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A Single Species of *pX* mRNA of Human T-Cell Leukemia Virus Type I Encodes *trans*-Activator $p40^x$ and Two Other Phosphoproteins

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Human T-cell leukemia virus type I (HTLV-I) contains the *pX* sequence which codes for the *trans*-activator of the long terminal repeat (LTR) and is thus postulated to be associated with leukemogenesis in adult T-cell leukemia. Overlapping open reading frames (ORF) in the *pX* sequence were recently found to code for $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ by ORF III, in addition to $p40^x$ coded for by ORF IV. The mechanism of expression of these newly identified proteins and their possible association with *trans*-activation were studied. On transfection of an expression plasmid that contains a cDNA sequence of the *pX* mRNA, products from both ORFs III and IV were detected in the cells. The RNA was synthesized in vitro from the cDNA clone by SP6 RNA polymerase and translated in a rabbit reticulocyte lysate. As translation products, two proteins, $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$, were detected in addition to $p40^x$. Elimination of the first and second ATG codons in ORF III resulted in loss of the ability to code for $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$, respectively, which indicated that the translations from these two ATG codons were independent. A mutant that lacked both ATG codons was fully active in *trans*-activation of chloramphenicol acetyltransferase gene expression directed by the LTR. These results indicate that a 2.1-kilobase *pX* mRNA of HTLV-I independently encodes three proteins, $p40^x$, $p27^{x\text{-}III}$, and $p21^{x\text{-}III}$, by different ORFs and that the last two proteins are not involved in *trans*-activation of the unintegrated LTR.

Human T-cell leukemia virus type I (HTLV-I) (19, 32) is an etiological agent of adult T-cell leukemia (ATL) (8, 9, 33) and an exogenous human retrovirus (21, 32). The viral genome has an extra sequence, *pX*, between the *env* gene and the 3′ long terminal repeat (LTR) (23). Expression of the *pX* sequence was suggested to be associated with leukemogenesis by HTLV-I from the following findings: (i) the absence of a specific site for provirus integration in leukemic cells, disproving a *cis*-acting function of the LTR (22); (ii) frequent in vitro immortalization of infected T cells, suggesting direct involvement of viral products (18, 20); and (iii) the *trans*-acting function of the *pX* product, which activates viral gene expression (6, 30).

The *pX* sequence contains four possible open reading frames (ORFs), I to IV (23) (Fig. 1A), and a product encoded by ORF IV was previously identified as $p40^x$ (10, 14, 17, 28). This product was proposed to be a *trans*-acting transcriptional activator (6, 30). We recently found, however, that two other phosphoproteins, $pp27^{x\text{-}III}$ and $pp21^{x\text{-}III}$, are also encoded by ORF III of the *pX* sequence (11). Because ORF III in the *pX* sequence mostly overlaps ORF IV, which encodes $p40^x$, the proposal that $p40^x$ alone is responsible for *trans*-activation (3, 5, 6, 29, 30) became uncertain. After finding these novel proteins, we proposed that $p40^x$ is, in fact, responsible for the *trans*-activation of the LTR as a result of studies in which a termination codon was introduced into each ORF (25, 26). However, our evidence was not conclusive because the mechanisms of expression of these novel proteins were not known. Tight binding of $p27^{x\text{-}III}$ to the nucleus (11) suggests some regulatory roles of viral gene expression, although their functions remain to be elucidated.

To elucidate the function of the newly identified proteins, we tried to determine their mechanism of expression. Attempts to isolate a cDNA clone with possible mRNA which express only ORF III were unsuccessful, and instead only clones of the 2.1-kilobase (kb) *pX* mRNA coding for $p40^x$ by ORF IV were isolated. The 2.1-kb *pX* mRNA is formed by double splicing (24, 31) and contains another ORF III, which can be initiated by the first ATG codon. This extended ORF III mostly overlaps the ORF IV for $p40^x$ (Fig. 1A) and has a capacity to encode a 21-kilodalton (kDa) protein. To examine the possibility that $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ are translated from ORF III in the 2.1-kb *pX* mRNA, in vivo and in vitro translation products were analyzed. We report here that a single species of *pX* mRNA encodes $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ as well as $p40^x$ with different ORFs and different initiation codons.

MATERIALS AND METHODS

Construction of a cDNA clone of *pX* mRNA. Isolation of a cDNA clone of 2.1-kb mRNA and expression plasmid pSVPX were described previously (24). For expression of exactly the same mRNA as that which was naturally spliced and terminated, plasmid pMTCX containing the metallothionine promoter and cDNA sequence was constructed (see Fig. 1B). In this cDNA sequence, the sequence upstream of the *Cla*I site was derived from a cDNA clone (24), and that downstream of the *Cla*I site was derived from the provirus clone pATK08 (23). Thus, the viral sequence contained a 3′ LTR and a small cellular fragment, but was defective in 36 bases in the leader sequence at the 5′ end of the cDNA sequence.

Construction of plasmids for RNA synthesis. A DNA fragment containing the whole cDNA sequence and a small DNA fragment of the LTR at the 3′ end was cut out with *Pst*I and *Hin*cII from the cDNA clone pMTCX (Fig. 1B) and inserted into the *Pst*I–*Sma*I site of pSP64 (16). The plasmid pSPCX so constructed was used for the preparation of RNA after linearization with *Eco*RI. A mutant pSPCX-ATG1 with

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a deletion of the first ATG codon (corresponding to position 5124 in the provirus genome) was constructed as follows. pMTCX was cleaved at the unique *Sph*I site, where the first ATG codon in the *pX* cDNA is located, and treated with the Klenow fragment of DNA polymerase I. Then 10-mer *Bgl*II linkers were ligated to the DNA. After religation of the plasmid at the new *Bgl*II site, the *Pst*I–*Hin*cII fragment was inserted into the *Pst*I–*Sma*I site of pSP64, as in the case of pSPCX. As a result, the cDNA had a six-nucleotide insertion, and so no reading frame was modified.

Plasmid pSPCX-ATG4, in which the fourth ATG codon (corresponding to position 7476 in the provirus genome) was deleted, was constructed as follows. Plasmid pSPCX was serially cleaved at the unique *Cla*I and *Mlu*I sites and treated with the Klenow fragment. Then an 8-mer *Bgl*II linker was ligated to the end. In this mutant also, the reading frame was not altered. pSPCX-ATG1&4 has a four-base deletion at the *Sph*I site (positions 5123 to 5126), which includes the first ATG codon and a single base substitution of T to C at the fourth ATG codon (position 7477). Thus the first two ATG codons in ORF III were eliminated. Similarly, pMTCX-ATG1&4 has the same mutations as pSPCX-ATG1&4; and pMTCX/IV^{ter}, which carried a termination codon in ORF IV, was constructed by replacing the small *Tth*III-I–*Mlu*I fragment of pMTCX with the corresponding fragment of the pMTPX/IV^{ter} (26). Each mutation was confirmed by nucleotide sequencing by the method of Maxam and Gilbert (15).

In vitro RNA synthesis and translation assay. The plasmid DNA was linearized at the *Eco*RI site (Fig. 1B) and incubated with SP6 RNA polymerase in the presence of the Cap analog (m^7GpppG), according to the instructions for the assay kit (SP6 system; Amersham Corp., Arlington Heights, Ill.). The RNA synthesized was purified by treatment with DNase followed by phenol extraction, gel filtration, and ethanol precipitation as described previously (16). Then the RNA (50 ng) was translated in 10 μl of a rabbit reticulocyte lysate (translation kit N90; Amersham) in the presence of [^{35}S]cysteine (10 μCi). The translation products were analyzed by 12% polyacrylamide gel electrophoresis, immediately or after immunoprecipitation as described previously (34), and were located by fluorography. The antisera against the synthetic peptides used were described previously (11).

Transfection, product analysis, and the chloramphenicol acetyltransferase assay. Plasmid transfection was carried out as described previously (6), and the viral proteins were analyzed by a blotting procedure with rabbit antisera against synthetic peptides and sera of ATL patients as described previously (10, 11). Activities for *trans*-activation of the LTR were assayed in HOS cells by cotransfection with pLTR-chloramphenicol acetyltransferase (CAT) and pMTCX or its mutants as described previously (6).

RESULTS

Expression of two overlapping ORFs by a cDNA clone of 2.1-kb *pX* mRNA. Previously, we reported that the *pX* sequence of HTLV-I is transcribed and spliced into 2.1-kb mRNA by double splicing (Fig. 1A) and translated into p40^x (24). This was shown directly by constructing expression plasmid pSVPX constituted from the simian virus 40 promoter and cDNA of this 2.1-kb mRNA and transfecting it into COS cells. Transient expression of p40^x was detected in the transfected cells by a blotting assay with patient serum or antiserum against a peptide corresponding to the C terminus of p40^x (24). However, p27$^{x\text{-}III}$ and p21$^{x\text{-}III}$ were not detected, probably because of their low levels of expression.

On the other hand, we have isolated two independent stable transformants of FL cells (6) with the same plasmid pSVPX and have detected p27$^{x\text{-}III}$ in addition to p40^x in these two transformants. A 40-kDa band was detected in a transformant with serum from an ATL patient (Fig. 2A, lane 3) but not with normal human serum (Fig. 2A, lane 2). To detect p27$^{x\text{-}III}$ and p21$^{x\text{-}III}$ in the same transformant, we used rabbit antiserum against a C-terminal peptide of p27$^{x\text{-}III}$, because sera of ATL patients were previously shown not to contain antibodies against p27$^{x\text{-}III}$ and p21$^{x\text{-}III}$ (11). With mixed antisera against p40^x and p27$^{x\text{-}III}$, 27- and 40-kDa proteins were detected in the transformant (Fig. 2B, lane 6), corresponding to p27$^{x\text{-}III}$ and p40^x in HTLV-I-infected MT-2 cells (Fig. 2B, lane 4). Although a faint band at 40 kDa was detected with preimmune rabbit serum (Fig. 2B, lane 5) and p21$^{x\text{-}III}$ was not detected in the transformant, the detection of both p27$^{x\text{-}III}$ and p40^x strongly suggests that an mRNA transcribed from plasmid pSVPX can code for at least two proteins coded for by different overlapping ORFs in vivo. However, it is possible that the transcript from the cDNA clone could be further spliced into a different mRNA than that which encodes p27$^{x\text{-}III}$.

Translation of *pX* mRNA synthesized by in vitro transcription. As described above, a cloned cDNA was shown to express at least two *pX* proteins in vivo. For determination of whether a single 2.1-kb *pX* mRNA was used for translation of both ORFs III and IV, the *pX* cDNA was inserted into an SP6 vector and was transcribed into RNA with SP6 RNA polymerase. The RNA produced gave a single band of exactly the predicted size on agarose gel electrophoresis. The RNA was translated in a rabbit reticulocyte lysate, and the translation products from pSPCX RNA were immunoprecipitated with a mixture of antisera to the C-terminal peptide of p27$^{x\text{-}III}$ and p40^x. Bands of 40, 27, and 21 kDa were detected (Fig. 3, lane 5), and these bands were observed only when the RNA was added to the reaction mixture (Fig. 3, lane 1). Small amounts of 40- and 27-kDa products were also precipitated nonspecifically with preimmune serum (Fig. 3, lane 2). The 27- and 21-kDa products should have the same antigenicities as p27$^{x\text{-}III}$ and p21$^{x\text{-}III}$ because the intensities of the bands were reduced to those with preimmune serum after preabsorption of the antisera with peptide III, but not with peptide IV (Fig. 3, lane 4). Similarly, the 40-kDa product was confirmed to be encoded by ORF IV (Fig. 3, lane 3). In this competition assay, two minor products of 30 and 25 kDa were found which were presumably initiated at internal AUG codons in ORF IV. Thus, the results show that ORFs III and IV are both translatable and code for 27- and 21-kDa proteins and a 40-kDa protein, respectively.

On electrophoresis of the in vitro translation products in parallel with extracts from HUT102 cells, 40-, 27-, and 21-kDa products comigrated with p40^x, p27$^{x\text{-}III}$, and p21$^{x\text{-}III}$, respectively (Fig. 3, lanes 5 and 6). Thus the in vitro translation products of *pX* mRNA were identical, or at least closely related, to the p40^x, p27$^{x\text{-}III}$, and p21$^{x\text{-}III}$ produced in vivo. Therefore, these results indicate that the in vitro products are not due to artificial translation in the rabbit reticulocyte lysate and indicate that a single species of 2.1-kb RNA can code for three proteins.

Identification of initiation sites for synthesis of p27$^{x\text{-}III}$ and p21$^{x\text{-}III}$. The 2.1-kb *pX* mRNA consists of three exons: the first from the LTR, the second from the 3′ region of the *pol* gene, and the third from the *pX* sequence (Fig. 1A). The first AUG codon in the mRNA, which was originally derived from the *pol* gene, can initiate ORF III, which has the

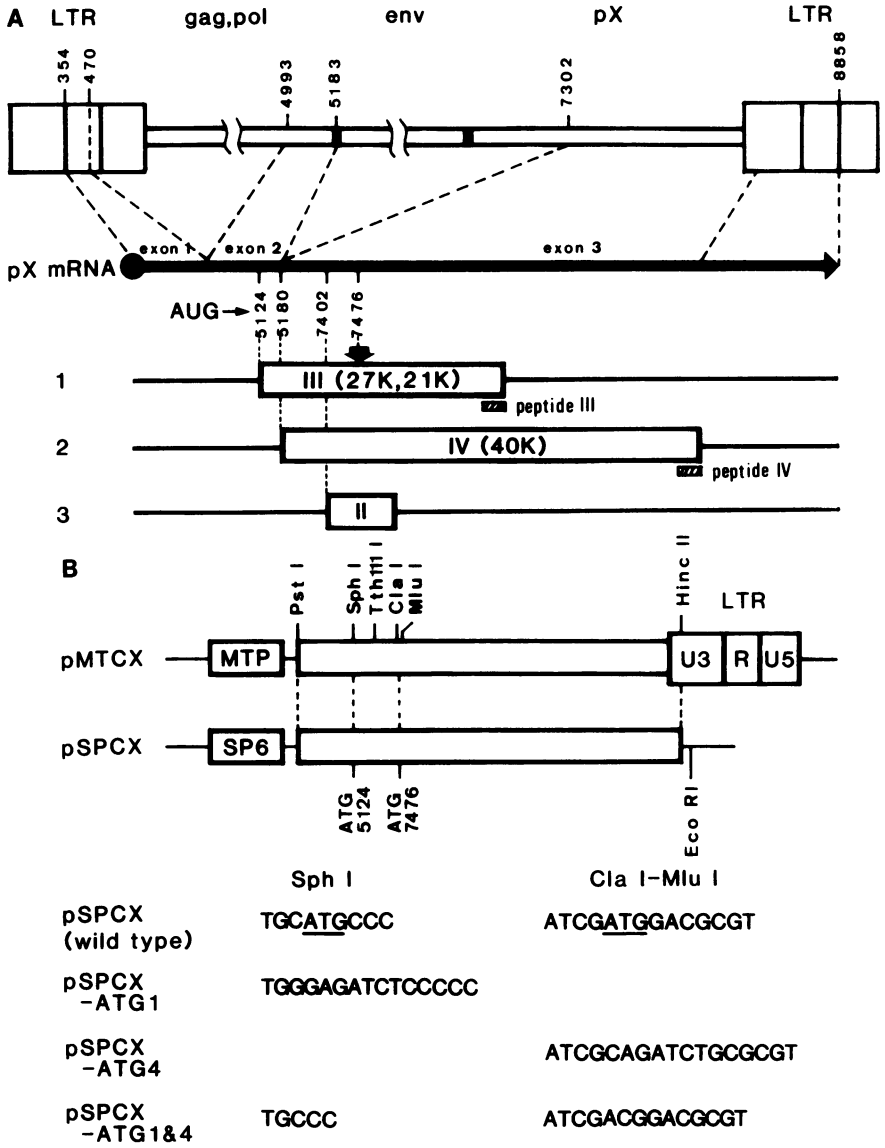

FIG. 1. (A) Constitution of the 2.1-kb *pX* mRNA of HTLV-I and arrangements of the ORFs. Three exons in *pX* mRNA are shown in comparison with the proviral genome (top). Three possible ORFs, II, III, and IV, are shown at the bottom in the extended form based on the original proposal for the provirus form (23). Thus, all the ORFs in this figure start with ATG. Lines 1 to 3 represent three possible translational registers. The locations of the synthetic peptides are also shown. The numbers on the provirus genome and mRNA represent nucleotide numbers from the 5′ end of the provirus genome. K is molecular weight, in thousands. (B) Constructions of pMTCX and pSPCX. The restriction sites for mutations and sequence alterations in the mutants are shown. The ATG codons underlined are the initiation codons for $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ synthesis.

capacity to encode a 21-kDa polypeptide. The second AUG codon is located 56 bases downstream of the first AUG codon and in ORF IV, which codes for $p40^{x}$ (24). $p40^{x}$ is efficiently initiated from the second AUG codon. Thus the first AUG codon was predicted to initiate $p21^{x\text{-}III}$ translation because of its coding capacity for the 21-kDa polypeptide. This possibility was examined by deleting the first ATG codon in pSPCX, thus constructing pSPCX-ATG1 (Fig. 1B). When the RNA from this plasmid was translated in vitro, a band of 21 kDa was seen, in contrast to the prediction, but there was no band of 27 kDa (Fig. 4A, lane 3), indicating that the 27-kDa product is initiated at the first AUG codon. Thus, it appears that ORF III can code for a 21-kDa protein, but that the product migrated as a 27-kDa band on polyacrylamide gel electrophoresis. The reason for this discrepancy is unknown, but it might be due either to its unusually high contents of proline (21%) and serine (15%) or phosphorylation of the product. The production of a 21-kDa protein without a 27-kDa protein indicates that the two proteins are translated independently and that the 21-kDa protein is not processed from the 27-kDa protein. Next, the second AUG codon in ORF III, that is, the fourth AUG codon in the mRNA, was deleted, thus constructing the deletion mutant pSPCX-ATG4 (Fig. 1B). Translation of the RNA gave the 27-kDa protein but not the 21-kDa protein (Fig. 4A, lane 4). Thus synthesis of the 21-kDa protein was initiated at the second AUG codon in ORF III in the 2.1-kb mRNA. Again, the molecular mass predicted from this translation was 12 kDa, which was markedly different from the 21 kDa that was found. However, although the 21-kDa protein was translated

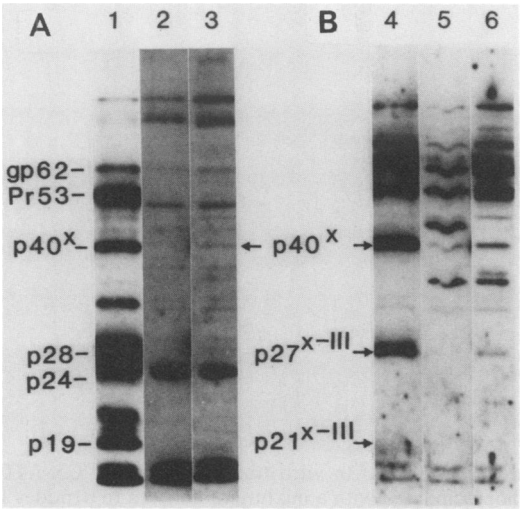

FIG. 2. Detection of *pX* proteins in cells transfected with pSVPX, a cDNA clone of 2.1-kb mRNA. FL cells were transfected with expression plasmid pSVPX (24) in which the full cDNA sequence for 2.1-kb *pX* mRNA is inserted downstream of the simian virus 40 promoter, and a cell clone expressing $p40^{x}$ was isolated. Cell extract (10 μg of protein) was applied to each slot and electrophoresed in a 12% polyacrylamide gel. $p40^{x}$ or $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ were detected by the blotting procedure with serum from an ATL patient (A) or rabbit antisera against synthetic peptides corresponding to the C termini of the respective proteins (B). Lanes 1 and 4, HTLV-I-producing cell line MT-2; lanes 2 and 5, FL cells; lanes 3 and 6, FL cells transfected with pSVPX.

independently, it is part of the 27-kDa protein, and thus the same explanation for the discrepancy between the predicted and apparent molecular masses of the 21-kDa protein may be applied as in the case of the 27-kDa protein; the proline and serine contents of the 21-kDa protein are 24 and 19%, respectively. These results show that the three proteins are translated independently from a single 2.1-kb mRNA, that is, $p27^{x\text{-}III}$ from the first AUG codon in ORF III, $p40^{x}$ from the second AUG codon in ORF IV, and $p21^{x\text{-}III}$ from the fourth AUG codon in ORF III.

Activity of a mutant that does not express $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$. The identification of the novel proteins $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ and determination of their mechanisms of expression indicate that previous studies on the identification of a *trans*-acting activator of the LTR were insufficient to eliminate the possible effects of these new proteins on LTR activation. For example even direct expression of $p40^{x}$ is not sufficient, because the construction inevitably contains the ATG codon for $p21^{x\text{-}III}$. Although we have proposed that $p40^{x}$ is essential for *trans*-activation from experiments with mutants carrying the ORF-specific termination codon, it is still possible that a fragment of $p27^{x\text{-}III}$ might be functional in activating the LTR. Therefore, we constructed a mutant pMTCX-ATG1&4, which has mutations at both ATG codons in ORF III. This mutant cannot produce even fragments of these proteins, as shown by translation in vitro (Fig. 4B). The mutant was cotransfected with pLTR-CAT into HOS cells, and the CAT activity produced was measured. The mutant pMTCX-ATG1&4 showed activity almost as high as that of the wild-type pMTCX (Fig. 5). On the other hand, pMTCX/IV^{ter}, a mutant carrying the termination codon in ORF IV, was completely inactive in *trans*-activation of the LTR. Thus, these results indicate that neither of the products translated from ORF III is involved in activation of the LTR in transient expression.

DISCUSSION

Expression of the *pX* sequence is known to mediate activation of its own LTR (3, 5, 25, 26, 29) and to be required for efficient viral gene expression, which is thought to play a key role in ATL development by activating some cellular genes. Recently, the *pX* sequence was found to code for three proteins, $p40^{x}$, $p27^{x\text{-}III}$, and $p21^{x\text{-}III}$ (11). Originally, $p40^{x}$ alone was proposed to mediate the *trans*-acting transcriptional activation because it was the only *pX* protein known, but when the other two proteins were found, this conclusion became dubious. From previous studies in which we introduced ORF-specific termination codons into the *pX* sequence, we concluded that ORF IV, and thus $p40^{x}$, is responsible for the *trans*-acting activation (25, 26). However, the possible contributions of $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ or their fragments could not be excluded because their mechanisms of expression were unknown.

Two *pX* proteins, $p40^{x}$ and $p27^{x\text{-}III}$, coded for by different ORFs were detected in cells transfected with a cDNA clone of 2.1-kb mRNA. This was good evidence that the cDNA clone had information for the translation of two different ORFs. On the other hand, in vitro translation of a single RNA species synthesized from the *pX* cDNA clone by SP6 RNA polymerase gave the three products, the 40-, 27-, and 21-kDa proteins, which were the same as those found in HTLV-I-infected cell lines. Therefore, it was concluded that the in vitro translation products are identical to the in vivo products. The failure to detect $p21^{x\text{-}III}$ in transfected cells (Fig. 2, lane 6) may be due to a low level of its expression, because it is present in HTLV-I-infected cells (Fig. 2, lane

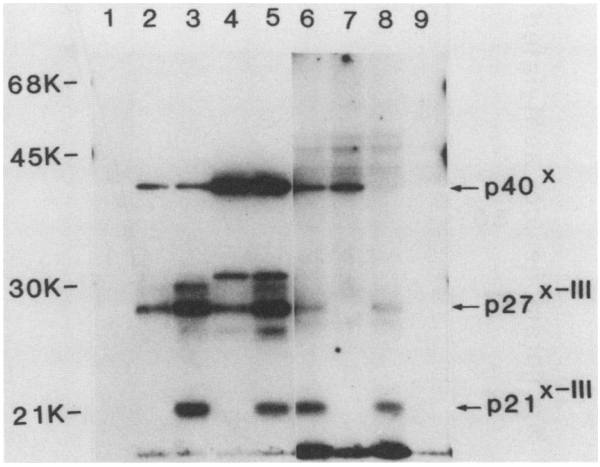

FIG. 3. In vitro translation of a single species of *pX* mRNA synthesized by SP6 RNA polymerase. In vitro translation products of the wild-type pSPCX RNA (lanes 2 through 5) were immunoprecipitated and compared with in vivo products in HUT102 cells (lanes 6 through 9). Electrophoresis was in a 12% polyacrylamide gel, and autoradiograms from the same gel with different exposures were reconstituted. Lane 1, a mixture of antisera to C-terminal peptides of $p27^{x\text{-}III}$ and $p40^{x}$ (peptides III and IV) without added RNA; lanes 2 and 9, preimmune rabbit serum; lanes 5 and 6, a mixture of antisera to peptides III and IV; lanes 4 and 7, a mixture of antisera preabsorbed with peptide III; lanes 3 and 8, a mixture of antisera preabsorbed with peptide IV. K is molecular weight, in thousands.

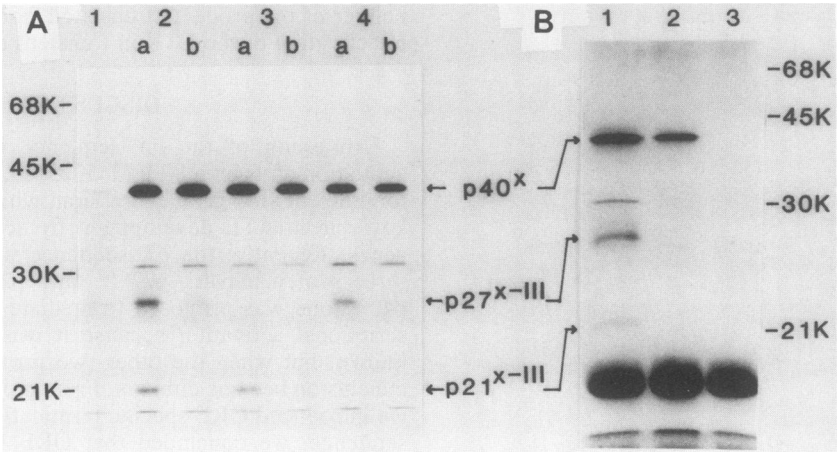

FIG. 4. In vitro translation of *pX* mRNA with deletions of possible initiation codons. (A) In vitro translations of pSPCX-ATG1 and pSPCX-ATG4 RNAs. Each translation mixture (with 25 ng of RNA) was immunoprecipitated with a mixture of antisera to peptides III and IV before (lanes a) and after (lanes b) its preabsorption with peptide III. Lanes 1, pSPCX RNA with normal rabbit serum; lanes 2, pSPCX RNA; lanes 3, pSPCX-ATG1; lanes 4, pSPCX-ATG4. (B) In vitro translation of pSPCX-ATG1&4 RNA. The translation mixture was directly analyzed by 15% polyacrylamide gel electrophoresis. Lane 1, pSPCX RNA; lane 2, pSPCX-ATG&4; lane 3, no added RNA. K is molecular weight, in thousands.

4). Thus it was concluded that a single species of mRNA can code for the three proteins $p40^{x}$, $p27^{x\text{-}III}$, and $p21^{x\text{-}III}$, excluding possible splicing in vivo. Deletions of the first and fourth ATG codons abolished the syntheses of $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$, respectively, indicating that the 2.1-kb *pX* mRNA encodes these three proteins independently and that they are initiated from different ATG codons in vivo. Thus *pX* mRNA appears to be polycistronic. This conclusion is consistent with the fact that we were previously unable to detect alternatively spliced mRNA coding for $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ by blotting analysis of cellular mRNA or to isolate the corresponding cDNA clone (unpublished data). Such polycistronic mRNA has not been found in eucaryotic cellular mRNA, but it has been found in some animal virus mRNAs (1, 2, 4, 7, 27).

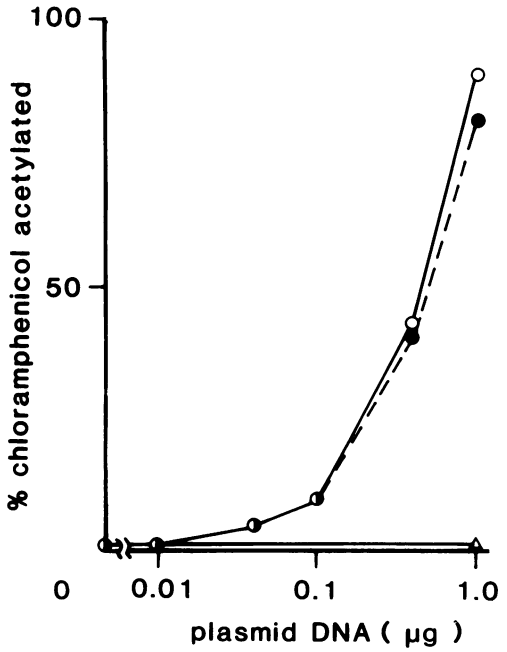

FIG. 5. Activation of CAT expression directed with pLTR-CAT by ATG-defective mutants of pMTCX. One microgram of pLTR-CAT was cotransfected with various amounts of plasmids into HOS cells, and CAT activity was measured as described previously (12). Symbols: ○, pLTR-CAT with wild-type pMTCX; ●, pLTR-CAT with pMTCX-ATG1&4, which has defects at both ATG codons in ORF III; △, pLTR-CAT with $pMTCX/IV^{ter}$, which carries a termination codon in ORF IV.

In eucaryotic mRNAs, the first AUG codon is used efficiently for translation (12). However, in *pX* mRNA, the second AUG codon, which codes for $p40^{x}$, is used most efficiently. This may be explained by the modified scanning model described by Kozak (13) which suggests that sequences flanking the AUG initiation codon affect the efficiency of codon recognition by 40S ribosomes. The consensus sequence for efficient initiation is PuNNAUGG (13) (where Pu is a purine nucleoside and N is any nucleoside). In the *pX* mRNA, the flanking sequences of the AUG codons for $p27^{x\text{-}III}$, $p40^{x}$, and $p21^{x\text{-}III}$ are UGCAUGC, ACCAUGG, and UCGAUGG, respectively, in that order on the mRNA, and the second AUG codon matches the consensus sequence. Accordingly, 40S ribosomes recognize the first AUG codon less efficiently and continue to scan until they find the second AUG codon, with which the ribosomes can form an initiation complex efficiently. Thus, the fourth AUG codon is used much less efficiently. This prediction is consistent with the finding that $p27^{x\text{-}III}$ is present in larger amounts than $p21^{x\text{-}III}$ in most cell lines, although the ratio of the two proteins varies in different cell lines (11). The reason for these variations is not understood, but it may be due to differences in some cellular factors.

The N-terminal region of $p27^{x\text{-}III}$, which is not present in $p21^{x\text{-}III}$, is highly basic, i.e., NH_2-Met-Pro-Lys-Thr-Arg-Arg-Arg-Pro-Arg-Arg-Ser-Gln-Arg-Lys-Arg-Pro. Thus $p27^{x\text{-}III}$, but not $p21^{x\text{-}III}$, may be a DNA-binding protein. Consistent with this prediction, $p27^{x\text{-}III}$ was found to be a nuclear protein (11).

The mutant pMTCX-ATG1&4 which could not express $p27^{x\text{-}III}$ or $p21^{x\text{-}III}$ or even their fragments was almost as active as the wild-type pMTCX in transient activation of the LTR function. These results exclude the possibility that even a fragment of $p27^{x\text{-}III}$ or $p21^{x\text{-}III}$ is involved in *trans*-activation of the LTR in transient assays. Thus $p40^{x}$ alone is responsible for this function. These results do not exclude the possibility that $p27^{x\text{-}III}$, $p21^{x\text{-}III}$, or both are required for

activation of the LTR integrated into the cellular genome. The nuclear association of the novel protein $p27^{x\text{-}III}$ and its phosphorylation suggest that at least $p27^{x\text{-}III}$ has some regulatory functions in viral gene expression, but the functions of $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$ are not yet known.

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