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The Human Immunodeficiency Virus Type 1 *gag* Gene Encodes an Internal Ribosome Entry Site

CHRISTOPHER B. BUCK,[1] XUEFEI SHEN,[2] MICHAEL A. EGAN,[2] THEODORE C. PIERSON,[3] CHRISTOPHER M. WALKER,[4] AND ROBERT F. SILICIANO[3]*

Program in Cellular and Molecular Medicine,[1] Program in Biochemistry, Cellular and Molecular Biology,[2] and Graduate Program in Immunology,[3] Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and Department of Virology, Chiron Corporation, Emeryville, California 94608[4]

Several retroviruses have recently been shown to promote translation of their *gag* gene products by internal ribosome entry. In this report, we show that mRNAs containing the human immunodeficiency virus type 1 (HIV-1) *gag* open reading frame (ORF) exhibit internal ribosome entry site (IRES) activity that can promote translational initiation of Pr55^{gag}. Remarkably, this IRES activity is driven by sequences within the *gag* ORF itself and is not dependent on the native *gag* 5′-untranslated region (UTR). This cap-independent mechanism for Pr55^{gag} translation may help explain the high levels of translation of this protein in the face of major RNA structural barriers to scanning ribosomes found in the *gag* 5′ UTR. The *gag* IRES activity described here also drives translation of a novel 40-kDa Gag isoform through translational initiation at an internal AUG codon found near the amino terminus of the Pr55^{gag} capsid domain. Our findings suggest that this low-abundance Gag isoform may be important for wild-type replication of HIV-1 in cultured cells. The activities of the HIV-1 *gag* IRES may be an important feature of the HIV-1 life cycle and could serve as a novel target for antiretroviral therapeutic strategies.

Like other complex retroviruses, human immunodeficiency virus type 1 (HIV-1) employs a variety of mechanisms to express numerous proteins from a single-genomic-length RNA transcript. One such mechanism is the regulated splicing of the primary transcript into more than 30 distinct mRNA species (65, 74–76). Other mechanisms for the production of different polypeptides from mature viral mRNA species rely on translational events that are atypical of normal host protein translation.

The initiation of translation of most eukaryotic mRNAs is thought to occur by a process involving ribosome scanning. In this model, the 40S ribosomal subunit binds to the 5′ end of a capped mRNA (80, 81), commences scanning toward the 3′ end, and initiates translation upon encountering an AUG codon in suitable context (often referred to as a "Kozak" context) (47, 49, 50). The 60S ribosomal subunit is then recruited, and translation of a single polypeptide begins.

Translation of some HIV-1 mRNAs can depart from this basic model in several ways. Translation of the *pol* open reading frame (ORF) requires a −1 frameshift at a "slippery" sequence within the *gag* ORF. This frameshift allows roughly 5 to 10% of translating ribosomes to bypass the normal *gag* stop codon, enabling translation of a Gag-Pol precursor protein (reviewed in reference 32). Other HIV-1 gene products, including Env and Nef, have been shown to be translated by "leaky" scanning of ribosomes past the *vpu* and *rev* AUGs, respectively (74, 75, 77).

Another alternative mechanism for translational initiation is driven by RNA structural elements referred to as internal ribosome entry sites (or segments) (IRESs). IRESs are thought to promote initiation of translation by directly binding ribosomes, with participation of other host cell factors, in a manner independent of the mRNA cap or of scanning through upstream sequences. Since the initial discovery of an IRES in the 5′ untranslated region (UTR) of picornaviruses (43, 66), IRES activity has been described in a wide variety of viral and cellular mRNAs (reviewed in references 41 and 71). Details of the molecular mechanisms through which these RNA structures promote the internal entry of ribosomes are the subject of much current interest.

IRESs have been described in several retroviruses (4, 5, 19, 63, 91), leading to speculation that HIV-1 mRNAs might exhibit IRES activity. The data presented in this report indicate that mRNAs containing the HIV-1 *gag* ORF exhibit IRES activity that can drive production of both the familiar Gag precursor protein, Pr55^{gag}, and a novel 40-kDa N-terminally truncated isoform of Gag (p40). We demonstrate that this novel Gag isoform is present in cultured cells infected with HIV-1. Our findings suggest that expression of p40 may be required for wild-type viral growth kinetics.

Unlike the majority of other IRESs, which have been found partially or wholly within the 5′ UTRs of mRNAs, the IRES activity reported here is driven by sequences entirely within the *gag* ORF. This surprising arrangement is similar to the IRES of Moloney murine leukemia virus (MMLV) (91). The observation of IRES activity in a variety of retroviral species suggests that such activity may be an important feature of retroviral biology. The HIV-1 *gag* IRES described here might thus serve as a novel target for viral attenuation or antiretroviral therapeutics.

* Corresponding author. Mailing address: Graduate Program in Immunology, Johns Hopkins University School of Medicine, 720 Rutland Ave., Ross 1059, Baltimore, MD 21205. Phone: (410) 955-2958. Fax: (410) 955-0964. E-mail: rsilicia@welch.jhu.edu.

MATERIALS AND METHODS

Vaccinia virus expression vectors. Vaccinia virus vvB2 was generated by ligation of a PCR product of the HXB2 *gag* gene from plasmid pHXB2neo (kindly provided by Xiao Fang Yu, Johns Hopkins University) into the *Kpn*I-*Nhe*I sites of vector pSC11MCS1 (32). This placed *gag* ATG$_1$ 8 nucleotides (nt) downstream of an ATG codon in strong Kozak context (AGT ACC <u>ATG</u> G) found in the multicloning site of this vector, resulting in somewhat diminished Pr55^{gag} expression compared to unobstructed Pr55^{gag}. Virus vvENVGAG was generated by first transferring the *Xma*I-*Xba*I fragment of plasmid vector pENVGAG (see below) into pSC11MCS1 *Xma*I-*Nhe*I vector species. The *Kpn*I-*Eco*NI *env* fragment of the resulting plasmid was replaced with the *Kpn*I-*Eco*NI *env* fragment of plasmid pPE15 (kindly provided by Patricia Earl, National Institutes of Health) in order to remove the two vaccinia virus early transcription termination signals found in this portion of *env* (20). The resulting construct, pvEG, encodes a hybrid *env* ORF derived from two closely related *env* sequences, HIV-1 LAI (pENVGAG) and HIV-1 BH8 (pPE15). Recombinant vaccinia viruses were generated by recombination into strain vVWR-LVar and subsequent plaque purification according to a method described previously (21).

Plasmid expression vectors. Plasmid construction was accomplished by standard methods. All PCR inserts and splice junctions were verified by sequencing. The vectors were based on the Pr55^{gag} gene of HIV-1 isolate HXB2 (HIV Sequence Database, 1997 [http://hiv-web.lanl.gov/], Los Alamos National Laboratory Theoretical Biology and Biophysics, Los Alamos, N.Mex.). The Pr55^{gag} gene was silently modified to introduce an *Xho*I site near its 5′ end (G$_9$→T, A$_{10}$→C) and to attenuate the slippery sequence which promotes ribosomal frameshifting to the *pol* reading frame as described earlier (70). The Kozak context surrounding the start codon Pr55^{gag} in these vectors is CACACC<u>ATG</u> G. The HIV-1 *env* gene is derived from provirus pLAI.

Vectors pEMCV-CAT, pCAT, pCAT-GAG, pENVGAG, pENV, pGAG, pENV-ΔUTR-GAG, pENV-Ψ-GAG, pΔENV-GAG, pENV-ΔΔGAG, and pENVCAT were all expressed from the backbone of pCI (Promega). Vectors pLACZ, pIGAG, pΔ40, pΔ55, pFS, and pFSΔ40 were expressed from the backbone of plasmid pCMV6C.p55gag-pre, which was kindly provided by Chiron Corp. (Emeryville, Calif.). All vectors contain cytomegalovirus (CMV) promoter and an intron in the 5′ UTR to boost expression. All of the vectors also bear the hepatitis B virus (HBV) posttranscriptional regulatory element (PRE) (corresponding to the roughly 570-bp *Sph*I-*Fsp*I fragment of HBV strain adw 7) in the 3′ UTR to help overcome the Rev-dependency of *gag* and *env* mRNAs (35, 36).

Plasmids pIGAG and pGAG contain the silently modified HXB2 Pr55^{gag} gene. pΔ40 bears an ATG$_{142}$→ATC mutation generated by PCR-directed mutagenesis. pΔ55 was made by intramolecular ligation of blunted *Nhe*I and *Xho*I sites surrounding ATG$_1$, resulting in a 7-nt truncation at the 5′ end of Pr55^{gag}. pFS bears a single G deletion from a string of five G residues at *gag* bases 28 to 32.

The control vector pCAT was generated by ligating the *Kpn*I-*Xba*I chloramphenicol acetyltransferase (CAT) gene fragment from vector pCDNA3.1CAT (Invitrogen) into vector pGAG such that CAT replaced Pr55^{gag} between the intron and the HBV PRE. Vector pENVGAG was generated by three-way ligation of a pLAI *env* gene fragment and a short PCR product into vector pCIGAG such that the *env* stop codon was replaced by a GGC CAC ACC (Gly-His-Thr) linker between the two genes. pENV-ΔUTR-GAG was generated by replacing the *Avr*II-*Bst*BI Pr55^{gag} fragment of pENVGAG with an *Nhe*I-*Bst*BI fragment of pCIGAG, resulting in a +1 frameshift between the *env* and *gag* ORFs. In this construct, the *env* ORF terminates at a stop codon found in the *gag* +1 frame at bp 23 to 25. To create vector pΔENV-GAG, a frameshift was introduced into vector pENV-GAG by blunting an *Acc*65I site found at nt 123 to 128 of the *env* ORF. Vector pENV-UTR-GAG was generated by ligating a PCR product bearing the entire 335-bp *gag* 5′ UTR upstream of the *gag* gene in the context of pENVGAG such that the *env* ORF terminates at a TGA codon at positions −308 to −310 bp upstream of Pr55^{gag} ATG$_1$. Vector pENV-Ψ-GAG was generated by transferring the *Bss*HII-*Sp*I fragment of the HXB2 provirus into pENVGAG, thereby replacing a fragment of the *gag* 5′ UTR starting from position −78 such that the *env* ORF terminates at positions −23 to −25. The *Bss*HII site used for making pENV-Ψ-GAG interrupts the kissing loop found in stem-loop 1 of the packaging (Ψ) signal but leaves intact the 5′ major splice donor and Ψ signal stem-loops 2, 3, and 4. pENVCAT was generated by removing *gag* from vector pENVGAG by replacing its *Xho*I-*Xba*I *gag* fragment with the *Bsr*BI-*Xba*I CAT fragment of pCDNA3.1CAT. Initiation of translation at the remaining Pr55^{gag} AUG$_1$ would thus generate a slightly amino-extended version of CAT predicted to weigh 27.5 kDa (as opposed to the predicted 25.6 kDa of wild-type CAT). Vector pCAT-GAG was generated by first transferring the *Nhe*I-*Xba*I CAT fragment of pCDNA3.1CAT (which includes the native CAT stop codon) into an *Nhe*I site upstream of *gag* in vector pCIGAG and the

replacing the *Xho*I-*Xba*I fragment of the resulting vector with the *Xho*I-*Xba*I Ψ-*gag* fragment of pENVΨGAG. pEMCV-CAT was generated by first transferring the *Spe*I-*Hinc*II fragment of pEP2AmetWT (a gift from Richard Lloyd, Baylor College of Medicine) into pCI *Xba*I-*Sma*I and then ligating the *Hind*III (blunted)-*Bsp*120 fragment pCDNA3.1CAT into the resulting vector trimmed *Tfi*I (blunted)-*Not*I.

p40^{sac} stable expression vector pSAC was generated by a series of ligations involving fragments from pLAI and pHygro. pHygro was generated by removing the *Hind*III fragments from vector pIRES1-Hygro (Clontech), thus removing hybrid intron and encephalomyocarditis virus (EMCV) IRES sequences. Intermediate vector pLΔRH was generated by ligating the *Sal*I-*Pvu*II fragment of pHygro into the *Sal*I-*Aat*II (blunted) vector fragment of pVATG (see below). A second intermediate vector, pLRH, was generated by transferring the Rev-responsive element (RRE)-bearing *Bsa*BI-*Xho*I fragment of pLAI into the *Stu*I-*Sal*I vector fragment of pLΔRH. Finally, vector pSAC was generated by ligating an enhanced green fluorescent protein (EGFP) (Clontech)-bearing PCR fragment into the *Sfo*I-*Cla*I vector fragment of pLRH. This PCR product contained a Pr55^{gag} ATG$_1$→ATC mutation and inserted an AG dinucleotide between the EGFP stop codon and the mutated Pr55^{gag} ATC$_1$. The finished vector thus contains, in 5′ to 3′ order, 5′ long terminal repeat (LTR), deletion of 5′ splice donor and Ψ signal, EGFP, Pr55^{gag} ATG$_1$→ATC mutation, *sac-pol*, RRE, CMV promoter, hygromycin B resistance gene, and bovine growth hormone poly(A) signal. Control vector pΔSAC was generated by cutting vector pSAC *Bsr*GI-*Acc*I, blunting, and religating, thus removing all *gag* and *pol* coding regions.

Northern blotting probe vector pBHg was generated by transferring the *Hind*III-*Mfe*I fragment of *gag* from vector pGAG into pBluescript trimmed with *Eco*RI-*Hind*III. This vector was linearized with *Xho*I prior to transcription with T7 RNA polymerase (see Northern blotting section, below). The resulting probe is homologous to bases 923 to 1183 of the Pr55^{gag} ORF and is also homologous to a 28-bp section of the 3′ UTR of all pCI-based vectors (including pCAT).

Large-scale plasmid preps for transfection purposes were made by EndoFree Plasmid Maxi kit (Qiagen).

Proviral plasmids. The *Sap*I-*Sph*I fragment of ATG$_{142}$→ATC mutant plasmid pΔ40 was transferred into plasmid-cut pLAI *Sap*I-*Sph*I to create provirus pVATC. Because this *Sap*I-*Sph*I fragment bears a nonsilent Ser$_{LAI}$→Asn$_{HXB2}$ mutation at codon 126, an isogenic control provirus, pVATG, was generated using the *Sap*I-*Sph*I fragment of unmutated pGAG.

Cell culture and transfection. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll purification of blood from laboratory volunteers, and maintained in STCM (RPMI, 10% fetal calf serum [FCS], interleukin-2 [100 U/ml], T-cell growth factor). PBMC were activated by treatment for 3 days with 0.25 mg of phytohemagglutinin (PHA) per ml. Activated PBMC cultures were CD8$^+$ depleted by treatment with anti-CD8 magnetic beads (Dynal). Epstein-Barr virus (EBV)-transformed B cells were derived from patient A42 (32). COS-7 and 293T cell lines were obtained from the American Type Culture Collection (ATCC) and maintained according to conditions described in the ATCC catalog.

COS-7 cells were transfected in 12-well plates with Lipofectamine Plus (Life Technologies, Inc.) and were subjected to radioimmunoprecipitation assay (RIPA) or p24 enzyme-linked immunosorbent assay (ELISA) at 24 h posttransfection. For poliovirus infection experiments, 3.5×10^6 293T cells were preplated overnight in a 25-cm^2 flask and then transfected with Lipofectamine 2000 (Life Technologies). At 24 h posttransfection, cells were divided into eight wells of a 12-well plate (4 cm^2/well) and then infected with poliovirus beginning 40 h posttransfection. Infectious HIV-1 supernatants were collected from provirus-transfected COS-7 cells 3 days after transfection.

The stable Jurkat lines Jurkat+sac and JurkatΔsac were generated by electroporation of Jurkat cells with 10 μg of *Bsp*HI-linearized pSAC or pΔSAC in RPMI at 270 mV and 960 μF. The transfected cells were incubated for 2 days and then subjected to selection in 350 μg of hygromycin B (Boehringer) per ml for 3 weeks. Cells were then sorted using a fluorescence-activated cell sorter for EGFP expression, and the brightest 10% of FL1$^+$ cells were collected (data not shown). The resulting polyclonal populations were maintained in the presence of 350 μg of hygromycin B per ml.

Immunoprecipitation. Pulse-chase experiments on vaccinia virus-infected cells were performed as described elsewhere (86). Briefly, 1 million A42 EBV cells were vaccinia virus infected for 4 h, starved for 30 min in starvation medium (Cys$^-$ Met$^-$ Dulbecco modified Eagle medium [DMEM], 5% dialyzed FCS [Life Technologies]), and then radiolabeled for 30 min in a small volume of starvation medium supplemented with 1.5 mCi of [^{35}S]Cys-Met ProMix (Amersham) per ml. Cells were chased with chase medium (DMEM, 10% FCS, and 450 mg of cysteine and 300 mg of methionine per ml) and then disrupted in Triton lysis
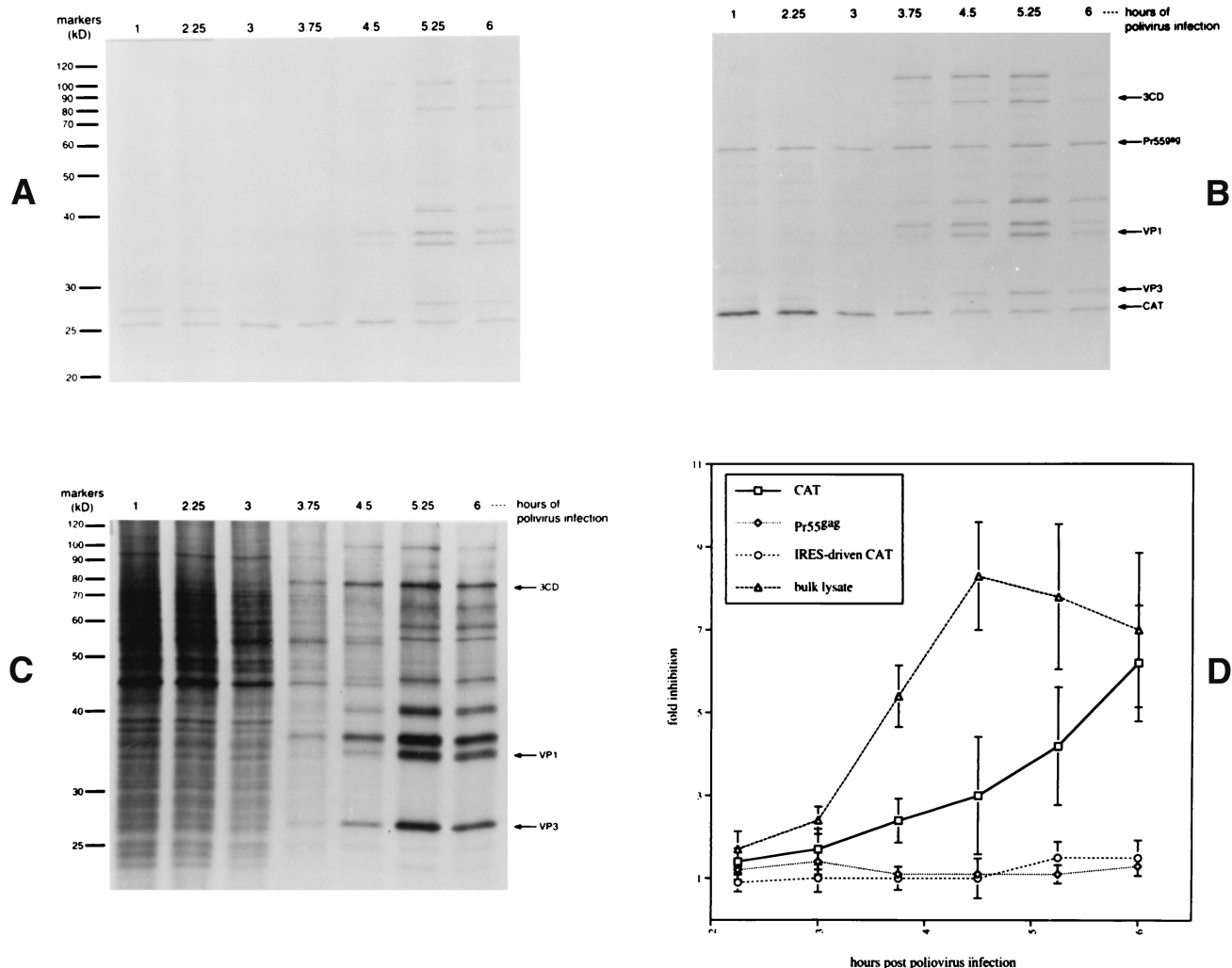
FIG. 1. RIPA analysis of Pr55*gag* from plasmid-transfected and poliovirus-infected 293T cells. Cells were transfected with pEMCV-CAT (A) or pCAT-GAG (B) for 40 h and then infected with freshly amplified poliovirus stock for the times shown. Cells were then detached, radiolabeled with [^{35}S]Cys-Met for 45 min, lysed, and subjected to immunoprecipitation with anti-HIV–anti-CAT antibody mixture. Unprecipitated bulk lysate of cells transfected with pCAT-GAG is shown in panel C. Panel D presents a summary of PhosphorImager analysis of five independent transfection-infection experiments. The *y* axis represents the fold inhibition of band intensity with respect to the band seen at 1 h post-poliovirus infection for the protein indicated. In two of the five experiments summarized in panel D, the CAT and Pr55*gag* bands were quantitated in cells cotransfected with pCAT and pENV-ΔUTR-GAG instead of pCAT-GAG. The intensity of signal of lysate bands was determined by examining a 28- to 33-kDa window found between prominent poliovirus VP3 and VP1 bands.

buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 0.1% sodium dodecyl sulfate [SDS]; 1 mM EDTA; 1% Triton X-100; protease inhibitor cocktail). Lysates were precleared with normal human immunoglobulin G (IgG; Sigma). Immunoprecipitation was accomplished using ~2 μg of anti-HIV IgIV, a human polyclonal antibody stock derived from HIV-1-seropositive individuals, and protein G-Sepharose beads (Pharmacia), followed by five washes with Triton lysis buffer.

RIPA of transfected COS-7 cells was performed in a similar manner. Approximately 5×10^5 transfected cells were suspended by treatment with phosphate-buffered saline (PBS)–EDTA (PBS, 0.5 mM EDTA, 5% dialyzed FCS) at 37°C for 15 min. Cells were then radiolabeled for 30 min in 50 μl of 1.5 mCi of [^{35}S]Cys-Met ProMix per ml, washed once with Tris-saline (50 mM Tris [pH 8.0], 150 mM NaCl), and then lysed in RIPA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail). The lysates were precleared with 50 μg normal human immunoglobulin G and immunoprecipitated with anti-HIV IgIV. The resulting beads were washed with two washes of IP wash (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% NP-40), one wash of Tris-saline (50 mM Tris [pH 8.0], 150 mM NaCl), and one wash of 50 mM Tris (pH 6.8). We chose to assess the presence or absence of Pr55*gag* and p40 in transfected cells by RIPA rather than by Western blot because

the HIV-IgIV antibody stock performs better in RIPA. However, similar results were also obtained by Western blotting (data not shown).

Detection of p40 in HIV-1-infected PBMC cultures was performed by pre-treating 2×10^6 PHA-activated, CD8$^+$-depleted PBMC infected with vLAI, vATG, or vATC with or without indinavir for 2 h. Indinavir was provided by The Merck Company. Cells were starved for 30 min in starvation medium with or without indinavir and then radiolabeled with 200 μCi of ProMix (with or without indinavir) for 1 h. Cells were washed and lysed using the same procedure as for COS-7 cells above, except that 50 μM indinavir was added to the lysis buffer and the lysate was immunoprecipitated with a polyclonal rabbit anti-p24 serum (NIAID ARRRP catalog no. 384) instead of anti-HIV IgIV to avoid possible detection of gp41.

In order to minimize background poliovirus bands, more stringent denaturing immunoprecipitation conditions were used to detect Pr55*gag* in transfected 293T cells infected with poliovirus. Transfected cells (see above) were infected with poliovirus by the addition of amplified poliovirus stock for the times shown in Fig. 1. Cells were then detached and radiolabeled in suspension for 45 min at 37°C. After radiolabeling, the cells were washed once in Tris-saline and then lysed by the addition of 35 μl of 2× SDS loading buffer (125 mM Tris, pH 6.8;
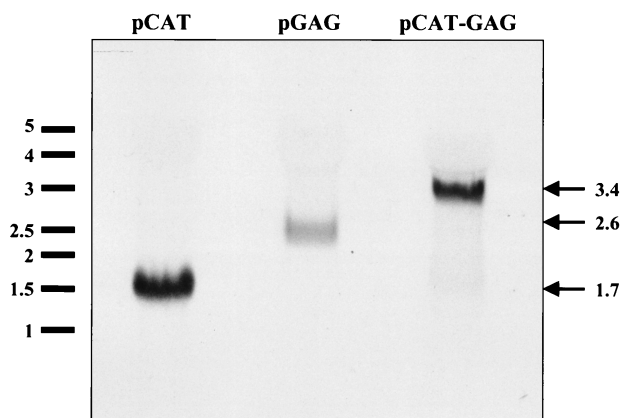
FIG. 2. Northern blotting of bicistronic vector pCAT-GAG. 293T cells were transfected with the plasmid expression vector indicated above each lane and subjected to Northern blotting. Kilobase RNA size markers are shown on the left. The predicted sizes the full-length transcript of each vector are given on the right.

20% glycerol; 2% β-mercaptoethanol; 4% SDS; 50 μg of bromophenol blue per ml). The lysate was incubated for 5 min at 100°C and then brought up in 1.5 ml of RIPA buffer. Samples were precleared with 50 μg of normal human immunoglobulin and then cleared with a mixture of 2 μg of anti-HIV IgIV and 1 μg of polyclonal rabbit anti-CAT (Eppendorf 5′, Inc.). The beads were then washed once with RIPA buffer, twice with EE wash (0.5 M NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA; 5% sucrose; 1% NP-40), once with IP wash, once with Tris-saline, and once with 50 mM Tris (pH 6.8).

Immunoprecipitated samples were analyzed by comparison to The Benchmark Unstained Protein Ladder (Life Technologies) by SDS-polyacrylamide gel electrophoresis (PAGE) on 8.5 or 12% gels.

Northern blotting. Cytoplasmic RNA was isolated (RNeasy kit; Qiagen) from 293T cells transfected for 40 h with the plasmid vector indicated in Fig. 2. Each RNA (5 μg) was subjected to Northern blotting (NorthernMax-Gly Kit; Ambion) according to the kit instructions. Blots were probed with a biotinylated RNA probe generated by T7 transcription of vector pBHg (Maxiscript Kit; Ambion) with a 60:40 ratio of UTP to biotin-16-UTP (Boehringer). Detection was carried out using Ambion's Brightstar Biodetect Kit according to manufacturer's instructions.

p24 ELISA. A commercially available anti-p24 ELISA kit (Coulter/Immunotech) was used to determine the levels of Gag in cell lysates and supernatants. Cells were washed twice with PBS-EDTA and then lysed in 300 μl of the lysis buffer provided with the kit. Cell lysates were diluted appropriately (typically, 1:100) to fall within the 7- to 125-pg/ml range of the assay. Lysates were standardized by quantitation of total protein using a detergent-compatible protein assay kit (Bio-Rad).

Edman microsequencing. Microsequencing of p40^{sac} was performed by the Johns Hopkins University School of Medicine Biosynthesis and Sequencing Facility using a Perkin-Elmer/Applied Biosystems Procise 492 Protein Microsequencer. p40^{sac} was isolated for microsequencing by immunoprecipitation of 5×10^7 vvB2-infected A42 EBV cells with HIV-IgIV (see above). The immunoprecipitated sample was electrophoresed by SDS-PAGE on a 12% gel and blotted overnight at 15 V onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was stained with Ponceau S (Bio-Rad), and the p40^{sac} band was excised and subjected to sequencing. Attempts to microsequence the 55-kDa species produced by vvB2-infected cells were performed in a similar manner except that the HIV-IgIV antibody was covalently conjugated to protein G-Sepharose beads prior to immunoprecipitation to avoid elution of interfering immunoglobulin heavy chain.

Virus culture. For visualization of p40^{sac} in HIV-1-infected cells, 2×10^6 activated/depleted PBMC were treated overnight with 1 ml of infectious supernatant collected from provirus-transfected COS-7 cells, incubated for 5 days, and then subjected to RIPA. For viral growth curves, provirus-transfected COS-7 supernatants were normalized by p24 ELISA so that roughly 50-ng equivalents (roughly 50 μl of supernatant) of p24 were added to 10^5 Jurkat cells or the stable cell lines Jurkat+sac or JurkatΔsac in a 1-ml volume in a 24-well plate. Cells were washed twice with 10 ml of culture medium 24 h postinfection. Supernatants

were sampled periodically and subjected to p24 ELISA. Infected cultures were split as necessary.

Cesium-banded high-titer Mahoney type 1 poliovirus stock was the generous gift of Ellie Ehrenfeld (National Institutes of Health). In addition to being more easily transfectable than the HeLa cells typically used in poliovirus shutoff experiments, 293T cells were found to exhibit more-protracted shutoff kinetics than HeLa cells (data not shown). Amplified infectious poliovirus stocks were generated by adding ~0.1 μl of high-titer poliovirus stock (10^{11} PFU/ml) to a confluent T-75 flask of 293T cells in 15 ml of culture medium for 24 h. The resulting cell lysate was clarified by centrifugation at $300 \times g$ for 10 min, and 0.5 ml of the clarified lysate was used to infect confluent 4-cm² wells of transfected 293T cells.

RESULTS

Expression of Pr55^{gag} in poliovirus-infected cells. To investigate the hypothesis that the HIV-1 *gag* ORF exhibits IRES activity, we undertook a series of experiments involving *gag* expression in poliovirus-infected cells. Because poliovirus mediates a rapid inhibition of cap-dependent translation in permissive host cells (23, 56), it has been widely used in the investigation of the cap dependency of translation of various mRNAs.

Figure 1 depicts a time course experiment in which 293T cells were first transfected with the indicated plasmid expression vector and then infected with poliovirus for various times. Bicistronic vector pCAT-GAG encodes the CAT reporter gene upstream of the Pr55^{gag} gene, including a 77-bp segment of the native Pr55^{gag} 5′ UTR. Control vector pEMCV-CAT encodes CAT under control of the EMCV IRES. After transfection and poliovirus infection, cells were radiolabeled with [^{35}S]Cys-Met for 45 min and subjected to immunoprecipitation with an anti-HIV–anti-CAT antibody mixture (Fig. 1A and B). Using this method, it was found that translation of EMCV IRES-driven CAT (Fig. 1A) and Pr55^{gag} (Fig. 1B) were not significantly impacted by poliovirus-mediated shutoff of cap-dependent translation. In contrast, the cap-dependent translation of the upstream CAT ORF from vector pCAT-GAG declined substantially over the course of the infection. Bulk lysates of the transfected cells (Fig. 1C) displayed a pattern in which the translation of host proteins diminished with duration of poliovirus infection, while a characteristic pattern of poliovirus-specific bands began to appear.

A summary of PhosphorImager (Molecular Dynamics) quantitation of five independent poliovirus time course experiments is shown in Fig. 1D. In two of these experiments, cells were cotransfected with a CAT expression vector, pCAT, and a different bicistronic vector, pENV-ΔUTR-GAG (see below), instead of pCAT-GAG. The results suggest that the expression of Pr55^{gag} from the constructs pCAT-GAG and pENV-ΔUTR-GAG was cap independent, a finding which supports the hypothesis that the HIV-1 *gag* gene encodes an IRES.

Northern blotting of pCAT-GAG. In addition to IRES activity, several possible explanations can be postulated to explain the expression of the second cistron of bicistronic constructs such as pCAT-GAG. These alternative scenarios include the existence of alternate splicing variants, RNA species derived from a cryptic promoter just upstream of the second cistron, or species of RNAs broken apart between the two cistrons. To rule out these possible explanations for the production of Pr55^{gag} from construct pCAT-GAG, we performed Northern blots on cells transfected with pCAT-GAG (Fig. 2). This Northern blotting shows that pCAT-GAG produces only one
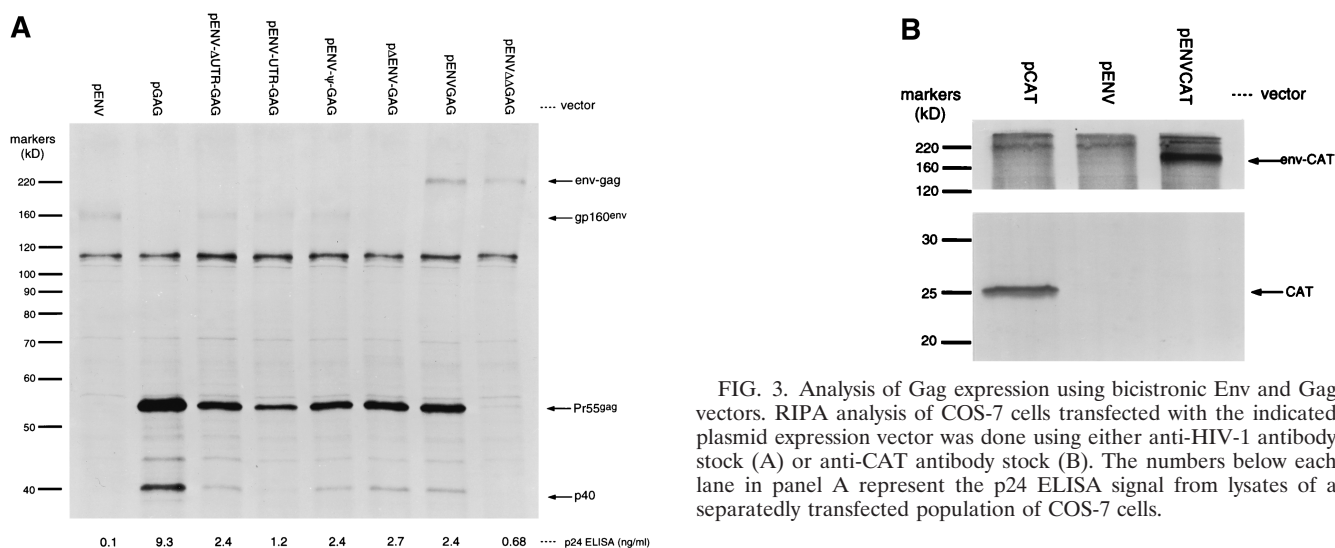
FIG. 3. Analysis of Gag expression using bicistronic Env and Gag vectors. RIPA analysis of COS-7 cells transfected with the indicated plasmid expression vector was done using either anti-HIV-1 antibody stock (A) or anti-CAT antibody stock (B). The numbers below each lane in panel A represent the p24 ELISA signal from lysates of a separately transfected population of COS-7 cells.

major RNA which is the predicted size (3.4 kb) of the full-length bicistronic CAT-GAG mRNA. This result argues against the possibility that the production of Pr55^{gag} is the result of a cryptic RNA species.

Env-Gag bicistronic vectors. To further investigate the IRES activity of the *gag* gene, we constructed a panel of bicistronic vectors. In these constructs, the HIV-1 *env* gene was used as an upstream reporter cistron, and the *gag* gene served both as the sequence to be tested for IRES activity and as a downstream reporter cistron. The *env* gene was chosen as an upstream reporter gene because it contains a large number of ATG codons and regions of highly structured RNA which would be expected to significantly disrupt scanning by any ribosomes bypassing the ATG codons within *env* (24). Expression of a novel 40-kDa *gag* isoform (p40), which results from translational initiation at an internal *gag* AUG codon, was also monitored in these experiments and will be discussed in greater detail below.

We first sought to determine what portion of the Pr55^{gag} 5′ UTR might be necessary for potentiating the translation of *gag* as a second cistron. Vector pENV-ΔUTR-GAG encodes only the *env* and *gag* reporter ORFs with the *gag* 5′ UTR entirely omitted. This construct was found to express Pr55^{gag} efficiently in poliovirus-infected cells (see above), suggesting that the native *gag* 5′ UTR is dispensable for the IRES activity of the *gag* ORF. In order to determine what impact the native *gag* 5′ UTR might have on *gag*'s translation as a second cistron, we constructed vector pENV-UTR-GAG, which contains the entire Pr55^{gag} 5′ UTR between the two ORFs. We also constructed vector pENV-Ψ-GAG, which contains a 77-bp fragment of the *gag* 5′ UTR encompassing most of the HIV-1 packaging (Ψ) signal. All three vectors were found to drive significant amounts of Pr55^{gag} expression when compared to vector pGAG, which encodes only Pr55^{gag} with an unobstructed vector-derived 5′ UTR (Fig. 3A). This result confirms the observation that the native *gag* 5′ UTR is not necessary to potentiate the expression of Pr55^{gag} as a second cistron. Indeed, placement of the entire *gag* 5′ UTR upstream of Pr55^{gag}

(in vector pENV-UTR-GAG) resulted in somewhat decreased levels of Pr55^{gag} expression relative to the expression seen from vector pENV-ΔUTR-GAG. This decrease could have been due to activity of HIV-1 polyadenylation signal (found in the R domain of the HIV-1 LTRs), the presence of the primer binding site, or the presence of the viral dimerization initiation signal, since removal of these sequences (in pENV-Ψ-GAG) resulted in higher Pr55^{gag} expression levels.

We generated several other vectors to investigate the conditions under which *gag* might function as a downstream cistron. In vector pΔENV-GAG, a frameshift was introduced near the 5′ end of the *env* ORF of vector pENV-ΔUTR-GAG in order to demonstrate that translation of the *env* ORF is not required for translation of the downstream *gag* ORF. Vector pENVGAG encodes an in-frame fusion of the *env* ORF (lacking the native *env* stop codon) to the *gag* ORF. Expression of Pr55^{gag} from this vector (Fig. 3A) argues against the possibility that the expression of Pr55^{gag} from vector pENVGAG is due to translational reinitiation following *env* translation, since the *env-gag* and *gag* ORFs are coterminal. A control vector, pENV-ΔΔGAG, encodes the same Env-Gag fusion protein as vector pENVGAG except that the Pr55^{gag} and p40 initiator codons have been mutated to ATC, a noninitiator Ile codon (79). As seen in Fig. 3A, mutation of these ATG codons abrogates Pr55^{gag} and p40 production, suggesting that their expression is the result of translation initiation at these internal sites.

In a control vector, pENVCAT, all but the first 12 bp of the *gag* sequence of pENVGAG were replaced with the CAT reporter gene. As seen in Fig. 3B, pENVCAT does not drive expression of any CAT-sized species. This result suggests that the internal translational initiation at Pr55^{gag} AUG$_1$ observed in vector pENVGAG is dependent on sequences within the *gag* ORF, since the CAT ORF was not sufficient to direct this activity.

In order to further exclude the possibility that Pr55^{gag} and p40 expression from vector pENVGAG was the result of cryptic splicing or cryptic promoter usage, we generated a recombinant vaccinia virus vector, vvENVGAG, which encodes the same Env-Gag fusion protein as plasmid pENVGAG. Because vaccinia viruses replicate in the cytoplasm of infected cells and
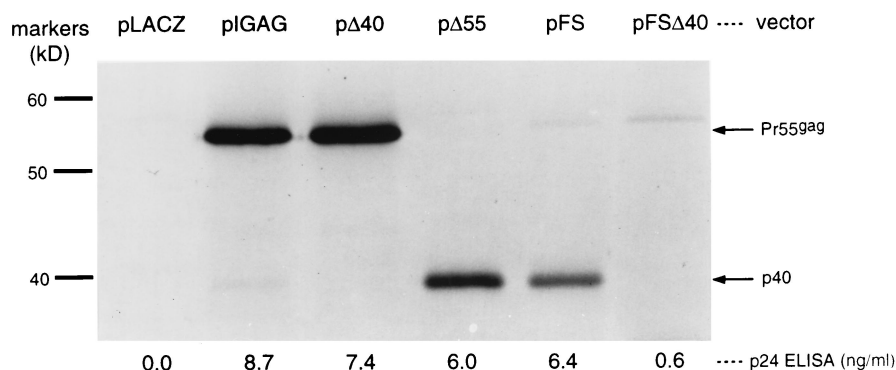
FIG. 4. Manipulation of p40 expression. Each lane represents comparable numbers of Cos-7 cells transfected with the indicated plasmid vector and subjected to RIPA with anti-HIV antibody stock. The numbers below each lane represent p24 ELISA signal from lysates of a separately transfected population of Cos-7 cells.

utilize virus-encoded transcription factors (59), it is unlikely that cryptic host promoters would have any activity in the context of a recombinant vaccinia virus. Splicing of vaccinia virus RNAs has not been reported. COS-7 cells infected with vvENVGAG exhibited Env-Gag, Pr55^{gag}, and p40 bands (data not shown), indicating that expression of Pr55^{gag} and p40 from these vectors was not due to cryptic splicing or cryptic promoter usage.

These results confirm that sequences within the *gag* ORF can promote internal initiation of translation of Pr55^{gag} even when the *gag* ORF is placed downstream of large numbers of ATG codons and highly structured RNA sequences. This activity is a feature of sequences within the *gag* ORF and functions even in the absence of the native *gag* 5′ UTR.

Characterization of a 40-kDa *gag* gene product. A wide variety of plasmid and vaccinia virus expression vectors encoding Pr55^{gag} consistently display a minor *gag* gene-specific 40-kDa band (Fig. 3 and 4 and data not shown). Because the *gag* gene contains an isolated AUG at codon 142 that could initiate translation of a 40-kDa protein, we considered the possibility that p40 was the result of an internal initiation event at this AUG. To help determine its origins, we immunoprecipitated p40 from cells infected with vvB2, a vaccinia virus vector encoding Pr55^{gag}, and subjected it to Edman microsequencing. This sequencing yielded the amino acid sequence NH$_2$-VHQAISPRT, a sequence identical to the predicted amino acid sequence of a polypeptide initiated at AUG$_{142}$, assuming its initiator methionine was cleaved away (82; reviewed in reference 1). Pulse-chase experiments were also performed on cells infected with vvB2. Pr55^{gag} and p40 exhibited no precursor-product relationship, arguing against the concept that p40 was derived through proteolytic cleavage of Pr55^{gag} (data not shown).

We also examined a panel of plasmid expression vectors (Fig. 4) to verify that p40 was the result of an independent translation initiation event and not simply a by-product of Pr55^{gag}. In two vectors, we independently mutated the Pr55^{gag} and p40 initiator ATG codons to ATC. We found that mutation of ATG$_{142}$ to ATC abrogated p40 expression, suggesting that this codon is used as an initiator codon for p40. Although the mutation of Pr55^{gag} ATG$_1$ to ATC prevented Pr55^{gag} expression, it did not abolish p40 expression. Similarly, introduc-

tion of a frameshift downstream of ATG$_1$ did not abolish p40 expression. Thus, p40 can be expressed independently of Pr55^{gag} through translational initiation at *gag* codon 142.

p40 expression in HIV-1-infected cells. To determine whether internal initiation at AUG$_{142}$ occurs during viral replication, we examined HIV-1-infected cells for the presence of p40. Answering this question was complicated by the activity of the HIV-1 protease. Met$_{142}$ lies only six amino acids downstream of the eight-amino-acid protease recognition site (codons 129 to 136) at the matrix/capsid boundary. Thus, a single HIV-1 protease cleavage of Pr55^{gag} yields a protein nearly identical in size to p40. Similarly, a single cleavage of Pr55^{gag} at the capsid-p2 boundary results in a protein of approximately 40 kDa. For clarity, we will hereafter refer to the 40-kDa protein generated by initiation at AUG$_{142}$ as p40^{sac} for "start at capsid." To determine whether p40^{sac} is produced in infected cells, we used indinavir (an HIV-1 protease inhibitor) to prevent the generation of Pr55^{gag} protease cleavage products (89). PHA-activated, CD8$^+$-depleted PBMC were infected with supernatants from COS-7 cells transfected with wild-type provirus pLAI, p40^{sac} knockout provirus pVATC (carrying an ATG$_{142}$→ATC [Met→Ile] mutation), or provirus pVATG, an isogenic wild-type control for pVATC (see Materials and Methods). Five days after infection, PBMC cultures were pretreated with 50 mM indinavir and then radiolabeled with [^{35}S]Cys-Met. Thus, all radiolabeled *gag* gene products in the indinavir-treated cells were generated in the functional absence of HIV-1 protease. The labeled cells were then lysed and subjected to immunoprecipitation with anti-p24 serum.

Cells infected with wild-type viruses vLAI and vATG expressed 40-kDa products both in the presence and in the absence of indinavir (Fig. 5). The absence of a p24 band in cultures treated with indinavir suggests a strong inhibition of HIV-1 protease in these cultures. Although some 40-kDa Pr55^{gag} protease cleavage products accumulated in vATC-infected cells in the absence of indinavir, the ATG$_{142}$→ATC mutation in this virus completely ablated expression of p40^{sac}, as shown by the absence of a 40-kD band in the presence of indinavir. The results support the conclusion that HIV-1-infected cells produce small amounts of a novel 40-kDa protein, p40^{sac}, through internal initiation at AUG$_{142}$.
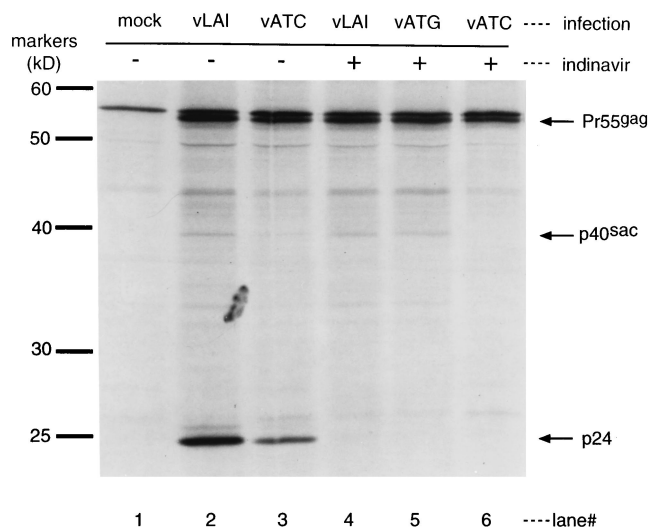
FIG. 5. p40*sac* expression in HIV-1-infected PBMCs. PHA-activated, CD8^{+}-depleted PBMC were mock infected (lane 1) or were infected with wild-type viruses vLAI or vATG (lanes 2, 4, and 5) or with p40*sac* knockout virus vATC for 5 days. Cells were treated with (+) or without (−) indinavir, an HIV-1 protease inhibitor, and then radiolabeled and subjected to RIPA with polyclonal anti-p24 serum.

Rescue of impaired replication of a p40*sac*-deficient HIV-1 mutant. In order to investigate whether p40*sac* might play a role in viral replication in culture, we examined the replication kinetics of the p40*sac* knockout virus vATC. This mutant virus displayed dramatically impaired replication compared to wild-type vLAI or isogenic wild-type control vATG in Jurkat and CD8-depleted PBMC cultures (Fig. 6; also data not shown). In light of the fact that ATG_{142} and the region surrounding it are highly conserved (60; HIV Sequence Database), even relatively conservative nonsilent mutations (such as the $Met_{142} \rightarrow Ile$ mutation found in vATC) might be expected to be deleterious to

viral growth through disruption of the normal functions of Pr55*gag*.

To determine whether the delayed replication kinetics of vATC were due to its inability to produce p40*sac* or due to the amino acid substitution it carries in Pr55*gag*, we generated a polyclonal stably transfected Jurkat line which expresses p40*sac* in *trans*. In the context of full-length HIV-1 mRNAs, translational initiation at Pr55*gag* codon 142 would be expected to result in production of p40*sac* and a 145-kDa protein, p145*sac-pol*, by virtue of frameshifting at the HIV-1 "slippery" site. In an effort to provide appropriate levels of p40*sac* and p145*sac-pol*, we made a Rev- and Tat- inducible *sac-pol* vector, pSAC, based on the pLAI provirus. The vector expresses p40*sac* and p145*sac-pol* from the HIV-1 LTR. To block ribosome scanning and to monitor expression, we inserted the EGFP gene upstream of Pr55*gag* codon 1, which was mutated from ATG to ATC to abrogate Pr55*gag* expression. A fragment containing the HIV-1 RRE was placed downstream of the *pol* stop codon, as was a fragment carrying the CMV promoter, hygromycin B phosphotransferase, and a poly(A) signal. A Jurkat line stably carrying a control vector, pΔSAC, in which the *sac-pol* gene was removed from pSAC, was also made. In the presence of Tat and Rev provided by an infecting virus, transcription and nuclear export of EGFP-p40*sac*-p145*sac-pol* mRNA expressed from these vectors should be induced. Consistent with this prediction, uninfected Jurkat cultures stably carrying these constructs displayed constitutive low levels of fluorescence (and low p24 ELISA signal for vector pSAC), while infected cultures of the lines showed a substantial population of highly fluorescent cells (data not shown). We named these cell lines Jurkat+sac and JurkatΔsac.

Although p40*sac* knockout virus vATC failed to grow to high titers in Jurkat and JurkatΔsac cultures, it was able to grow in the Jurkat+sac line (Fig. 6). When cultures were infected with much higher inoculums, vATC could eventually grow to high titers in Jurkat and JurkatΔsac cultures but with dramatically
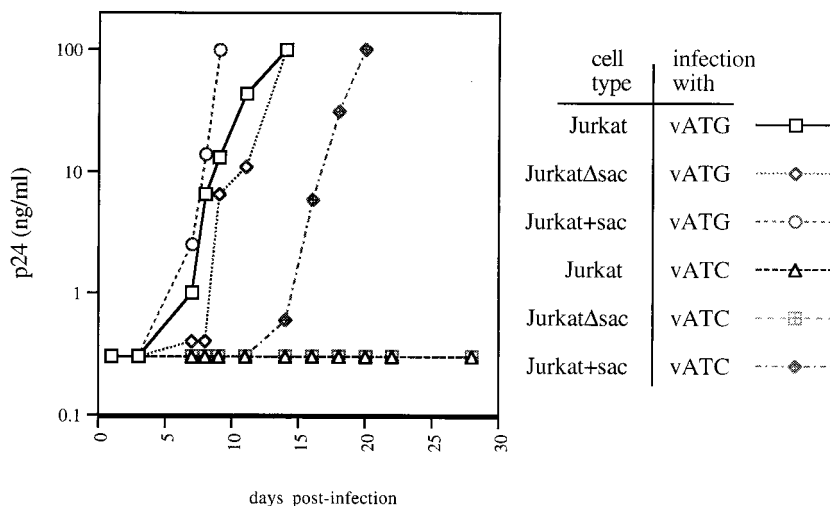

cell type	infection with	
Jurkat	vATG	—□—
JurkatΔsac	vATG	·····◇·····
Jurkat+sac	vATG	----○----
Jurkat	vATC	----△----
JurkatΔsac	vATC	- -⊞- -
Jurkat+sac	vATC	---◈---

FIG. 6. Rescue of codon 142 mutant virus vATC by expression of p40*sac* and p145*sac-pol* in *trans*. Infectious supernatants from 293T cells transfected with codon 142 mutant provirus pVATC or isogenic wild-type control provirus pVATG were normalized for p24 content and added to Jurkat cells, the p40*sac* and p145*sac-pol* stable Jurkat cell line Jurkat+sac, or the control Jurkat cell line JurkatΔsac. Cells were washed at 24 h postinfection, and the supernatants were sampled at the times shown and subjected to p24 ELISA.

slower kinetics than seen in Jurkat+sac cultures (data not shown). Although vATC grew well in the Jurkat+sac line, growth was somewhat attenuated compared to wild-type virus vATG. This could reflect some impact of the Pr55^{gag} ATG$_{142}$→ATC mutation in vATC, inappropriate timing of p40^{sac} and p145$^{sac-pol}$ expression, or inappropriate levels of p40^{sac} and p145$^{sac-pol}$ in the Jurkat+sac line.

These results are consistent with the concept that the defective growth phenotype of mutant virus vATC can be overcome, at least in part, by expression of p40^{sac} and p145$^{sac-pol}$ in trans. Thus, expression of p40^{sac} (or p145$^{sac-pol}$) may be required for normal growth kinetics of HIV-1 in culture.

DISCUSSION

Our results demonstrate that the HIV-1 gag gene encodes an IRES. One line of evidence that Pr55^{gag} can be translated in a cap-independent fashion via an IRES-like mechanism comes from experiments involving poliovirus-infected cells. Poliovirus mediates a rapid reduction in cap-dependent translation shortly after infection of permissive cells. This host cell translational shutoff coincides with the cleavage by poliovirus 2A protease of the eIF4G subunit of eIF4F (also known as the cap-binding complex), and the cleavage of poly(A) binding protein by poliovirus proteases 2A and 3C (44, 46). The IRES-mediated translation of poliovirus RNAs under these conditions is either unimpaired or enhanced. Poliovirus-infected cells (45) and extracts of cells infected with poliovirus have been widely used to demonstrate the presence of IRES activity within various mRNA sequences (23). As seen in Fig. 1, the translation of Pr55^{gag} in cells transfected with bicistronic vector pCAT-GAG was insensitive to poliovirus-mediated shutdown. This supports the concept that Pr55^{gag} can be translated through a cap-independent translation mechanism, such as an IRES, located within the gag ORF. Northern blots of cells transfected with pCAT-GAG were found to exhibit only one full-length mRNA species, arguing against the possibility that this cap-independent expression of Pr55^{gag} was due to alternative splicing, cryptic promoter usage, or broken RNA species.

A separate line of evidence which suggests that gag encodes an IRES comes from a series of bicistronic vectors where cap-dependent ribosome scanning was obstructed by placement of the HIV-1 env ORF upstream of gag. As seen in Fig. 3A, placement of the env ORF upstream of gag does not abrogate Pr55^{gag} expression. This result held true whether the native gag 5′ UTR was present or not, whether the env ORF was translated or not, and also when the env ORF was translated as an in-frame fusion with the gag ORF. Utilization of Pr55^{gag} AUG$_1$ as an internal initiator codon required the presence of the gag ORF itself, as evidenced by the absence of expression of any CAT-length products by vector pENVCAT, in which CAT replaces most of the gag ORF downstream of env (Fig. 3B). These results argue against modified ribosome scanning mechanisms for Pr55^{gag} or p40^{sac} expression such as leaky scanning (49, 77), translational reinitiation (28, 54), ribosome shunting (72, 96), broken RNA, or ribosome "stutter" (18, 95). The expression of Pr55^{gag} both from vaccinia virus vector vvENVGAG and plasmid vector pENVGAG argues against the possibility that expression of these proteins is due to cryptic promoter usage or cryptic splicing. Thus, two separate lines of evidence indicate that Pr55^{gag} can be translated as an internal or downstream cistron by virtue of an IRES within the gag ORF.

In addition to potentiating the translation of Pr55^{gag}, the HIV-1 gag IRES also facilitates translation of a novel 40-kDa protein, p40^{sac}, whose translation initiates at an AUG codon near the 5′ end of the p24^{capsid} domain of Pr55^{gag}. Several features of HIV-1 biology may have prevented the previous identification of this novel Gag isoform. As demonstrated in Fig. 5, intermediate products of the cleavage of Pr55^{gag} by the HIV-1 protease are roughly 40 kDa in mass and can obscure the presence of p40^{sac} (26). In past instances when infected cells treated with HIV-1 protease inhibitors have exhibited a 40-kDa band, the band may have been discounted as reflecting incomplete inhibition of protease activity by the drug (89, 92). The fact that p40^{sac} is expressed at much lower levels than Pr55^{gag} has also perhaps hidden it from identification even when Pr55^{gag} has been expressed in the absence of HIV-1 protease (25, 57, 73).

Despite its low abundance, our results suggest that p40^{sac} may play a vital role in viral replication in vitro. HIV-1 mutant virus vATC, which fails to generate p40^{sac}, and, presumptively, p145$^{sac-pol}$ replicates with dramatically impaired growth kinetics in cultured cells (Fig. 6). The replication defect of vATC can be partially overcome by the expression of p40^{sac} or p145$^{sac-pol}$ in trans, suggesting that the defect stems, at least in part, from the mutant's inability to generate these novel proteins. This observation is consistent with the concept that p40^{sac} and/or p145$^{sac-pol}$ play an important role in viral replication in culture.

It has been shown that sequences within the native gag 5′ UTR can inhibit cap-dependent translation of reporter proteins. The Tat-responsive element (TAR) is an RNA secondary structural element that is responsible for binding HIV-1 Tat protein and may play a role in the inhibition of cellular interferon-induced double-stranded RNA-activated kinase (12, 22). The TAR is located at the 5′ end of all HIV-1 RNA transcripts. Because the presence of secondary structure at or near the 5′ end of RNAs reduces the accessibility of the 5′ cap to eIF4F (49), it is thought that this feature of HIV-1 mRNAs can inhibit their cap-dependent translation (64, 78). Other stable HIV-1 RNA structural elements involved in regulating the splicing, polyadenylation, dimerization, packaging, primer binding, and reverse transcription of viral RNAs are thought to inhibit translation by blocking the scanning of 40S ribosomes (and their associated cofactors) through the gag 5′ UTR (29, 57). Thus, a possible function of the HIV-1 gag IRES might be to serve as a mechanism to bypass the structural barriers to cap-dependent translation by recruiting ribosomes directly to the gag ORFs.

It has been demonstrated that infection with HIV-1 can arrest cells in the G$_2$ phase of the cell cycle (reviewed in reference 13). The fact that cap-dependent translation is inhibited during some phases of the cell cycle (8) led Vagner and colleagues to speculate that an HIV-1 IRES might allow for enhanced HIV-1 gene expression under growth-arrest conditions where the cap-dependent translation of host cell mRNAs is inhibited (91). Recent reports have found that the IRESs of hepatitis C virus (HCV) and host mRNAs encoding ornithine decarboxylase and p58^{PITSLRE} are significantly more active

during the G_2/M phase of the cell cycle than in the G_1 or S phases (11, 34, 69). The possible interplay between HIV-1 cell cycle arrest and IRES utilization deserves future investigation.

In addition to the IRESs found in the 5′ UTRs of a variety of plus-stranded RNA viruses (3, 9, 31, 42, 43, 66, 88) and a number of eukaryotic cellular mRNAs (6, 27, 37, 38, 61, 62, 84, 90), IRESs have recently been described in several type C retroviruses. These include MMLV (91), Friend murine leukemia virus (FMLV) (4, 19), the Rat VL30 region of the Harvey murine sarcoma virus (5), and avian reticuloendotheliosis virus type A (53). A variety of other retroid family members, including human T-cell leukemia virus type 1 (2), human foamy virus (51), LINE-1 and VL30 (two groups of endogenous vertebrate retro-elements) (39, 52, 56, 87), and *Drosophila* R2 retrotransposable elements (30), have also been shown to exhibit possible IRES-like activities. These data, when taken together with our observation in this report that HIV-1 encodes an IRES, suggest the possibility that IRES-mediated translation is a typical feature of the retroviridae.

Murine leukemia viruses encode two different Gag proteins. Glyco-Gag, a nonstructural transmembrane glycoprotein important for viral infectivity, is initiated from a CUG codon located upstream of and in frame with the AUG-initiated *gag* gene (67, 68). The IRES of MMLV has been found to be situated entirely within the viral *glyco-gag* ORF in a 126-bp sequence just upstream of the *gag* AUG. The MMLV IRES was shown to initiate translation only at the *gag* AUG, whereas the FMLV IRES, which has not been fully localized, was shown to be capable of directing translation of both Gag and Glyco-Gag.

Localization of the MMLV *gag* IRES to a region entirely contained within an ORF was striking in that it (and perhaps the IRES of FMLV) was the first naturally occurring (10) IRES entirely contained within a translated ORF. More recently, a similar ORF-localized IRES has been described for the host mRNA encoding p110^{PITSLRE} and p58^{PITSLRE} (11). The HIV-1 Pr55^{gag} IRES activity described here is thus conceptually similar to the MMLV and PITSLRE IRESs in that it is located entirely within an ORF and is similar to the FMLV IRES in that it can direct translation of two different *gag* gene products.

Miele and colleagues have argued that the HIV-1 packaging signal does not encode an IRES (57). Using a series of bicistronic constructs in which the entire *gag* 5′ UTR and as much as the first 200 nt of the *gag* ORF were inserted between two reporter cistrons, these investigators found no evidence for potentiation of translation of the downstream reporter cistron. In contrast to Miele and colleagues' findings, a recent report from Ohlmann and colleagues has found that sequences in the 5′ UTR of the *gag* gene of another primate lentivirus, SIVmac, do exhibit IRES activity (63). Thus, it may be that IRES activity is typical of the lentiviridae, while the exact location of sequences required for the activity varies.

Perhaps the most surprising feature of the HIV-1 *gag* IRES is that it directs translational initiation at (or, conceivably, beyond) its 5′ border. Precedents for translational enhancement by sequence elements located 3′ of the ORF they control include the mRNAs of certain viruses (14, 85, 94), and the 3′ UTR of the mRNA for the β subunit of mitochondrial H^+-ATP synthase (40). Important future work on this topic will include identification of the minimal sequence elements required for HIV-1 *gag* ORF IRES activity and the potential mechanisms underlying its surprising, seemingly backward, arrangement.

Das et al. have demonstrated that constitutive expression of a small inhibitor RNA (IRNA) molecule isolated from yeast (15) can effectively protect cultured cells from productive poliovirus infection (17) without significantly impacting cell growth or morphology. The suppression of poliovirus replication in cells expressing this IRNA is likely the result of competition between the IRNA and the poliovirus IRES for access to host proteins required for viral IRES-mediated translation (16, 93). It is thus formally possible that similar antagonism of the HIV-1 *gag* IRES could disrupt the replication of HIV-1 or other IRES-bearing retroviruses.

REFERENCES

1. **Arfin, S. M., and R. A. Bradshaw.** 1988. Cotranslational processing and protein turnover in eukaryotic cells. Biochemistry **27:**7979–7984.
2. **Attal, J., M. C. Theron, F. Taboit, M. Cajero-Juarez, G. Kann, P. Bolifraud, and L. M. Houdebine.** 1996. The RU5 ('R') region from human leukaemia viruses (HTLV-1) contains an internal ribosome entry site (IRES)-like sequence. FEBS Lett. **392:**220–224.
3. **Belsham, G. J., and J. K. Brangwyn.** 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. **64:**5389–5395.
4. **Berlioz, C., and J. L. Darlix.** 1995. An internal ribosomal entry mechanism promotes translation of murine leukemia virus Gag polyprotein precursors. J. Virol. **69:**2214–2222.
5. **Berlioz, C., C. Torrent, and J. L. Darlix.** 1995. An internal ribosomal entry signal in the rat VL30 region of the Harvey murine sarcoma virus leader and its use in dicistronic retroviral vectors. J. Virol. **69:**6400–6407.
6. **Bernstein, J., O. Sella, S. Y. Le, and O. Elroy-Stein.** 1997. PDGF2/c-sis mRNA leader contains a differentiation-linked internal ribosomal entry site (D-IRES). J. Biol. Chem. **272:**9356–9362.
7. **Blum, H. E., E. Galun, T. J. Liang, F. von Weizsacker, and J. R. Wands.** 1991. Naturally occurring missense mutation in the polymerase gene terminating hepatitis B virus replication. J. Virol. **65:**1836–1842.
8. **Bonneau, A. M., and N. Sonenberg.** 1987. Involvement of the 24-kDa cap-binding protein in regulation of protein synthesis in mitosis. J. Biol. Chem. **262:**11134–11139.
9. **Borman, A., and R. J. Jackson.** 1992. Initiation of translation of human rhinovirus RNA: mapping the internal ribosome entry site. Virology **188:**685–696.
10. **Chen, C. Y., and P. Sarnow.** 1995. Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. Science **268:**415–417.
11. **Cornelis, S., Y. Bruynooghe, G. Denecker, S. Van Huffel, S. Tinton, and R. Beyaert.** 2000. Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Mol. Cell **5:**597–605.
12. **Cullen, B. R.** 1993. Does HIV-1 Tat induce a change in viral initiation rights? Cell **73:**417–420.
13. **Cullen, B. R.** 1998. HIV-1 auxiliary proteins: making connections in a dying cell. Cell **93:**685–692.
14. **Danthinne, X., J. Seurinck, F. Meulewaeter, M. Van Montagu, and M. Cornelissen.** 1993. The 3′ untranslated region of satellite tobacco necrosis virus RNA stimulates translation in vitro. Mol. Cell. Biol. **13:**3340–3349.
15. **Das, S., P. Coward, and A. Dasgupta.** 1994. A small yeast RNA selectively inhibits internal initiation of translation programmed by poliovirus RNA: specific interaction with cellular proteins that bind to the viral 5′-untranslated region. J. Virol. **68:**7200–7211.
16. **Das, S., D. J. Kenan, D. Bocskai, J. D. Keene, and A. Dasgupta.** 1996. Sequences within a small yeast RNA required for inhibition of internal initiation of translation: interaction with La and other cellular proteins

influences its inhibitory activity. J. Virol. **70:**1624–1632.

17. **Das, S., M. Ott, A. Yamane, W. Tsai, M. Gromeier, F. Lahser, S. Gupta, and A. Dasgupta.** 1998. A small yeast RNA blocks hepatitis C virus internal ribosome entry site (HCV IRES)-mediated translation and inhibits replication of a chimeric poliovirus under translational control of the HCV IRES element. J. Virol. **72:**5638–5647. (Erratum, **72:**9419.)

18. **Dasso, M. C., S. C. Milburn, J. W. Hershey, and R. J. Jackson.** 1990. Selection of the 5′-proximal translation initiation site is influenced by mRNA and eIF-2 concentrations. Eur. J. Biochem. **187:**361–371.

19. **Deffaud, C., and J. L. Darlix.** 2000. Characterization of an internal ribosome entry segment in the 5′ leader of murine leukemia virus env RNA. J. Virol. **74:**846–850.

20. **Earl, P. L., A. W. Hugin, and B. Moss.** 1990. Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia virus. J. Virol. **64:**2448–2451.

21. **Earl, P. L., and B. Moss.** 1993. Generation of recombinant vaccinia viruses, p. 16.17.1–16.17.12. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. John Wiley and Sons, Boston, Mass.

22. **Edery, I., R. Petryshyn, and N. Sonenberg.** 1989. Activation of double-stranded RNA-dependent kinase (dsl) by the TAR region of HIV-1 mRNA: a novel translational control mechanism. Cell **56:**303–312.

23. **Ehrenfeld, E.** 1996. Initiation of translation by picornavirus RNAs, p. 549–573. *In* J. W. B. Hershey, M. B. Matthews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

24. **Ellerbrok, H., N. Serpente, G. Pancino, C. Vanhee, L. D'Auriol, M. Sitbon, and C. Vaquero.** 1993. Sequences in the rev-responsive element responsible for premature translational arrest in the human-immunodeficiency-virus-type-1 envelope. Eur. J. Biochem. **216:**459–467.

25. **Erickson-Viitanen, S., J. Manfredi, P. Viitanen, D. E. Tribe, R. Tritch, C. A. D. Hutchison, D. D. Loeb, and R. Swanstrom.** 1989. Cleavage of HIV-1 Gag polyprotein synthesized in vitro: sequential cleavage by the viral protease. AIDS Res. Hum. Retrovir. **5:**577–591.

26. **Evans, L. A., J. M. Homsy, W. J. Morrow, I. Gaston, C. D. Sooy, and J. A. Levy.** 1988. Human monoclonal antibody directed against gag gene products of the human immunodeficiency virus. J. Immunol. **140:**941–943.

27. **Gan, W., and R. E. Rhoads.** 1996. Internal initiation of translation directed by the 5′-untranslated region of the mRNA for eIF4G, a factor involved in the picornavirus-induced switch from cap-dependent to internal initiation. J. Biol. Chem. **271:**623–626.

28. **Geballe, A. P.** 1996. Regulation of translation by upstream AUG codons, p. 173–197. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

29. **Geballe, A. P., and M. K. Gray.** 1992. Variable inhibition of cell-free translation by HIV-1 transcript leader sequences. Nucleic Acids Res. **20:**4291–4297.

30. **George, J. A., and T. H. Eickbush.** 1999. Conserved features at the 5′ end of *Drosophila* R2 retrotransposable elements: implications for transcription and translation. Insect Mol. Biol. **8:**3–10.

31. **Glass, M. J., X. Y. Jia, and D. F. Summers.** 1993. Identification of the hepatitis A virus internal ribosome entry site: in vivo and in vitro analysis of bicistronic RNAs containing the HAV 5′ noncoding region. Virology **193:**842–852.

32. **Hammond, S. A., R. P. Johnson, S. A. Kalams, B. D. Walker, M. Takiguchi, J. T. Safrit, R. A. Koup, and R. F. Siliciano.** 1995. An epitope-selective, transporter associated with antigen presentation (TAP)-1/2-independent pathway and a more general TAP-1/2-dependent antigen-processing pathway allow recognition of the HIV-1 envelope glycoprotein by CD8⁺ CTL. J. Immunol. **154:**6140–6156.

33. **Hatfield, D., and S. Oroszlan.** 1990. The where, what and how of ribosomal frameshifting in retroviral protein synthesis. Trends Biochem Sci. **15:**186–190.

34. **Honda, M., S. Kaneko, E. Matsushita, K. Kobayashi, G. A. Abell, and S. M. Lemon.** 2000. Cell cycle regulation of hepatitis C virus internal ribosomal entry site-directed translation. Gastroenterology **118:**152–162.

35. **Huang, J., and T. J. Liang.** 1993. A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. Mol. Cell. Biol. **13:**7476–7486.

36. **Huang, Z. M., and T. S. Yen.** 1995. Role of the hepatitis B virus posttranscriptional regulatory element in export of intronless transcripts. Mol. Cell. Biol. **15:**3864–3869.

37. **Iizuka, N., C. Chen, Q. Yang, G. Johannes, and P. Sarnow.** 1995. Cap-independent translation and internal initiation of translation in eukaryotic cellular mRNA molecules. Curr. Top. Microbiol. Immunol. **203:**155–177.

38. **Iizuka, N., L. Najita, A. Franzusoff, and P. Sarnow.** 1994. Cap-dependent and Cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **14:**7322–7330.

39. **Ilves, H., O. Kahre, and M. Speek.** 1992. Translation of the rat LINE bicistronic RNAs in vitro involves ribosomal reinitiation instead of frameshifting. Mol. Cell. Biol. **12:**4242–4248.

40. **Izquierdo, J. M., and J. M. Cuezva.** 2000. Internal-ribosome-entry-site functional activity of the 3′-untranslated region of the mRNA for the beta subunit of mitochondrial H⁺-ATP synthase. Biochem. J. **346:**849–855.

41. **Jackson, R. J., and A. Kaminski.** 1995. Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA **1:**985–1000.

42. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63:**1651–1660.

43. **Jang, S. K., H. G. Krausslich, M. J. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636–2643.

44. **Joachims, M., P. C. Van Breugel, and R. E. Lloyd.** 1999. Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. J. Virol. **73:**718–727.

45. **Johannes, G., and P. Sarnow.** 1998. Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites. RNA **4:**1500–1513.

46. **Kerekatte, V., B. D. Keiper, C. Badorff, A. Cai, K. U. Knowlton, and R. E. Rhoads.** 1999. Cleavage of Poly(A)-binding protein by coxsackievirus 2A protease in vitro and in vivo: another mechanism for host protein synthesis shutoff? J. Virol. **73:**709–717.

47. **Kozak, M.** 1986. Bifunctional messenger RNAs in eukaryotes. Cell. **47:**481–483.

48. **Kozak, M.** 1989. Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. Mol. Cell. Biol. **9:**5134–5142.

49. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell. **44:**283–292.

50. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.

51. **Lochelt, M., and R. M. Flugel.** 1996. The human foamy virus *pol* gene is expressed as a Pro-Pol polyprotein and not as a Gag-Pol fusion protein. J. Virol. **70:**1033–1040.

52. **Lopez-Lastra, M., S. Ulrici, C. Gabus, and J. L. Darlix.** 1999. Identification of an internal ribosome entry segment in the 5′ region of the mouse VL30 retrotransposon and its use in the development of retroviral vectors. J. Virol. **73:**8393–8402.

53. **Lopezlastra, M., C. Gabus, and J. L. Darlix.** 1997. Characterization of an internal ribosomal entry segment within the 5′ leader of avian reticuloendotheliosis virus type a RNA and development of novel Mlv-Rev-based retroviral vectors. Hum. Gene Ther. **8:**1855–1865.

54. **Luukkonen, B. G., W. Tan, and S. Schwartz.** 1995. Efficiency of reinitiation of translation on human immunodeficiency virus type 1 mRNAs is determined by the length of the upstream open reading frame and by intercistronic distance. J. Virol. **69:**4086–4094.

55. **Macejak, D. G., and P. Sarnow.** 1991. Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. Nature **353:**90–94.

56. **McMillan, J. P., and M. F. Singer.** 1993. Translation of the human LINE-1 element, L1Hs. Proc. Natl. Acad. Sci. USA **90:**11533–11537.

57. **Miele, G., A. Mouland, G. P. Harrison, E. Cohen, and A. M. Lever.** 1996. The human immunodeficiency virus type 1 5′ packaging signal structure affects translation but does not function as an internal ribosome entry site structure. J. Virol. **70:**944–951.

58. **Morley, S. J., P. S. Curtis, and V. M. Pain.** 1997. eIF4G: translation's mystery factor begins to yield its secrets. RNA **3:**1085–1104.

59. **Moss, B.** 1996. Poxviridae: the viruses and their replication, p. 1171–1176. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fundamental virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.

60. **Myers, G., B. Korber, S. Wain-Hobson, K. T. Jeang, L. E. Henderson, and G. N. Pavlakis.** 1994. Human retroviruses and AIDS. Los Alamos National Laboratory Theoretical Biology and Biophysics, Los Alamos, N.Mex.

61. **Nanbru, C., I. Lafon, S. Audigier, M. C. Gensac, S. Vagner, G. Huez, and A. C. Prats.** 1997. Alternative translation of the proto-oncogene c-*myc* by an internal ribosome entry site. J. Biol. Chem. **272:**32061–32066.

62. **Oh, S. K., M. P. Scott, and P. Sarnow.** 1992. Homeotic gene Antennapedia mRNA contains 5′-noncoding sequences that confer translational initiation by internal ribosome binding. Genes Dev. **6:**1643–1653.

63. **Ohlmann, T., M. Lopez-Lastra, and J. L. Darlix.** 2000. An internal ribosome entry segment promotes translation of the simian immunodeficiency virus genomic RNA. J. Biol. Chem. **275:**11899–11906.

64. **Parkin, N. T., E. A. Cohen, A. Darveau, C. Rosen, W. Haseltine, and N. Sonenberg.** 1988. Mutational analysis of the 5′ non-coding region of human immunodeficiency virus type 1: effects of secondary structure on translation. EMBO J. **7:**2831–2837.

65. **Pavlakis, G. N., S. Schwartz, D. M. D'Agostino, and B. K. Felber.** 1991. Structure, splicing, and regulation of expression of HIV-1: a model for the general organization of lentiviruses and other complex retroviruses, p. 41–63. *In* R. Kennedy, F. Wong-Staal, and W. C. Koff (ed.), Annual review of AIDS research. Marcel Dekker, Inc., New York, N.Y.

66. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of

eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature **334:**320–325.

67. **Pillemer, E. A., D. A. Kooistra, O. N. Witte, and I. L. Weissman.** 1986. Monoclonal antibody to the amino-terminal L sequence of murine leukemia virus glycosylated Gag polyproteins demonstrates their unusual orientation in the cell membrane. J. Virol. **57:**413–421.

68. **Prats, A. C., G. De Billy, P. Wang, and J. L. Darlix.** 1989. CUG initiation codon used for the synthesis of a cell surface antigen coded by the murine leukemia virus. J. Mol. Biol. **205:**363–372.

69. **Pyronnet, S., L. Pradayrol, and N. Sonenberg.** 2000. A cell cycle-dependent internal ribosome entry site. Mol. Cell **5:**607–616.

70. **Reil, H., H. Kollmus, U. H. Weidle, and H. Hauser.** 1993. A heptanucleotide sequence mediates ribosomal frameshifting in mammalian cells. J. Virol. **67:**5579–5584.

71. **Sachs, A. B., P. Sarnow, and M. W. Hentze.** 1997. Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell **89:**831–838.

72. **Schmidt-Puchta, W., D. Dominguez, D. Lewetag, and T. Hohn.** 1997. Plant ribosome shunting in vitro. Nucleic Acids Res. **25:**2854–2860.

73. **Schneider, R., M. Campbell, G. Nasioulas, B. K. Felber, and G. N. Pavlakis.** 1997. Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. J. Virol. **71:**4892–4903.

74. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64:**2519–2529.

75. **Schwartz, S., B. K. Felber, E. M. Fenyo, and G. N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. **64:**5448–5456.

76. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1991. Expression of human immunodeficiency virus type 1 Vif and Vpr mRNAs is Rev-dependent and regulated by splicing. Virology **183:**677–686.

77. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1992. Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. Mol. Cell. Biol. **12:**207–219.

78. **SenGupta, D. N., B. Berkhout, A. Gatignol, A. M. Zhou, and R. H. Silverman.** 1990. Direct evidence for translational regulation by leader RNA and Tat protein of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **87:**7492–7496.

79. **Shastri, N., V. Nguyen, and F. Gonzalez.** 1995. Major histocompatibility class I molecules can present cryptic translation products to T-cells. J. Biol. Chem. **270:**1088–1091.

80. **Shatkin, A. J.** 1976. Capping of eucaryotic mRNAs. Cell **9:**645–653.

81. **Shatkin, A. J.** 1985. mRNA cap binding proteins: essential factors for initiating translation. Cell **40:**223–224.

82. **Sherman, F., J. W. Stewart, and S. Tsunasawa.** 1985. Methionine or not methionine at the beginning of a protein. Bioessays **3:**27–31.

83. **Stein, I., A. Itin, P. Einat, R. Skaliter, Z. Grossman, and E. Keshet.** 1998. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. Mol. Cell. Biol. **18:**3112–3119.

84. **Teerink, H., H. O. Voorma, and A. A. Thomas.** 1995. The human insulin-like growth factor II leader 1 contains an internal ribosomal entry site. Biochim. Biophys. Acta **1264:**403–408.

85. **Timmer, R. T., L. A. Benkowski, D. Schodin, S. R. Lax, A. M. Metz, J. M. Ravel, and K. S. Browning.** 1993. The 5′ and 3′ untranslated regions of satellite tobacco necrosis virus RNA affect translational efficiency and dependence on a 5′ cap structure. J. Biol. Chem. **268:**9504–9510.

86. **Tobery, T. W., and R. F. Siliciano.** 1997. Targeting of HIV-1 antigens for rapid intracellular degradation enhances cytotoxic T lymphocyte (CTL) recognition and the induction of de novo CTL responses in vivo after immunization. J. Exp. Med. **185:**909–920.

87. **Torrent, C., C. Berlioz, and J. L. Darlix.** 1996. Stable MLV-VL30 dicistronic retroviral vectors with a VL30 or MoMLV sequence promoting both packaging of genomic RNA and expression of the 3′ cistron. Hum. Gene Ther. **7:**603–612.

88. **Tsukiyama-Kohara, K., N. Iizuka, M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66:**1476–1483.

89. **Vacca, J. P., B. D. Dorsey, W. A. Schleif, R. B. Levin, S. L. McDaniel, P. L. Darke, J. Zugay, J. C. Quintero, O. M. Blahy, E. Roth, et al.** 1994. L-735,524: an orally bioavailable human immunodeficiency virus type 1 protease inhibitor. Proc. Natl. Acad. Sci. USA **91:**4096–4100.

90. **Vagner, S., M. C. Gensac, A. Maret, F. Bayard, F. Amalric, H. Prats, and A. C. Prats.** 1995. Alternative translation of human fibroblast growth factor 2 mRNA occurs by internal entry of ribosomes. Mol. Cell. Biol. **15:**35–44.

91. **Vagner, S., A. Waysbort, M. Marenda, M. C. Gensac, F. Amalric, and A. C. Prats.** 1995. Alternative translation initiation of the Moloney murine leukemia virus mRNA controlled by internal ribosome entry involving the p57/PTB splicing factor. J. Biol. Chem. **270:**20376–20383.

92. **Venaud, S., N. Yahi, J. L. Fehrentz, N. Guettari, D. Nisato, I. Hirsch, and J. C. Chermann.** 1992. Inhibition of HIV by an anti-HIV protease synthetic peptide blocks an early step of viral replication. Res. Virol. **143:**311–319.

93. **Venkatesan, A., S. Das, and A. Dasgupta.** 1999. Structure and function of a small RNA that selectively inhibits internal ribosome entry site-mediated translation. Nucleic Acids Res. **27:**562–572.

94. **Wang, S., K. S. Browning, and W. A. Miller.** 1997. A viral sequence in the 3′-untranslated region mimics a 5′ cap in facilitating translation of uncapped mRNA. EMBO J. **16:**4107–4116.

95. **Williams, M. A., and R. A. Lamb.** 1989. Effect of mutations and deletions in a bicistronic mRNA on the synthesis of influenza B virus NB and NA glycoproteins. J. Virol. **63:**28–35.

96. **Yueh, A., and R. J. Schneider.** 1996. Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells. Genes Dev. **10:**1557–1567.