JOURNAL OF VIROLOGY, Feb. 1996, p. 944–951 Vol. 70, No. 2
0022-538X/96/$04.00+0

The Human Immunodeficiency Virus Type 1 5′ Packaging Signal Structure Affects Translation but Does Not Function as an Internal Ribosome Entry Site Structure

GINO MIELE,[1] ANDREW MOULAND,[2] GEOFFREY P. HARRISON,[1]
ÉRIC COHEN,[2] AND ANDREW M. L. LEVER[1]*

University of Cambridge Department of Medicine, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom,[1] and Laboratoire de rétrovirologie humaine, Département de microbiologie et immunologie, Université de Montréal, Montréal, Canada H3C 3J7[2]

Received 28 August 1995/Accepted 7 November 1995

The role of the RNA secondary structure in the 5′ packaging signal region of human immunodeficiency virus type 1 (HIV-1) in initiating translation of *gag* mRNA has been investigated both in vitro and in the presence of cellular cofactors in vivo. Heat denaturation of the structure and mutagenic deletion both lead to an increase in levels of translated products, indicating that the structure is a significant inhibitor of translation. The proximity of the *gag* AUG to the packaging signal structure suggested that it might function as an internal ribosome entry site. However, in both a cell-free system and eukaryotic cells, translation will initiate at a novel upstream initiation codon introduced within the 5′ noncoding region. This codon is utilized exclusively, resulting in *gag* protein products with an extra 11 amino acids at the amino terminus, which, when expressed in T lymphocytes, are confined intracellularly, probably because of the lack of an N-terminal glycine myristoylation signal. Deletion of the secondary structure abolishes *gag* production even in the presence of *tat* and *rev* in *trans*. Using dicistronic constructs containing the HIV-1 5′ leader cloned between two heterologous open reading frames, we were unable to detect any significant expression of the second open reading frame that would have been supportive of an internal ribosome entry site mechanism. Using mutant proviruses either lacking the entire packaging signal structure region or containing the introduced upstream initiation codon in long-term replication studies, we were unable to detect reverse transcriptase activity in culture supernatants. The 5′ packaging signal structure of HIV-1 does not serve as an internal ribosome entry site. The translation of *gag* is consistent with ribosomal scanning. However, the packaging signal structure causes significant translational inhibition.

The 5′ leader sequence of human immunodeficiency virus type 1 (HIV-1) has multiple *cis*-acting functions which are essential in the virus life cycle. These include transcriptional control through the *tat*-responsive element (TAR) (5, 45, 56, 61, 65), binding of the tRNA primer (31, 53), dimerization of the genomic RNA (11), and splicing (13). A signal important to genomic RNA encapsidation in HIV-1 has been identified in this region (3, 10, 30, 39). In COS cell transfection studies a deletion within the 5′ end of the *gag* open reading frame has also been suggested to affect packaging (40).

We and others have demonstrated that a stable secondary structure exists in the region between the primer binding site and the *gag* AUG (4, 21, 22, 58). Deletions in this region which reduce the efficiency of packaging would also disrupt this predicted secondary structure. Secondary structure predictions vary somewhat among investigators; however, there are some regions which are identified by all investigators. In particular, the stem-loop from bases 763 to 780 is consistently predicted.

Expression of many eukaryotic genes is regulated at the level of translation (64, 66). A scanning hypothesis of translation initiation has been proposed (35), but the mechanism is not yet fully elucidated. According to this model, after the 25-kDa cap recognition protein (known as p25 eIF-4E or CBP1) has bound to the mRNA cap, the 40S subunit of the ribosome (in combination with Met-tRNA and other factors) binds somewhere along the 5′ end of the untranslated sequence and then migrates along the RNA, unwinding the RNA secondary structure (dependent on ATP), until an initiator AUG is encountered (32, 35, 66). For efficient translation initiation, this AUG should be in an appropriate sequence context (RccAUGG), the purine (R) at position −3 being particularly important in determining the strength of initiation together with, to a lesser extent, the G at position +4 (33). Ribosomal scanning is known to be inhibited by strong secondary structures (16, 17, 19, 20, 23, 32, 34, 36, 43, 44, 69). Therefore, if translation of HIV-1 *gag* is initiated by the ribosomal scanning mechanism, the predicted secondary structure in this region would be expected to be inhibitory.

Internal ribosome entry has been demonstrated in a number of prokaryotic and eukaryotic systems, including picornaviruses (1, 24–28, 50, 63), a plant potyvirus (8), cowpea mosaic virus (67), the immunoglobulin (Ig) heavy chain-binding protein (41), homeotic gene antennapedia mRNA (48), and hepatitis C virus (68, 70). Most recently, internal initiation has been shown with murine leukemia virus-based constructs in which competing AUG and CUG initiation codons exist (6). In HIV-1 the proximity of the predicted secondary structure to the *gag* gene raised the possibility that it might also act as an internal ribosome entry site (IRES).

In this study we have investigated how the secondary structure in the HIV-1 5′ untranslated region affects translation of the *gag* and *gag/pol* open reading frames.

* Corresponding author. Mailing address: University of Cambridge Department of Medicine, Addenbrooke's Hospital, Hills Rd., Cambridge CB2 2QQ, United Kingdom. Phone: (1223) 336844. Fax: (1223) 336846.

MATERIALS AND METHODS

Vector construction. A summary of plasmid constructions is given below. All proviral clones were derived from pSVC21, an infectious proviral clone of the HIV-1 (HTLV-IIIB) isolate originally from a plasmid (pHXBc2) supplied by R. Gallo and F. Wong-Staal (14). pSVC21 incorporates a simian virus 40 origin of replication to ensure efficient gene expression in COS cells. The numbering of the sequence follows that of the Los Alamos database (47). Restriction sites refer to positions in the HXBc2 genome.

An HIV-1 *Bgl*II fragment (positions 475 to 2096) was subcloned into the *Bam*HI site of pBluescript KS+ (Stratagene), in the orientation of the T3 promoter, to serve as a template for mutagenesis. Site-directed mutagenesis (38) was carried out with the following oligonucleotides: A4, 5′-CCGCTAGTGA AACCATTTTGCGTACTC-3′; A14, 5′-TCTCGCACCCATCTCTGGTTTCCC TTTCGCTTT-3′; and D2, 5′-CCTTCTAGCCTAAAATAGTCA-3′. Subclones containing the required mutations were identified by dideoxy sequencing (59). The *Nar*I to *Apa*I fragment (HIV-1 positions 637 to 2010) was cloned back into the HXBc2 provirus, and each mutation was resequenced in the proviral clones.

Mutation A4. The construction of the mutation A4 generated a new initiation codon 33 bases upstream of the native *gag* initiation codon, which is at position 790. The G at position 763 was mutated to C in order to remove a nonsense codon, so that the use of the new AUG would add an extra 11 amino acids at the 5′ end of the *gag* open reading frame. These 11 amino acids are MVSLAEAR-RRE, with a total predicted molecular mass of 1,498 Da. To create a favorable Kozak consensus, the C at position 753 was mutated to an A and the U at position 759 was mutated to a G (Fig. 1B).

Mutation A14. The construction of mutation A14 generated a deletion from bases 677G to 790A, removing the entire secondary structure, including the major splice donor signal. The resulting leader sequence retains a translation context identical to that of the wild type (Fig. 1C).

Mutation D2m. The construction of mutation D2m resulted from a mismatch between the mutagenic template and oligo D2. An AUG initiation codon at position 767 was created out of frame with the native AUG but in frame with a stop codon at position 776 (Fig. 1D).

The RNA cap site for the *gag* mRNA is at position 454, just 5′ of the *Bgl*II site at position 474, which was used to excise the packaging signal region for mutagenesis. To generate mRNAs which included the RNA cap site, a *Sca*I fragment (positions 313 to 2751) from the mutated proviral clones was subcloned into the *Eco*RV site of pBluescript KS+ in the orientation of the T7 promoter. These clones are referred to as the pKSScaI series. All proviral clones were propagated in *Escherichia coli* DH5α, and plasmids were prepared by cesium chloride purification (42). pBluescript clones which were required for in vitro transcription (in the uncoupled system) were propagated in dam(−) Epicurian-coli SCS110 cells (Stratagene) to allow linearization with the methylation-sensitive restriction endonuclease *Bcl*I, which cleaves at HIV-1 position 2429. The linearized plasmid therefore contains the first 178 bases of the *pol* gene.

Dicistronic constructs. Dicistronic constructs (Fig. 2) were prepared to examine directly internal ribosome entry on HIV-1 mRNAs. The luciferase gene was excised from pGEM-*luc* (Promega) with *Bam*HI and *Xho*I and cloned into the mammalian expression vector pcDNA3 (Invitrogen) to generate pcDNA3-*luc* (kindly provided by Nahum Sonenberg, McGill University). Recombinant PCR was used to generate HIV (molecular clone pSVC21), poliovirus (type II Lansing), or control intercistronic sequences upstream of chloramphenicol acetyl transferase (CAT) sequences with 5′ oligonucleotides that contain an *Xho*I site and a 3′ oligonucleotide containing an *Apa*I site in CAT 10 nucleotides (nt) downstream of the stop codon. These were directionally cloned into pcDNA3-*luc* such that *gag* mRNA, including the cap (at position +1) up to an including the *gag* initiation codon, a poliovirus leader sequence (positions +1 to 735), or a negative control sequence from the pcDNA vector (nt +3578 to +3909) was placed upstream of CAT. In a similar manner, dicistronic constructs were made with the *env* leader as the test sequence, and the effect of the *gag* coding sequence on *gag* mRNA translation initiation was evaluated by including 100 and 200 nt of the *gag* coding sequence in addition to the *gag* mRNA leader. The following PCR primers were used.

pcDNA3 HIV-1: XhoIHIV5′, 5′-ATCACTCGAGGGGTCTCTCTGGTTAG AC-3′; and HIV-CAT (antisense), 5′-TCCAGTGATTTTTTTCTCCATCTCT CTCCTTCTAGCCTC-3′

pcDNA3 Polio: XhoIP255, 5′-ATCACTCGAGTTAAAACAGCTCTGGGGT-3′; and P25-CAT (antisense), 5′-GCTTCCTTAGCTCCTGAAAAGATATCTT AACAATGAGGT-3′

pcDNA3 Neg: XhoIpcDNA35′, 5′-ATCACTCGAGTCAAAGGCGGTAATA CGG-3′; and pcDNA3-CAT (antisense), 5′-GCTTCCTTAGCTCCTGAAAA CTGAGATACCTACAGCGTG-3′

pcDNA3-env: HIV285-env 5525 (antisense), 5′-TTCGTCGCTGTCTCCGCTT CAGTCGCCGCCCCTCGCC-3′; and HIV 5525 (sense), 5′-AAGCGGAGACA GCGACGAA-3′

HIV 5525-CAT (antisense): 5′-TCCAGTGATTTTTTTCTCCATTGCCACTG TCTTCTGCT-3′

All constructs: CAT 5′ (sense), 5′-ATGGAGAAAAAAATCACTGGA-3′; and ApaICAT3′, 5′-ACAGGGCCCTTAAAAAAATTACGCCCC-3′

pcDNA3 gag100: HIV446-CAT (antisense), 5′-TCCAGTGATTTTTTTCTCCA TGCTTGCCCATACTATATGTTT-3′

pcDNA3 gag200: HIV534-CAT (antisense), 5′-TCCAGTGATTTTTTTCTCCA TAAGGGATGGTTGTAGCTGTCC-3′

The plasmids used were pKSBglII WT, a wild-type HIV-1 sequence from positions 474 to 2095 in Bluescript KS+, as a mutagenic template; pKSBglII A4, like pSKBglII WT but with a novel AUG in the Kozak consensus; pKSScaI WT, the wild-type HIV-1 sequence from positions 313 to 2751 in Bluescript KS+; pKSScaI A4, like pKSScaI WT but with a novel AUG in the Kozak consensus; pKSScaI A14, like pKSScaI WT but with the deletion generated by oligonucleotide A14; pSVC21 A4, a proviral HIV-1 sequence with the A4 mutation; pSVC21 A14, a proviral HIV-1 sequence with the A14 mutation; pSVC21 D2m, a proviral HIV-1 sequence with the D2m mutation; pCDNA3 HIV-1, a dicistronic construct containing the HIV-1 *gag* leader sequence; pCDNA3 Polio, a dicistronic construct containing the poliovirus leader sequence; pCDNA3 Neg, a dicistronic construct containing a random sequence; pCDNA3 env, a dicistronic construct containing the HIV-1 *env* leader sequence; pCDNA3gag100, a dicistronic construct containing the HIV-1 *gag* leader sequence plus the first 100 nucleotides of the *gag* coding region; pCDNA3gag200, a dicistronic construct containing the HIV-1 *gag* leader sequence plus the first 200 nucleotides of the *gag* coding region.

In vitro transcription and translation. One microgram of supercoiled plasmid DNA from the pKSBglII WT and pKSBglII A4 clones was transcribed and translated in a coupled rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions with T3 RNA polymerase and 0.8 mCi of translation-grade [^{35}S]methionine (Amersham) per ml. One-tenth of the reaction product was boiled in sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) at 100°C for 4 min and loaded onto a denaturing polyacrylamide gel. Following electrophoresis, the gel was fixed, soaked in Amplify (Amersham) for 30 min, and dried and autoradiography was performed.

Since it is not known whether the wild-type RNA secondary structure can be fully formed prior to translation in the coupled transcription and translation reticulocyte system, transcription and translation reactions were also performed in an uncoupled system in which these processes were temporally separated.

Synthesis of capped RNA. Completely linearized pKSScaI plasmids (5 μg) were transcribed in 50 μl of 40 mM Tris-HCl (pH 7.5)–6 mM $MgCl_2$–2 mM spermidine–10 mM NaCl–10 mM dithiothreitol–0.5 mM each recombinant nucleoside triphosphate (except GTP, which was used at a final concentration of 0.05 mM)–0.5 mM m^7G (5′) ppp (5′) G (Boehringer Mannheim) with 50 U of RNasin (Promega) and 40 U of T7 RNA polymerase (Promega). Following DNase treatment (RQ1 DNase; at 37°C for 10 min), the RNA was extracted with phenol-chloroform, precipitated with ethanol, and then redissolved in double-distilled autoclaved water (diethyl pyrocarbonate treated). To quantify the transcription products, aliquots were analyzed by agarose gel electrophoresis and with DNA dipsticks (Invitrogen).

Two micrograms of each of the test-capped mRNA samples was used to program micrococcal nuclease-treated rabbit reticulocyte lysate (Promega). Translation reactions were carried out at 30°C for 1.5 h in the presence of 0.8 mCi of translation grade [^{35}S]methionine (Amersham) per ml. The RNA secondary structure was disrupted by heating the RNA samples to 67°C for 10 min. After denaturation, the RNAs were either translated immediately (to translate an unstructured mRNA template) or allowed to cool to room temperature and then to 0°C over a period of 1 h to translate a reannealed mRNA template. Portions equivalent to one-tenth of the translation reaction mixtures were heated in sample buffer at 100°C for 4 min and loaded onto a denaturing polyacrylamide gel. Following electrophoresis, the gel was fixed, soaked in Amplify (Amersham) for 30 min, and dried and autoradiography was performed. Bands were quantitated by densitometry.

Size differentiation of *gag* proteins was performed by SDS-polyacrylamide gel electrophoresis with linear gels of 12.5% or gradient gels of 7.5 to 15% (42).

In vitro transcription and translation of dicistronic constructs were performed after linearizing with *Apa*I. The reaction buffer consisted of 40 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM spermidine, 100 μg of bovine serum albumin, 5 mM (each) ATP, CTP, and UTP and 100 mM GTP, 1 mM m^7GpppG, 100 mM dithiothreitol, 0.4 U of RNasin per μl, and 0.5 μl of T7 RNA polymerase (New England Biolabs). [^{3}H]cytidine (10 nCi) was added to monitor incorporation. Reaction mixtures were treated with RQ1 DNase and then passed through a G-50 spin column. The eluate was precipitated with ethanol. Equal numbers of counts per minute from each reaction were used to program protein synthesis in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate (Promega) (52). Because CAT was not identifiable by gel electrophoresis because of background, an aliquot of the reaction mixture was used in a standard CAT assay (42). Both a blank (reticulocyte lysate alone) and a positive CAT expressor (pSP6 [46]) were used.

Cell culture. Cells were transfected with DEAE dextran (62) for monolayer and suspension cells. COS-1 cells were cultured in Eagle's minimal essential medium, and Jurkat-*tat* T lymphocytes (55) were grown in RPMI 1640. Cell lines

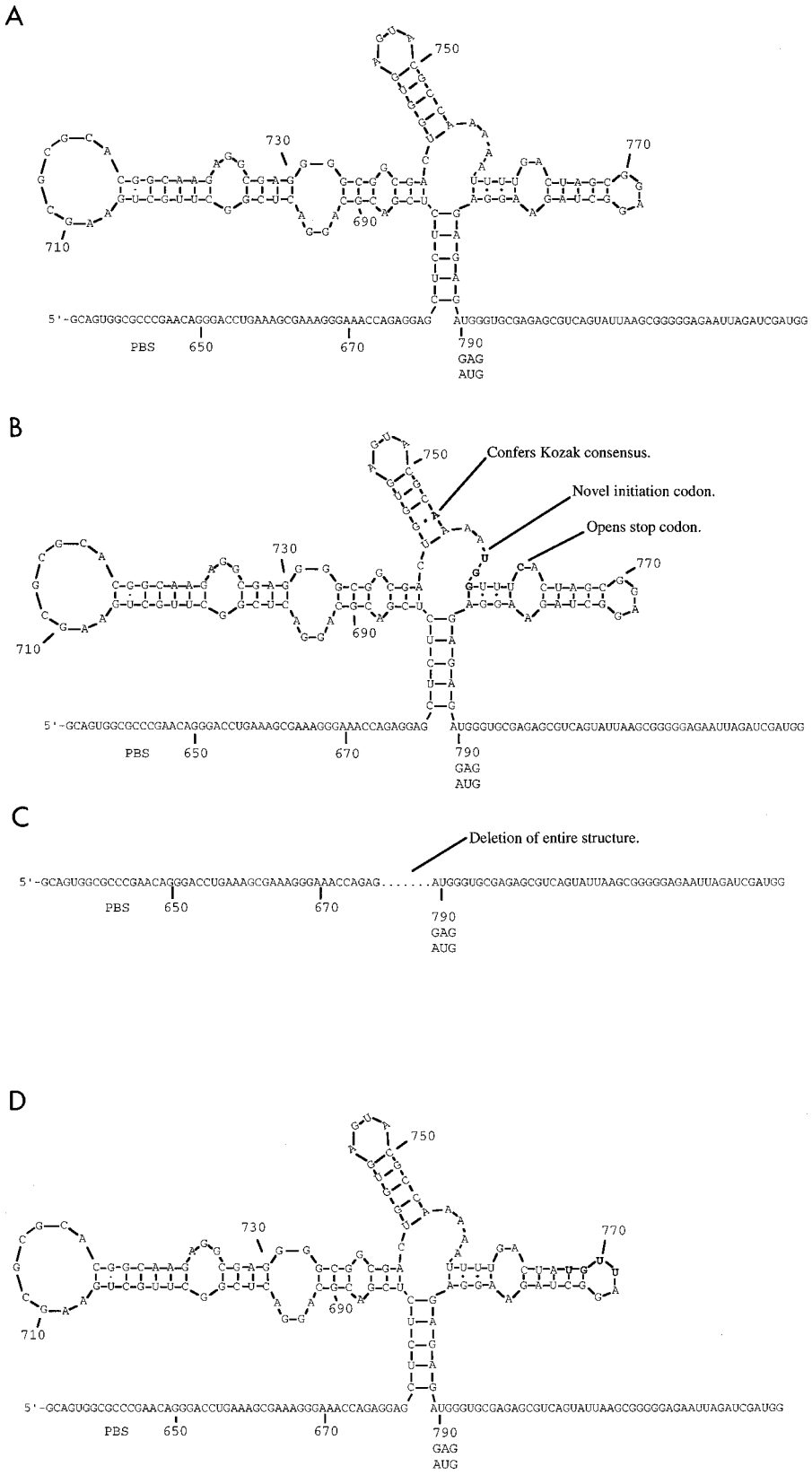

FIG. 1. Schematic of previously proposed HIV-1 packaging signal structure, where panel A illustrates the wild-type sequence and panels B, C, and D illustrate the positions of mutations A4, A14, and D2m, respectively. Mutation A4 inserts an upstream initiation codon in the Kozak consensus, which if utilized, results in a *gag* protein product of circa 56.5 kDa that is also myristoylation defective. Mutation A14 deletes the entire structure, leaving the native *gag* AUG and the AUB in the Kozak consensus intact. Mutation D2m creates an upstream AUG which is out of frame with *gag* and also contains a nearby stop codon.

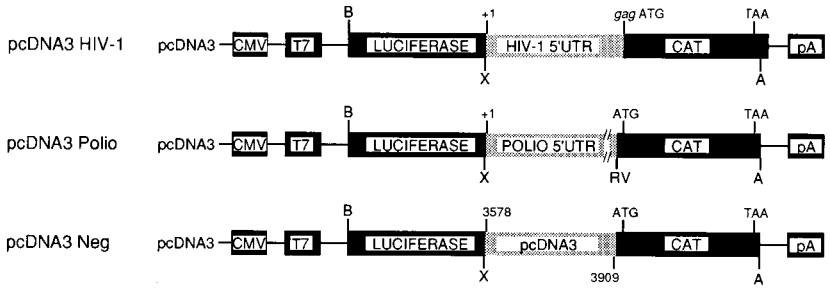

FIG. 2. Dicistronic constructs used to examine internal ribosome entry on HIV-1 mRNA. Dicistronic constructs with luciferase and CAT reporter genes as the first and second cistrons, respectively, were prepared as described in Materials and Methods. The HIV-1 5′ untranslated region (UTR) from clone pSVC21, including the cap (+1) to the *gag* ATG, the poliovirus (type II Lansing), including the cap (+1) to the *Eco*RV site (+735), and a control sequence from Invitrogen's pcDNA3 vector (nt 3578 to 3909) were placed within the intercistronic region to generate pcDNA3 HIV-1, pcDNA3 Polio, and pcDNA3 Neg, respectively. CMV, cytomegalovirus promoter; T7, T7 RNA polymerase promoter; B, *Bam*HI; X, *Xho*I; A, *Apa*I; pA, growth hormone polyadenylation signal of mammalian expression vector pcDNA3; ATG and TAA, initiation and stop codons of CAT mRNA.

were provided by the Medical Research Council (MRC) AIDS Directed Programme. Cell culture media were supplemented with 10% fetal calf serum, 10^5 IU of penicillin per ml, and 100 μg of streptomycin per ml.

COS-1 cells were harvested after 72 h and heated to 100°C in sample buffer for 4 min to extract and denature cellular proteins, and equal quantities were loaded onto gradient SDS-polyacrylamide gel. After electrophoresis, proteins were transferred overnight at 250 mA onto Hybond-C extra nitrocellulose paper (Amersham). The filters were probed with a monoclonal antibody (ADP315 provided by the MRC) to the p17 matrix protein at a concentration of 30 μg/ml for 2 h at room temperature. Bands were visualized by anti-mouse Ig horseradish peroxidase-linked whole antibody (from sheep) and enhanced chemiluminescence Western detection reagents (Amersham) according to the manufacturer's instructions. At 72 h posttransfection sufficient Jurkat-*tat* T cells were harvested to enable loading protein equivalent to 650,000 cells per lane on an 12.5% SDS–polyacrylamide gel. The cultures were maintained to constitute the long-term replication studies. Virions were prepared by incubating the culture supernatant overnight with a half volume of 30% polyethylene glycol 8000 in 0.4 M NaCl. The precipitate was collected by centrifugation at 2,000 rpm for 45 min at 4°C in an MSE 43124-129 rotor and resuspended in 0.5 ml of TNE (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA [pH 7.5]). This material was layered over equal volumes of TNE containing 20% sucrose and centrifuged at 98,000 × *g* for 2 h at 4°C. Virion preparations and cellular samples were loaded onto the same gel. Transfer and probing were as described above, except that a monoclonal antibody (ADP313, provided by the MRC) to the p24 capsid (CA) protein was used at a concentration of 3 μg/ml for 1 h at room temperature.

HeLa and COS-1 cells were transiently transfected with the dicistronic constructs by lipofection (46), and the CAT gene product was assayed by the standard thin-layer chromatography assay (42). Luciferase was assayed according to the manufacturer's instructions (Promega).

To study the long-term replication kinetics of the mutant proviral clones, the transfected Jurkat-*tat* cells were cultured for a period of 21 days and split on the basis of cell concentration (approximately 1:10) to maintain equivalent cell densities in the cultures. Prior to splitting, reverse transcriptase activity in the culture supernatants was determined by the Potts method (51).

RESULTS

Translation in a coupled transcription and translation reticulocyte system. Coupled transcription and translation of plasmid DNA from the (cap site-negative) pKSBglII series gives *gag* precursor proteins of the appropriate size (Fig. 3).

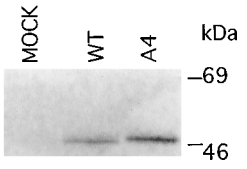

FIG. 3. One microgram of plasmid DNA from the pKSBglII series was used to program a coupled transcription and translation system according to the manufacturer's instructions (Promega). One-tenth of the reaction product was boiled in sample buffer for 4 min at 100°C and then electrophoresed on an SDS–12.5% polyacrylamide gel at 50 mA for 4 h. The positions of molecular mass markers (Amersham) are shown. Mock, no template.

These are not full length, as the *Bgl*II site at position 2096 used for subcloning the gene is within the coding region of *gag*. In pKSBglII A4 it is clear that translation has been initiated from the novel upstream AUG. The lack of an RNA cap site in these clones does not appear to impair translation.

The use of the novel upstream AUG might have been artifactual, resulting from the use of the coupled system in which the secondary structure necessary for IRES activity may not have had time to form. To resolve this problem, we separated the transcription and translation reactions (programming rabbit reticulocyte lysate with in vitro-synthesized capped RNA) to allow the RNA secondary structure to form prior to translation.

Uncoupled transcription and translation. The translation of in vitro-transcribed capped mRNA clearly demonstrated that with some constructs the *gag* products were more abundant when mRNAs were heat denatured and translated immediately (Fig. 4). This is particularly evident when comparing the pKSScaI WT p55 translation products. In both sets of samples, the production of the precursor protein was greater with pKSScaI A14 RNA than with pKSScaI A4, which was also greater than the pKSScaI WT *gag* protein. Heat denaturation of the mRNAs immediately prior to translation increased the amounts of translation product from the wild-type RNA and the pKSScaI A4 by approximately 25 and 36%, respectively, and increased that from pKSScaI A14 by less than 15%. The differences in the quantities of the products of translation likely relate to the lower level of secondary structure in pKSScaI A14, with additional heat disruption of the secondary structure in all three mRNA templates being most marked in pKSScaI A4 and pKSScaI WT. RNA was quantitated after denaturation and cooling or denaturation alone to ensure that degradation of the message had not occurred, which would account for the reduced quantities of protein production in the former (data not shown). pKSScaI A4 produced a greater quantity of *gag* protein than pKSScaI WT, probably because less RNA structure needed to be unwound before the ribosome encountered the initiation codon. Minor decreases in levels of stability caused by the downstream mutations to remove nonsense codons may also have contributed to this effect.

Since the leader sequence of HIV-1 is believed to contain the dimer linkage site (11), it was possible that the introduction of a mutant sequence such as A4 affected dimerization and thus the ability of the RNA to be translated. However, in vitro analysis of the RNA prior to translation confirmed that in all cases the vast majority of the RNA was monomeric (data not shown).

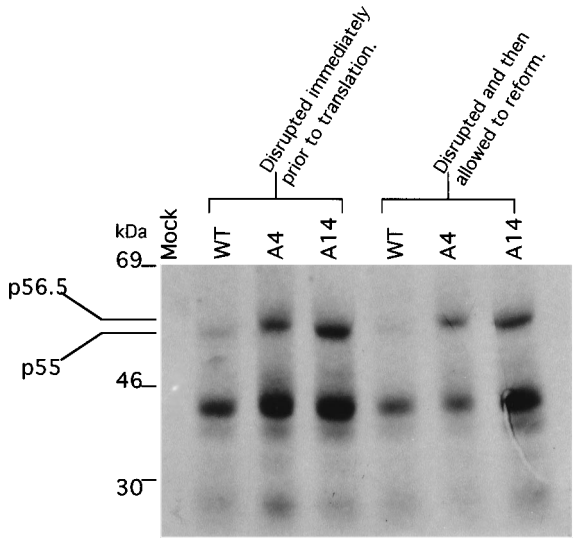

FIG. 4. Two micrograms of in vitro-synthesized capped mRNA was translated at 30°C for 1.5 h, incorporating [^{35}S]methionine. Secondary structure disruption prior to translation was effected by heating the mRNA samples to 67°C for 10 min. The samples were then either translated immediately or allowed to cool slowly to room temperature and then to 4°C (to reform the structure). One-tenth of the translation reaction product was denatured by boiling it in sample buffer for 4 min at 100°C, and then it was electrophoresed on a 7.5 to 15% denaturing polyacrylamide gradient gel at 50 mA for 4 h. The positions of molecular mass markers (Amersham) are shown. Quantitation relative to the A14 mutant heat disrupted immediately prior to translation (100%): disrupted, WT, 25%; A4, 90%; A14, 100%; disrupted and reannealed, WT, 19%; A4, 66%; A14, 83%.

Translation initiation in intact cells. A cellular factor known as the polypyrimidine tract-binding protein has been shown to be essential for internal ribosomal initiation in encephalomyocarditis virus (29). To assay the phenotype of the proviral clones in the presence of cellular factors, we transfected the pSVC21 A4 and pSVC21 WT proviral clones into COS-1 cells and the pSVC21 A4, pSVC21 A14, and pSVC21 WT clones into Jurkat-*tat* T lymphocytes.

Transfection of the mutant A4 proviral plasmid into COS-1 cells gave rise to an 18.5-kDa p17 matrix cleavage product of the *gag* precursor protein (Fig. 5). Transfection of the same pSVC21 A4 mutant provirus into T cells resulted in the production of a *gag* precursor protein larger than that of the wild type (visible after further electrophoresis; data not shown) which did not appear to be proteolytically cleaved (Fig. 6). In the T-cell transfections of pSVC21 A4, *gag* could not be detected in the supernatants. Transfection of the pSVC21 A14 mutant proviral clone resulted in no detectable expression of *gag*, intra- or extracellularly. This could have been due to the lack of spliced viral products such as *tat* and *rev* since the major splice donor is absent in this construct. To investigate this, the A14 mutant was transfected into a cell line stably expressing *tat* and *rev* from a plasmid HVCP, described previously (54), and probed with a monoclonal antibody to *gag* p55/p24. Even with *tat* and *rev* expressed in *trans*, there was no expression of *gag* from pSVC21 A14 (Fig. 7). RNase protection data demonstrated the presence of the cytoplasmic A14 message (data not shown); therefore, RNA instability was not the reason for the absence of *gag*.

Confirmation of the lethality of either removal of the structure or introduction of the upstream AUG was obtained in replication studies in which neither mutant A4 nor mutant A14 was shown to be replication competent (data not shown).

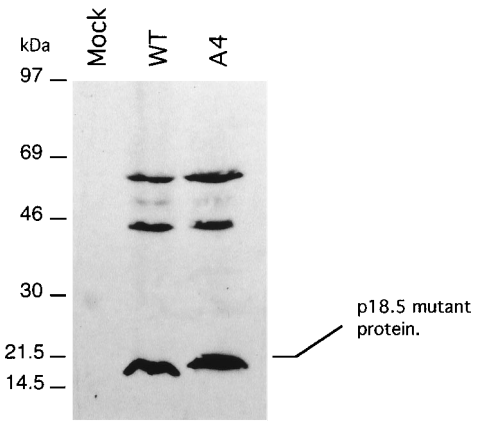

FIG. 5. Western blot (immunoblot) of transiently transfected COS-1 cells. Cells were harvested after 72 h, and equal numbers were loaded per track (650,000) following boiling in sample buffer for 4 min at 100°C to extract cellular protein. Electrophoresis was carried out on a 7.5 to 15% denaturing polyacrylamide gel at 50 mA for 4 h. Proteins were transferred overnight at 250 mA onto a Hybond-C extra nitrocellulose filter (Amersham) which was subsequently probed with a monoclonal antibody to the p17 cleavage product of HIV-1 *gag* at a concentration of 30 μg/ml for 2 h at room temperature. Bands were visualized with anti-mouse Ig horseradish peroxidase-linked whole antibody (from sheep) and the ECL Western detection reagents. The positions of molecular mass markers (Amersham) are shown.

Transfection of the proviral pSVC21 D2m construct resulted in the expression of trace amounts of *gag* (data not shown), consistent with scanning of the first-encountered AUG decreasing usage of the second (native) initiation codon.

Translation of dicistronic constructs. Data from the A4 and D2m mutants strongly favor the ribosomal scanning model for translation of HIV-1. It is possible, however, that the secondary structure upstream of the novel A4 AUG is itself capable of conferring internal ribosome entry. To exclude this possibility, the 5′ leader sequence was cloned into a dicistronic construct in which significant translation of the downstream

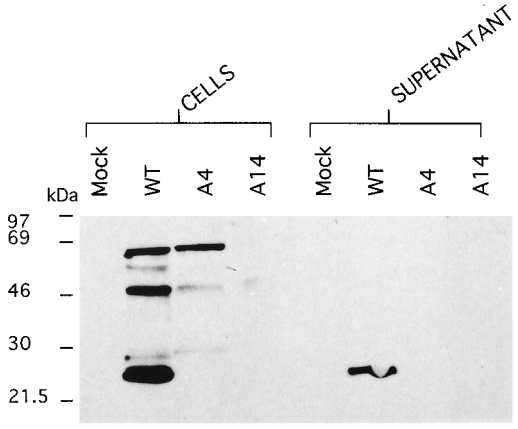

FIG. 6. Western blot of transfected CD4$^+$ Jurkat-*tat* cells (corresponds to day 3 of the long-term study). Cells were harvested after 72 h, and equal numbers were loaded per track (650,000) following boiling in sample buffer for 4 min at 100°C to extract cellular protein. Electrophoresis was carried out on a 12.5% denaturing polyacrylamide gel at 50 mA for 4 h. Proteins were transferred overnight at 250 mA onto a Hybond-C extra nitrocellulose filter which was subsequently probed with a monoclonal antibody to the p24 cleavage product of HIV-1 *gag* at a concentration of 3 μg/ml. Bands were visualized with anti-mouse Ig horseradish peroxidase-linked whole antibody (from sheep) and the ECL Western detection reagents (Amersham). The positions of molecular mass markers (Amersham) are shown.

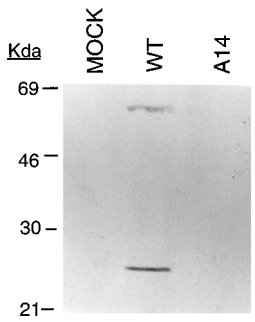

FIG. 7. The pSVC21 A14 proviral clone was transfected into the HVCP Jurkat-*tat* cells. Cells were harvested after 72 h, and equal numbers were loaded per track (650,000) following boiling in sample buffer for 4 min at 100°C to extract cellular protein. Electrophoresis was carried out on a 12.5% denaturing polyacrylamide gel at 50 mA for 4 h. Proteins were transferred overnight at 250 mA onto a Hybond-C extra nitrocellulose filter which was then subsequently probed with a monoclonal antibody to the p24 cleavage product of HIV-1 *gag* at a concentration of 3 μg/ml. Bands were visualized with anti-mouse Ig horseradish peroxidase-linked whole antibody (from sheep) and the ECL Western detection reagents (Amersham). The positions of molecular mass markers (Amersham) are shown.

exon would imply internal ribosomal entry. With a positive control poliovirus IRES structure, translation of the downstream CAT exon could be detected easily in COS and HeLa cell transfections (Fig. 8A), but as expected, the level of expression was less with reticulocyte lysates (Fig. 8B). By contrast, there was no evidence of CAT activity when the HIV-1 leader sequence was present. Identical findings occurred with MT4 cells, and the addition of 100 or 200 bases of *gag* sequence had no effect (data not shown). An *env* sequence was likewise negative. The failure of the HIV-1 leader to act as an IRES might be attributable to the use of the splice donor to skip the downstream exon. This was shown not to be the case with a T7/vaccinia virus expression system, in which nuclear processes would not come into play and in which Northern (RNA) analysis confirmed the presence of a single transcript of correct size in all cells, indicating no exon skipping had occurred (data not shown).

DISCUSSION

The evidence presented here clearly documents translational inhibition by the intact 5′ leader region of HIV-1. The heat sensitivity of this effect and the increased quantity of translation products when the structure is deleted support the concept that, in vitro, this area significantly slows translation. Indeed, the observation of translational inhibitory effects of the HIV-1 leader is in accordance with the findings of several other groups (12, 18, 49, 61). Dimerization of the RNA in our studies was not responsible for the effects of or for the differences among the constructs. The degree of translational inhibition is clearly related to the extent of secondary structure the ribosome encounters immediately upstream of the first AUG, as shown by the difference in inhibition levels between mutant A4 and the wild type.

Although the formation of the RNA secondary structure is believed to be very rapid, our studies suggest that it does not occur immediately after in vitro transcription in the coupled system. This is in accordance with our previous studies of RNA structure using single- and double-stranded probes in which the presence and stability of a structure were to some extent time dependent (21). It has been reported that the structural properties of the TAR region of the HIV-1 leader influence gene expression in both a *tat*-dependent (61) and -independent manner (12, 57). Clearly the packaging signal structure also plays an integral role in the regulation of *gag* expression. The packaging signal's proximity to the *gag* initiation codon suggested that this structure may function as an IRES, although there is no AUG motif upstream of the native initiation codon. The translation of constructs in a cap-independent manner, although suggestive of an IRES, likely relates to the cation concentration being at a level which does not accurately discriminate between cap-dependent and cap-independent functions (i.e., the reticulocyte system is optimized for efficient translation of the mRNA templates regardless of whether they contain cap sites). Moreover, the introduction of an upstream AUG in the Kozak consensus clearly provides evidence that ribosomal scanning is the mode of translation. The use of this novel initiation codon also demonstrates that ribosome shunting, a third mechanism of translation by which the scanning complex shunts from an upstream shunt site to a downstream acceptor site without scanning the intervening region (as with cauliflower mosaic virus [15]), does not occur. Introduction of a novel out-of-frame AUG immediately upstream of the native codon leads to a significant reduction in *gag* translation. Both leaky scanning and reinitiation of translation have previously been shown to occur with HIV-1 transcripts (40a, 60, 60a).

As a definitive test for the presence of an IRES, a dicistronic construct containing two assayable reporter genes with the sequence of interest cloned between them was used (in a cellular environment). The results with such a dicistronic construct, in transient transfections, unequivocally support the fact that the HIV-1 packaging signal structure does not function as

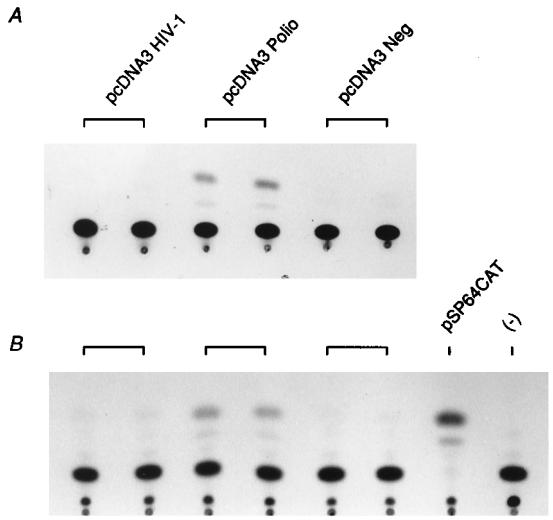

FIG. 8. The poliovirus mRNA leader can mediate internal ribosome entry but the *gag* leader cannot. Dicistronic constructs were as described in the legend to Fig. 2. Luciferase and CAT assays were performed as described in Materials and Methods. Plasmids were transfected into HeLa cells, and equal numbers of luciferase units were used for CAT assays. (A) CAT assay with HeLa cells (similar results were obtained with COS and MT4 cells). CAT activity was not detectable with pcDNA3gag 100 and pcDNA3gag 200 or pcDNA3env constructs (data not shown). Percent acetylation, reading from left to right (duplicate assays): pcDNA3 HIV-1, 2 and 1.3%; pcDNA3 Polio, 21 and 23%; pcDNA Neg, 1.2 and 1.2%. (B) CAT assay of reticulocyte lysates. Internal ribosome entry is achieved with the pcDNA3 Polio construct (30 and 33% acetylation). By contrast, virtually no CAT activity was detectable in duplicate assays with the pcDNA3 HIV-1 (7.6 and 9.7% acetylation) and pcDNA3 Neg (4.5 and 6.0% acetylation) control lanes. pSP64CAT (46) was transcribed in vitro with SP6 RNA polymerase, and the RNA was translated as described above and used as a positive CAT control (97% acetylation). (−) indicates the control lane containing reticulocyte lysate alone (5.6% acetylation).

an IRES. Using these in vitro methods, we would have been able to detect an IRES effect of a magnitude at least 10-fold lower than that demonstrated by the poliovirus leader had one existed.

The inability to detect any intra- or extracellular *gag* following transfection of T cells with the pSVC21 A14 proviral clone is evidently a direct consequence of the profound effect of deleting the entire 5′ packaging signal structure. Since in vitro translation with this region was actually enhanced by the deletion, the result implicates the 5′ packaging signal structure as having an important role in the translation of *gag* mRNA. The absence of intracellular $p24^{gag}$ cleavage products in T cells transfected with pSVC21 A4 may reflect the effect of the extra 11 amino acids (initiating from the upstream AUG) on proteolytic processing. The observation that A4 *gag* is present intracellularly in T cells but not in the supernatant is further evidence that the upstream novel initiation codon is being utilized, since translation from the upstream AUG would result in a myristoylation-defective protein that would be expected to be intracellularly confined. In light of the evidence from the T-cell transfections and the 21-day replication studies, it is clear that the A4 provirus is able to generate *gag* protein intracellularly yet extracellular export is completely abolished. The A14 provirus is, however, unable to sustain protein synthesis in cells, as evidenced by the lack of *gag* p55, of intracellular cleavage products, or of extracellular reverse transcriptase activity. However, whether this is a direct result of the inability of the provirus to integrate into the host cell genome or due to the deletions of a packaging signal and splice donor site requires further elucidation. The difference in the abilities of COS and Jurkat cells to cleave pSVC21 A4 *gag* precursor is striking and emphasizes the cellular dependence of many viral processes and how results obtained with COS cells may not be relevant to events occurring in cells for which the virus is tropic.

The packaging signal structure of HIV-1 is present only in unspliced RNAs. Translation of spliced RNAs, including those of the regulatory proteins Tat and Rev, would thus not be subject to translational inhibition as exemplified by the A4 mutant. It is possible that this inhibition is merely an unavoidable consequence of the necessity for some form of RNA structure to allow packaging and splicing of genomic RNA. Alternatively, the packaging signal structure may actually contribute to the switch from early to late gene expression. Translation of unspliced RNA is likely to be inhibited in favor of spliced products (even if nuclear export does occur), and the accumulation of regulatory proteins to a high level before structural protein synthesis may contribute to the explosive burst of viral production of which HIV is capable. The structure may also affect encapsidation by acting as a negative influence on translation, thus aiding in the sequestration of full-length genomic RNA molecules for packaging in the budding particle. It has been suggested that the structure is a favored site for interaction with the viral nucleocapsid protein (11, 58). It is possible that after translation of some full-length RNA the newly synthesized *gag* protein will then bind to this region, inhibiting translation of a proportion of the unspliced RNAs, thus ensuring the availability of the full-length message for packaging.

The 5′ leader region of HIV-1 may not necessarily be exclusively responsible for translational regulation, and it should be noted that translation suppressors have been discovered in the 3′ untranslated regions of many RNAs (2, 7, 9, 37). Despite the location of these suppressors at the 3′ end of the genome, they affect the 5′ leader by an as-yet-undefined mechanism.

In this communication we have clearly shown that the 5′ HIV-1 packaging signal structure inhibits the translation of *gag* in vitro yet is essential in vivo and that initiation occurs via the scanning model of translational initiation and not by internal ribosome entry.

Further elucidation of the characteristics of the HIV-1 packaging signal structure and the effect of disruptive, compensatory, and deletion mutations is currently under study in order to determine the relevance of this phenomenon to the viral life cycle.

ACKNOWLEDGMENTS

This work was supported by the MRC (United Kingdom) AIDS Directed Programme and the Sykes Trust. Part of this work was supported by grants from the MRC of Canada and the National Health Research Development Program (NHRDP) of Canada to E.C., who is a recipient of a Career Award from the NHRDP. A.J.M. is a fellow of the Fonds pour la Recherche en Santé du Québec.

We thank R. B. Ferns for donating the p55/p24 and p55/p17 monoclonal antibodies, through the MRC ADP, and Richard Jackson for helpful advice throughout the work. We are indebted to Nahum Sonenberg, Andrew Craig, and Yuri Svitkin for plasmids and advice.

REFERENCES

1. **Agol, V. I.** 1991. The 5′ untranslated region of picornaviral genomes. Adv. Virus Res. **40:**103–108.
2. **Ahringer, J., and J. Kimble.** 1991. Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the fem-3 3′ untranslated region. Nature (London) **349:**346–348.
3. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64:**1920–1926.
4. **Baudin, F., R. Marquet, C. Isel, J. L. Darlix, B. Ehresmann, and C. Ehresmann.** 1993. Functional sites in the 5′ region of human immunodeficiency virus type 1 RNA from defined structural regions. J. Mol. Biol. **229:**382–397.
5. **Berkhout, B., R. H. Silverman, and K. T. Jeang.** 1989. *Tat* transactivates the human immunodeficiency virus through a nascent RNA target. Cell **59:** 273–282.
6. **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.
7. **Braun, R. E.** 1990. Temporal translational regulation of the protamine 1 gene during mouse spermatogenesis. Enzyme (Basel) **44:**120–128.
8. **Carrington, J. C., and D. D. Freed.** 1990. Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. **64:**1590–1597.
9. **Ch'ng, J., C. Lai, D. L. Shoemaker, P. Schimmel, and E. W. Holmes.** 1990. Reversal of creatine kinase translational repression by 3′ untranslated sequences. Science **248:**1003–1006.
10. **Clavel, F., and J. M. Orenstein.** 1990. A mutant of human immunodeficiency virus with reduced RNA packaging and abnormal particle morphology. J. Virol. **64:**5230–5234.
11. **Darlix, J. L., C. Gabus, M. T. Nugeyre, F. Clavel, and F. Barre-Sinoussi.** 1990. *Cis* elements and *trans*-acting factors involved in the RNA dimerisation of the human immunodeficiency virus HIV-1. J. Mol. Biol. **216:**689–699.
12. **Edery, I., R. Petershyn, 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.
13. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46:**807–817.
14. **Fisher, A. G., E. Collati, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biological activity. Nature (London) **316:** 262–265.
15. **Fulterer, J., Z. Kisslaszio, and T. Hohn.** 1993. Non-linear ribosome migration on cauliflower mosaic virus 35S RNA. Cell **73:**789–802.
16. **Ganoza, M. C., E. C. Kofoid, P. Marliere, and B. G. Louis.** 1987. Potential secondary structure at translation initiation sites. Nucleic Acids Res. **15:** 345–360.
17. **Garrel, C. M., A. R. McKenzie, M. M. Patino, W. E. Walden, and E. C. Theil.** 1991. Ferritin mRNA: interactions of iron regulatory element with translational regulator protein P-90 and the effect on base paired flanking regions. Proc. Natl. Acad. Sci. USA **88:**4166–4170.
18. **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.
19. **Gold, L.** 1988. Post-transcriptional regulatory mechanisms in *Escherichia coli*. Annu. Rev. Biochem. **57:**199–233.

20. **Hall, M. N., J. Gabay, M. Debarbouille, and M. Schwartz.** 1982. A role for mRNA secondary structure in the control of translation initiation. Nature (London) **295:**616–618.
21. **Harrison, G. P., and A. M. L. Lever.** 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor have a conserved stable secondary structure. J. Virol. **66:**4144–4153.
22. **Hayashi, T., Y. Ueno, and T. Okamoto.** 1993. Elucidation of a conserved RNA stem-loop structure in the packaging signal of human immunodeficiency virus type 1. FEBS Lett. **327:**213–218.
23. **Iserentant, D., and W. Fiers.** 1980. Secondary structure of mRNA and efficiency of translation. Gene **9:**1–12.
24. **Jackson, R. J.** 1991. Initiation without an end. Nature (London) **353:**14–15.
25. **Jackson, R. J., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15:** 477–483.
26. **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 vitro. J. Virol. **63:**1651–1660.
27. **Jang, S. K., H.-G. Kräusslich, M. J. H. 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.
28. **Kaminski, A., M. T. Howell, and R. J. Jackson.** 1990. Initiation of encephalomyocarditis virus RNA translation: the authentic initiation site is not selected by a scanning mechanism. EMBO J. **9:**3753–3759.
29. **Kaminsky, A., S. L. Hunt, and R. J. Jackson.** Personal communication.
30. **Kim, H. J., K. Lee, and J. J. O'Rear.** 1994. A short sequence upstream of the 5′ major splice site is important for encapsidation of HIV-1 genomic RNA. Virology **198:**336–340.
31. **Kleiman, L., S. Gaudry, F. Boulerice, M. A. Wainberg, and M. A. Parniak.** 1991. Incorporation of tRNA into normal and mutant HIV-1. Biochem. Biophys. Res. Commun. **174:**1272–1280.
32. **Kozak, M.** 1986. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. USA **83:**2850–2854.
33. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiation codon that modulates translation by eukaryotic ribosomes. Cell **44:** 283–292.
34. **Kozak, M.** 1988. Leader length and secondary structure modulate mRNA function under conditions of stress. Mol. Cell. Biol. **8:**2737–2744.
35. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
36. **Kozak, M.** 1989. Circumstances and mechanisms of inhibition of translation by secondary structure in eukaryotic mRNAs. Mol. Cell. Biol. **9:**5134–5142.
37. **Kruys, V., B. Beutler, and G. Huez.** 1990. Translational control mediated by UA-rich sequences. Enzyme (Basel) **44:**193–202.
38. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
39. **Lever, A. M. L., H. Gottlinger, W. Haseltine, and J. Sodroski.** 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 into virions. J. Virol. **63:**4085–4087.
40. **Luban, J., and S. P. Goff.** 1994. Mutational analysis of *cis*-acting packaging signals in human immunodeficiency virus type 1 RNA. J. Virol. **68:**3784–3793.

40a. **Luukonan, B. G. M., W. Tan, and S. Schwartz.** 1995. Efficiency of reinitiation on human immunodeficiency virus type 1 mRNAs is determined by the length of the upstream open reading frame and intercistronic distance. J. Virol. **69:**4086–4094.

41. **Macejak, D. G., and P. Sarnow.** 1991. Internal initiation of translation mediated by the 5′ leader of a cellular RNA. Nature (London) **353:**90–94.
42. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Manzella, J. M., and P. J. Blackshear.** 1990. Regulation of rat ornithine decarboxylase mRNA translation by its 5′ untranslated region. J. Biol. Chem. **265:**11817–11822.
44. **Manzella, J. M., W. Rychlik, R. E. Rhoads, J. W. B. Hershey, and P. J. Blackshear.** 1991. Insulin induction of ornithine decarboxylase: importance of mRNA secondary structure and phosphorylation of eukaryotic initiation factors eIF-4B and eIF-4E. J. Biol. Chem. **266:**2383–2389.
45. **Meusing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein. Cell **48:**691–701.
46. **Mouland, A. J., S. Bevan, J. H. White, and G. N. Hendy.** 1994. Human chromogranin A gene: molecular cloning, structural analysis and neuroendocrine cell-specific expression. J. Biol. Chem. **269:**6918–6926.
47. **Myers, G., B. Korber, R. F. Berofsky, R. F. Smith, and G. N. Pavlakis.** 1991. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.
48. **Ou, S. K., M. P. Scott, and P. Sarnow.** 1982. Homeotic gene antennapedia mRNA contains 5′-noncoding sequences that confer translational initiation by internal ribosome binding. Genes Dev. **6:**1643–1653.
49. **Parkin, N., A. Darveau, R. Nicholson, and N. Sonenberg.** 1988. *cis*-acting translational effects of the 5′ noncoding region of c-*myc* mRNA. Mol. Cell. Biol. **8:**2875–2883.
50. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334:**320–325.
51. **Potts, B. J.** 1990. "Mini" reverse transcriptase (RT) assay, p. 103–106. *In* A. Aldovini and B. D. Walker (ed.), Techniques in HIV research. Stockton Press, New York.
52. **Promega.** 1991. Promega protocols and applications guide. Promega, Madison, Wis.
53. **Rhim, H., J. Park, and C. D. Morrow.** 1991. Deletions in the tRNA (Lys) primer binding site of human immunodeficiency virus type 1 identify essential regions for reverse transcription. J. Virol. **65:**4555–4564.
54. **Richardson, J. H., L. A. Child, and A. M. L. Lever.** 1993. Packaging of human immunodeficiency virus type 1 RNA requires *cis*-acting sequences outside the 5′ leader region. J. Virol. **67:**3997–4005.
55. **Rosen, C. A.** 1991. Regulation of HIV gene expression by RNA-protein interactions. Trends Genet. **7:**9–14.
56. **Rosen, C. A., J. G. Sodroski, K. Campbell, and W. A. Haseltine.** 1986. Construction of recombinant murine retroviruses that express the human T-cell lymphotrophic virus type III transactivator genes. J. Virol. **57:**379–384.
57. **Roy, S., M. Agy, A. G. Hovanessian, N. Sonenberg, and M. G. Katze.** 1991. The integrity of the stem structure of human immunodeficiency virus type 1 Tat-responsive sequence RNA is required for interaction with the interferon-induced 68,000-M_r protein kinase. J. Virol. **65:**632–640.
58. **Sakaguchi, K., N. Zambrano, E. T. Baldwin, B. A. Shapiro, J. W. Erickson, J. G. Omichinski, G. M. Clore, A. M. Gronenborn, and E. Apella.** 1993. Identification of a binding site for human immunodeficiency virus type 1 nucleocapsid protein. Proc. Natl. Acad. Sci. USA **90:**5219–5223.
59. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
60. **Schwartz, S., B. K. Felber, E.-M. Fenyö, 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.

60a. **Schwartz, S. B., 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.

61. **Sengupta, D. N., B. Berkhout, A. Gatinol, A. 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.
62. **Sodroski, J., W. C. Goh, and C. Rosen.** 1986. Replicative and cytopathic potential of HTLV-III/LAV with *sor* gene deletions. Science **321:**1549–1553.
63. **Sonenberg, N.** 1990. Picornavirus RNA translation continues to surprise. Trends Genet. **7:**105–106.
64. **Standart, N., and T. Hunt.** 1990. Control of translation of masked RNAs in clam oocytes. Enzyme (Basel) **44:**106–119.
65. **Steffy, K., and F. Wong-Staal.** 1991. Genetic regulation of human immunodeficiency virus. Microbiol. Rev. **55:**193–205.
66. **Thach, R. E.** 1992. Cap recap: the involvement of eIF-4F in regulating gene expression. Cell **68:**177–180.
67. **Thomas, A. A. M., E. Ter Haar, J. Wellink, and H. O. Voorma.** 1991. Cowpea mosaic virus middle component RNA contains a sequence that allows internal binding of ribosomes and that requires eukaryotic initiation factor 4F for optimal translation. J. Virol. **65:**2953–2959.
68. **Tsukiyamakohara, K., N. Iizuka, M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66:**1476–1483.
69. **Van De Guchte, M., T. Van Der Lende, J. Kok, and G. Venema.** 1991. A possible contribution of mRNA secondary sructure to translation initiation efficiency in *Lactococcus lactis*. FEMS Microbiol. Lett. **81:**201–208.
70. **Wang, C., P. Sarnow, and A. Siddiqui.** 1993. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J. Virol. **67:**3338–3344.