted GST-hCRM1 (aa 805–1071) was concentrated to 1 ml using an Ultra- free-15 Centrifugal Filter Device (Millipore). Electro-eluted GST-hCRM1 (aa 805–1071) was used to raise polyclonal antibodies in sheep and New Zealand White rabbits by standard techniques. 500 ml of pGE1X32-RanWT cultures were induced with 0.02 mM IPTG for 5 h. GST-RanWT was bound to 1 ml of GSH-agarose (Sigma) and eluted with 10 mM GSH (Sigma); 50 mM Tris-HCl, pH 8. Purified GST-RanWT was incubated with 2 mM GTP at 4 °C for 4 h, followed by addition of 10 mM MgCl2 to load GTP. Coupling of Proteins to Affi-Gel—1.5 mg of bovine γ-globulin or 1.2 mg of Rev was incubated with 150 μl of Affi-Gel-10 (Bio-Rad) at 4 °C with mixing for 4 h. Beads were blocked in 1 ml of 100 mM ethanamine/HEPES, pH 7.9, at 4 °C with mixing for 1 h.

In Vitro Binding Assay—In vitro translated 35S-labeled dCRM1 was generated from pCR-BLUNT-dCRM1 using a coupled reticulocyte lysate system (TNT; Promega) and incubated with 7.5 μl of Affi-10 bovine-γ-globulin or Affi-10-Rev beads and 2 μg of GST-Ran-GTP in 50 μl of binding buffer (50 mM HEPES, pH 7.9, 200 mM NaCl, 5 mM MgCl2, 100 μM GTP) at 4 °C with mixing for 1 h. Beads were pelleted, washed twice with 100 μl of binding buffer, and resuspended in 20 μl of binding buffer. Protein was eluted from the beads using sample buffer and analyzed by SDS-PAGE and fluorography.

Immunofluorescence Analysis—Independent stable Drosophila S2 lines expressing dCRM1-FLAG or Rev-Tg cells were seeded onto cover slips in serum-free Shields & Sang M3 Insect Medium (Sigma) for 4 h, induced with 0.5 mM CuSO4, and grown overnight at 23 °C. Rev-Tg cells were incubated with 25 mM leptomycin B for 3 h and followed by the addition or absence of 5 μg/ml actinomycin D (Calbiochem) for 5 h. Cells were fixed in 10% paraformaldehyde, permeabilized in 0.2% Triton X-100 and blocked with 5% bovine serum albumin. dCRM1-FLAG and Rev-Tg were detected with an anti-FLAG monoclonal antibody (Kodak) at 2 μg/ml followed by a goat anti-mouse antibody conjugated to FITC (Sigma). Samples were counterstained with 0.5 μg/ml DAPI and visualized using an Olympus IX70 immunofluorescence microscope.

RESULTS

Functional Homologues of Rev, but Not Rev Mutants, Support Envelope Expression in Drosophila Cells—We and our colleagues have previously shown that, in transfected Drosophila cells, expression of HIV-1 RRE-containing envelope constructs is highly dependent upon co-expression of the viral Rev protein. Our results suggested to us that Rev functions in Drosophila cells in a manner that is identical to that observed in primate systems and that the fundamental mechanism by which Rev acts is highly conserved. To confirm and extend these observations, we examined the mechanism of Rev-dependent gene expression in the Drosophila system in more detail. In particular, we examined the ability of functional homologues of Rev or mutant Rev proteins to support HIV-1 envelope expression in the Drosophila system. Drosophila cells were transfected with the previously described HIV-1 gp160 envelope expressing construct pM160Δ32, in the presence or absence of vectors expressing human T-cell leukemia virus type-1 (HTLV-1) Rex (pMTRev-Tg) or mutant Rev proteins (pMTRev-M5, or pMTRev-M10). Envelope protein expression was monitored by Western blotting of cell free supernatants from these stably transfected cell lines. As observed in the primate systems (50), HTLV-1 Rex, a NES-containing functional homologue of Rev, was able to partially substitute for Rev function and supported envelope protein expression in Drosophila cells (Fig. 1A). In contrast, a mutant Rev protein, Rev M5, that encodes a substitution within the RNA-binding domain of Rev was severely compromised in its ability to support envelope expression (Fig. 1B). This type of mutant fails to bind the RRE in vitro and lacks Rev function in mammalian cell-based assays (34). Moreover, we found that the classical NES-mutant Rev M10 also failed to support envelope expression in Drosophila system (Fig. 1C). In such cases expression of the relevant Rex or Rev protein was confirmed by Western blot analysis of whole cell extracts (Fig. 1D). Rev M10 is the definitive trans-dominant Rev mutant identified by Malim et al. (34) and previously characterized in mammalian cells. Importantly, we found that Rev M10 could also inhibit the function of “wild type” Rev protein in a dose-dependent manner in the Drosophila system (Fig. 1E). Thus, defective Rev
mutants. Expression is supported by Rev and HTLV-1 Rex, but not by Rev by leptomycin B (Fig. 2A). In contrast, expression from an RRE-deficient Rev-independent envelope construct, pMt120A32 (45), was not significantly affected by leptomycin B (Fig. 2B), indicating that the effect of LMB is highly specific for the Rev-dependent pathway of gene expression. However, it should be noted that, at the highest dose of LMB used, a small decrease in Rev-independent gene expression was also observed (Fig. 2B). We suspect that this is an indirect effect of LMB on general nuclear export processes because of the pleiotropic effects of inhibiting CRM1 function.

Several independent but converging lines of investigation have identified the target of leptomycin B as the NES receptor protein CRM1 (37, 42, 43), and homologues of CRM1 have been identified in both yeast and man (37, 38, 41). To determine whether Drosophila cells express a counterpart of hCRM1, we raised antisera against a recombinant carboxyl-terminal fragment of hCRM1 and used these polyclonal antisera to probe Western blots of cell extracts obtained from Drosophila S2 and primate cell lines. Two independently generated antibodies (S67 and R278) were shown to be reactive against human and primate CRM1 (Fig. 3), and both of these antibodies detected a 110-kDa protein immunologically related to CRM1. Antibody R278 also recognized a protein of approximately 66 kDa (Fig. 3). The 110-kDa antigen is not recognized by pre-immune sera (data not shown) and was tentatively designated Drosophila CRM1 (dCRM1). In addition to the 110-kDa protein, antibody R278 also recognized a protein of approximately 66 kDa (Fig. 3B). Although we cannot rule out the possibility that this lower signal represents a proteolytic fragment of the 110-kDa protein, we believe, and our preliminary results are consistent with the view, that the 66-kDa signal represents a novel cellular protein that shares a common antigenically related epitope with CRM1. We are currently characterizing this 66-kDa protein.

Cloning Drosophila CRM1—Our results demonstrated that Rev functions in Drosophila cells in a manner that is indistinguishable from that observed in primate cells and that Rev-mediated gene expression can be inhibited in a dose-dependent manner both by trans-dominant Rev mutants and by the antiviral compound leptomycin B. Moreover, a protein immunologically related to the leptomycin B-sensitive NES receptor CRM1 can be detected in Drosophila cell extracts, and a weakly
hybridizing band can be detected in Northern blots of *Drosophila* RNA when probed with an hCRM1 cDNA fragment (data not shown). To clone dCRM1, we searched the available data bases for sequences related to a conserved peptide GVQDMAC-DTPIKI found in human, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* CRM1 homologues. A partial genomic fragment with homology to CRM1 was identified (AC004423), and a putative dCRM1 DNA fragment was amplified by RT-PCR from total RNA isolated from *Drosophila* S2 cells using primers based upon the data base sequence. The PCR product was sequenced, confirming its relationship to hCRM1, and used as a probe to screen a *Drosophila* embryonic cDNA library. We obtained 16 positive clones, the longest of which encodes a partial 2.5-kb dCRM1 cDNA (nt 939–3384). We used 5'-RACE to obtain the 5'-end of the dCRM1 coding region and 5'-untranslated region, and multiple clones were obtained and sequenced.

Complete sequencing of the composite dCRM1 clone on both strands and analysis of the derived amino acid sequence (Fig. 4, A and B) revealed a 3732-base pair full-length dCRM1 cDNA with a predicted open reading frame encoding a 1063-amino acid protein with a molecular mass of 122.8 kDa (accession number AF190557). Comparison of the predicted protein sequence with that of CRM1 from yeast and man indicated 71.3% identity with human CRM1 and 54% with the yeast protein. Notably, dCRM1 also exhibits weak regions of homology (41.3% similarity) with the nuclear import factor importin-β, a feature previously recognized in hCRM1, and also the carboxy-terminal region of dCRM1 between amino acids 950–1063 exhibits weak homology (47.6% similarity) with the amino-terminal domain of the nuclear import factor importin α-4. During this work the genomic sequence of dCRM1 was completed and made available through the *Drosophila* genome project sequence data base. Comparison of our cloned dCRM1 cDNA with that of the genomic DNA sequence revealed two amino acid differences, V1015E and T1017A (Fig. 4), between the genomic sequence defined by AC004423 and the cDNA sequence obtained in our study, respectively. The variant amino acid residues within the cDNA clone isolated in our study were confirmed by sequencing multiple independent clones. Moreover, comparison of our cloned dCRM1 cDNA to the hCRM1 sequence showed conserved Glu and Ala residues at these positions and that the cDNA isolated in our study is more typical of the CRM1 consensus at these amino acid positions. The amino acid variation at positions 1015 and 1017 between the clones identified in our study compared with those observed in the genomic clone may represent allelic variation within *Drosophila* populations.

**Fig. 3.** *Drosophila* cells express a CRM1 related antigen. *D. melanogaster* S2 whole cell lysates were assayed for CRM1 expression by Western blotting and probed with sheep primary anti-hCRM1 serum S867 (A) or rabbit primary anti-hCRM1 serum R278 (B). Antibodies were raised against a recombinant carboxyl-terminal protein fragment of human CRM1.

**Fig. 4.** Gene structure and protein sequence of dCRM1. A, the structure of cloned the dCRM1 cDNA is depicted by the boxed exons; the introns, predicted by comparison of the cDNA sequence obtained in this study and genomic sequence deposited in the data base, are illustrated as lines extending below and joining the exonic regions. Numbers above the boxed regions represent the nucleotide coordinates of the cDNA sequence obtained in this study (GenBank™ accession number AF190557); the nucleotide coordinates of the ATG initiating translation are shown in bold. Sequences below the boxed regions highlight the splice junctions, and the genomic coordinates (AC002747) for the splice junctions are shown in the table below the main figure. B, the predicted amino acid sequence of dCRM1 is shown in one-letter amino acid code.

The 5'-end of the cDNA sequence and the presence of an intron within the 5'-untranslated region were confirmed by sequencing multiple independent 5'-RACE clones, obtained both from cultured *Drosophila* S2 cells and mixed stage *D. melanogaster* embryos. Additional analysis of the dCRM1 cDNA sequence obtained in this study with the data base-derived genomic sequence indicates that dCRM1 gene structure likely comprises seven exons and six introns (Fig. 4A). Interestingly, one of the most highly conserved regions between dCRM1 and hCRM1 is encoded entirely within exon 3. Only three nonconservative changes are found within this coding region, suggesting that considerable functional constraint impinges upon the sequence divergence of this exon.

We next examined *Drosophila* S2 cells and *Drosophila* embryos for dCRM1 expression by probing a Northern blot of *Drosophila* poly(A)+ RNA isolated both from S2 cells and mixed stage embryos. We identified a single RNA species of 3.8 kb that is expressed both in tissue culture cells and embryos (Fig. 5). The size of this transcript is consistent with the predicted structure of the dCRM1 mRNA when a poly(A)+ tail of 70–150 nucleotides is included. In our analysis we found no evidence...
for alternatively spliced CRM1 transcripts under the conditions used.

dCRM1 Binds to Rev in Vitro—The protein coding potential of the composite cDNA was confirmed by in vitro translation, and a single labeled product of approximately 110 kDa was identified by SDS-PAGE analysis. The 110-kDa protein could be immunoprecipitated from the extract using our anti-hCRM1 antisera (Fig. 6A), confirming the identity of the labeled protein product. To examine the ability of the in vitro translated dCRM1 to bind to Rev, we immobilized recombinant Rev protein on Sepharose beads and incubated the beads with in vitro translated 35S-labeled dCRM1 protein. After extensive washing, the bound material was eluted and analyzed by SDS-PAGE and fluorography. We found that dCRM1 bound to immobilized Rev protein in the presence of Ran-GTP but did not bind efficiently to an immobilized bovine γ-globulin control (Fig. 6B).

Localization of dCRM1 in Drosophila Cells—CRM1 is a nuclear protein (41), and because dCRM1 bound to immobilized Rev and LMB inhibited Rev-dependent gene expression, we wished to determine the subcellular localization of dCRM1. Stably transfected Drosophila S2 cells were generated that express dCRM1 fused at the carboxyl terminus to the FLAG-epitope tag (dCRM1-Flg). Cells expressing dCRM1-Flg under control of the metallothionein promoter were grown on coverslips, and dCRM1-Flg expression was induced by the addition of 500 μM CuSO4. Following overnight culture, the cells were fixed, permeabilized, and probed with the anti-FLAG epitope monoclonal antibody M2 and FITC-labeled anti-mouse secondary antibody. Indirect immunofluorescence microscopy of the stained cells revealed extensive dCRM1-Flg associated punctate staining of Drosophila S2 cell nuclei (Fig. 7). Little if any detectable cytoplasmic staining was observed. At present, the resolution of our immunofluorescence staining technique does not allow us to distinguish between a punctate staining pattern spread throughout the nucleoplasm or a staining pattern that is restricted to the nuclear periphery. Nevertheless, our data indicate that dCRM1 localizes primarily to the nuclear compartment of Drosophila cells.

Leptomycin B Prevents Export of NES-containing Proteins from Drosophila Nuclei—Our data indicate that Drosophila cells express a homologue of hCRM1 that localizes to the nucleus, that dCRM1 binds to Rev in vitro, and Rev-dependent gene expression can be inhibited in transfected Drosophila cells by leptomycin B, a known inhibitor of CRM1-mediated nuclear export activity (37, 42, 43). These results are consistent with the notion that, in Drosophila cells, dCRM1 plays an analogous, if not identical, role to hCRM1 in human cells. To further test these ideas, we determined if the leptomycin B-induced inhibition of Rev-dependent gene expression in Drosophila cells is because of a block in nuclear export of Rev. We examined the localization of FLAG epitope-tagged Rev (Rev-Tg) in cells coexpressing RRE-RNA by indirect immunofluorescence microscopy of fixed and permeabilized cells. The FLAG-tagged Rev protein was detected with the anti-FLAG murine monoclonal antibody M2 and FITC-labeled anti-murine secondary antibody. As expected, in transfected and induced Drosophila S2 cells, Rev-tg staining was observed both within the nuclei and cytoplasm (Fig. 8A) Importantly, in mammalian cells nuclear accumulation of Rev requires ongoing RNA synthesis. Consistent with this, indirect immunofluorescence analysis indicated that most of the expressed Rev-Tg localized to the cytoplasmic compartment of Drosophila S2 cells in the presence of the RNA polymerase II inhibitor actinomycin D (Fig. 8D). In contrast, in the presence of 25 μM leptomycin B, translocation of Rev to the cytoplasm was almost completely blocked, and the majority of the Rev-tg protein remained within the nuclei (Fig. 8G). Thus, leptomycin B potently inhibits nuclear export of Rev in Drosophila cells (Fig. 8) and consequently abrogates Rev-dependent gene expression in this insect cell system (Fig. 2). These results establish the sensitivity of dCRM1-mediated nuclear export of NES-containing nuclear shuttling proteins to LMB, and underscore the highly conserved nature of the nuclear export processes between insect and mammalian systems.

CRM1 May Be Ubiquitous in Eukaryotes—Finally as discussed above, our results combined with a growing body of published data indicate that CRM1 function has been highly conserved over eukaryotic evolution. We therefore examined cells and tissue samples from a variety of metazoans for CRM1 expression by Western blotting using the anti-CRM1 antibody R278. We found that all of the eukaryotes examined, including primate, rodent, avian, and amphibian representatives, expressed CRM1-related antigens (Fig. 9).

DISCUSSION

We previously demonstrated that expression of the HIV-1 envelope glycoproteins in transfected Drosophila cells is highly
dependent upon co-expression of Rev (44, 45). In the absence of Rev, envelope proteins cannot be expressed from RRE-containing transcripts, and this block to expression is because of a failure of the envelope transcripts to locate to the cytoplasm of transfected insect cells. However, the block to expression can be overcome by supplying Rev in trans; whereupon, env transcripts are released from the nucleus and accumulate within the cytoplasm of transfected cells where they are efficiently translated. This pattern of Rev activity appears to faithfully recapitulate the Rev-dependent effects on HIV-1 gene expression that are normally observed both in infected human cells and in transiently transfected heterologous mammalian cell
systems. From these observations we suggested that the cellular factors and pre-mRNA processing pathways accessed by Rev are highly conserved. We have now shown that the mechanism of Rev action in Drosophila cells is functionally indistinguishable from that observed in mammalian systems. Specifically, we have demonstrated that in insect cells Rev function is absolutely dependent upon an intact RNA-binding domain and that defects in the leucine-rich effector domain of Rev severely impair Rev function, as observed previously for the mammalian systems (34). These results are consistent with a requirement for binding of Rev to RRE-RNA and for interaction with cellular factors that support Rev activity. In fact, we show here that an effector domain-deficient Rev mutant (Rev M10) not only fails to support a Rev-response but also exhibits a trans-dominant negative phenotype in insect cells. Moreover, a divergent but functionally related NES-containing RNA-binding protein, Rex from HTLV-1, can also promote HIV envelope protein expression in insect cells. These data indicate that Rev functions in Drosophila cells in an identical manner to that observed in mammalian cells and that the insect cellular factors, which support Rev function, also support the activity of other NES-containing nuclear shuttling proteins.

Our observation that Rev-dependent gene expression in insect cells is highly sensitive to leptomycin B prompted us to screen for homologues of the LMB-target protein CRM1 in Drosophila. We have now identified, cloned, and undertaken a preliminary characterization of dCRM1. Drosophila CRM1 is a 1063-amino acid protein that exhibits extensive homology (71.4% identity) with hCRM1 and significant but somewhat lower degree of conservation with the orthologous yeast protein. Like the mammalian and yeast counterparts, dCRM1 contains nuclear localization signal and a potent nuclear export sequence (9, 10). Importantly, dCRM1 function is sensitive to LMB, and treatment of S2 cells with this compound prevents Rev-dependent gene expression and potently blocks nuclear export of Rev. These observations, coupled with the amino acid similarity of the human and Drosophila CRM1 proteins, indicate that the cellular components with which CRM1 interacts, and the fundamental biochemical mechanism by which nuclear export of NES-containing proteins is achieved, are highly conserved throughout eukaryotic evolution.

The evidence to date indicates that CRM1 is not a general mRNA export pathway. Instead the nuclear export pathway accessed by Rev and defined by CRM1 is primarily involved in the export of protein substrates from the nucleus to the cytoplasm. In mammalian systems, a variety of effectors of cellular gene expression (4, 5), cell cycle progression (6–8), and cell signaling (51, 52) employ the CRM1 pathway to modulate their nuclear localization. Restricting the accumulation of effector molecules within sub-cellular compartments may be a crucial and perhaps widespread mechanism employed by eukaryotic cells to regulate important cellular processes (8, 11). From the observations reported here, it seems highly likely that similar regulatory circuits exist in Drosophila and that balancing the nuclear import and export of effector molecules will play a significant role not only in maintaining cellular homeostasis and controlling cellular proliferation but also in defining the pattern of Drosophila development. In this respect, it is interesting to note that two groups recently demonstrated that nuclear accumulation of the homeobox protein extradenticle (Exd) and its developmentally regulated cofactor homothorax (Hth) is governed by the opposing activities of a nuclear localization signal and a potent nuclear export sequence (9, 10). Interestingly, the export of Exd from the nucleus can be inhibited by LMB, implicating the CRM1 pathway in modulating the localization and therefore functional activity of these regulators of fly development.

We have previously used the Drosophila system to demonstrate that a negative element contained within the RRE is directly responsible for the nuclear retention and, therefore, for the Rev-responsiveness of env transcripts (45). Importantly, the negative element identified in our studies is functional in all of the primate cell systems tested and is fully functional in human CD4+ T-cells (45). Nuclear retention of viral mRNA is a prerequisite for Rev-dependent CRM1-mediated gene expression. Therefore, given the highly conserved nature of the Rev/CRM1-dependent activation pathway in these insect cells, the efficient RRE-mediated nuclear retention of transcripts in both mammalian and insect systems (45, 46), and the observed conservation between mammalian and insect pre-mRNA processing pathways (53–56), we suspect that the cellular factors and fundamental mechanism responsible for the nuclear retention of RRE-containing transcripts are also highly conserved. Moreover, our demonstration that the RRE confers nuclear retention and Rev-dependence to env transcripts in both human and Drosophila cells and that CRM1 function is conserved within these systems, indicates that conclusions and predictions based upon data derived from Drosophila S2 cells are equally valid for modeling the events that underpin Rev-dependent gene expression in mammalian systems.

The CRM1-defined nuclear export pathway is primarily involved in the export of protein substrates from the cell nucleus, and evidence suggests that this pathway is not used by the vast majority of cellular mRNAs (32). A view that is supported by our observation that protein expression from an RRE-deficient Rev-independent envelope construct is largely unaffected by treatment of cells with LMB. Accumulated data from a number of laboratories indicate that there are multiple routes for export of mRNA substrates from the nucleus and that these initial independent pathways converge upon the nuclear pore via interactions with common protein cofactors. While HIV transcripts access the CRM1 pathway via Rev and its interaction with the RRE, the RNA-encoded signals that are required for active export of cellular mRNAs have remained poorly defined. However, in addition to the RRE, a number of RNA elements involved in nuclear export of viral mRNA have been identified. The constitutive transport elements (CTE) of the type D simian retroviruses such as Mazon-Pfizer monkey virus (57), the PRE of hepatitis B virus (58), and an element within the herpes simplex thymidine kinase mRNA (59) have all been implicated as determinants of mRNA nuclear export. These elements can rescue expression of HIV reporter genes in a manner that is Rev-independent and, importantly, insensitive to inhibition by LMB, suggesting that these RNA elements do not access the CRM1 pathway. RNA elements with similar properties have not been identified in cellular mRNAs, but a strong candidate for this type of element has recently been identified within the 3’-untranslated region of the developmentally regulated Caenorhabditis elegans tra-2 mRNA (60). Taken together, such results indicate that RNA-encoded export
elements are widespread, access a variety of export pathways, and confer additional levels of regulatory control upon nascent transcripts.

In addition to CRM1/Rev and the nuclear export determinants encoded within RNAs, a number of protein cofactors required for mRNA export have been identified in mammals and yeast (61–63). In particular, Tap (61, 62) and hnRNP A1 (64) have been studied in detail. Tap binds to the CTE of transcripts.

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