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# Abundant Synthesis of Functional Human T-Cell Leukemia Virus Type I p40$^x$ Protein in Eucaryotic Cells by Using a Baculovirus Expression Vector

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**The human T-cell leukemia virus type I (HTLV-I) p40$^x$ protein is a 40-kilodalton polypeptide encoded in the 3′-terminal region of the virus. This protein is responsible for positive transcriptional *trans*-activation of promoter elements located within the HTLV-I long terminal repeat. We introduced the protein-coding region of HTLV-I p40$^x$ into the genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus. After infection of the insect *Spodoptera frugiperda* (SF9) cell line, this recombinant strain of baculovirus produced approximately 200 mg of intact p40$^x$ protein per 2.5 × 10$^8$ cells. The protein was biologically active in *trans*-activation of an HTLV-I long terminal repeat-human β-globin construct. Biochemical analyses of the protein suggest that the p40$^x$ polypeptide underwent posttranslational modification in these eucaryotic SF9 cells.**

Human T-cell leukemia virus type I (HTLV-I) is a T-cell lymphotropic virus associated with one particular form of adult T-cell leukemia (13, 16, 37, 38). At the 3′ end of the HTLV-I genome is a long open reading frame (LOR) which encodes a protein designated p40$^x$ (22, 29). The mRNA coding for this p40$^x$ polypeptide is generated through a double splicing event which unites the initiating methionine codon of the *env* gene with one additional guanosine nucleotide to the p40$^x$ coding LOR (27, 35).

Numerous recent studies have demonstrated that the p40$^x$ product is a potent transcriptional *trans*-activator of the promoter element contained within the HTLV-I long terminal repeat (LTR) (2, 5, 8, 28, 33). To obtain sufficient protein for investigating the structure and mechanism of action of the p40$^x$ protein, we placed the complete HTLV-I p40$^x$ coding sequence downstream of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin promoter (30). This hybrid gene was inserted by cellular recombination into the baculovirus genome (32) to produce a recombinant virus (vAcPx) that synthesized abundant amounts of the p40$^x$ polypeptide after infection of invertebrate SF9 cells.

We found that the HTLV-I p40$^x$ protein synthesized from the baculovirus expression vector was indistinguishable in size, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, from that synthesized in lymphoid (22) or bacteria cells (10). Furthermore, the p40$^x$ protein synthesized in the eucaryotic insect cells appeared to be biologically active. Despite significant overproduction (up to 200 mg of p40$^x$ synthesized per 2.5 × 10$^8$ cells) in SF9 cells, the baculovirus-produced p40$^x$ was quantitatively compartmentalized into the nucleus, as was the p40$^x$ produced in lymphoid cells (17). As a further reflection of biological activity, the p40$^x$ protein synthesized in SF9 cells also strongly *trans*-activated transcription from the HTLV-I LTR. By the above criteria, the p40$^x$ polypeptide synthesized in insect cells behaved similarly to that produced in HTLV-I-transformed T cells.

Since the baculovirus-SF9 cell system is capable of a variety of eucaryotic posttranscriptional modifications, including glycosylation (32), extracellular secretion (31, 32), accurate signal peptide cleavage (31), and phosphorylation (24), we anticipate that the synthesis of p40$^x$ protein in infected SF9 cells will faithfully reflect that produced in HTLV-I-infected lymphocytes.

## MATERIALS AND METHODS

**Cells and viruses.** *Spodotera frugiperda* SF9 cells were obtained from Max Summers, Texas A&M University, and propogated either as a suspension or as monolayer cultures in Grace medium (K. C. Biologicals) supplemented with 10% fetal calf serum (GIBCO) at 28°C. A recombined baculovirus (vAcPx) containing the p40$^x$ coding sequence driven by a polyhedrin promoter was produced by cotransfection of plasmid pAcPx (Fig. 1) with wild-type AcNPV strain $E_2$ DNA into SF9 cells. At 72 h after cotransfection, virions extruded into the supernatant medium were used to inoculate fresh monolayers of SF9 cells that were subsequently overlaid with 1% low-melting-point agarose (M. D. Summers and G. E. Smith, Tex. Agric. Exp. Stn. Bull. 1555, in press). Five days later, the agarose overlay was removed and preserved, and the infected monolayer was blotted onto a nitrocellulose disk (BA85, Schleicher & Schuell) and subsequently hybridized to a nick-translated (25) p40$^x$-specific probe derived from the viral LTR. Hybridization was performed in buffer containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 40% formamide, 10% dextran sulfate, 0.5% SDS, and 5× Denhardt solution at 45°C for 24 h. The filters were extensively washed in 2× SSC–0.5% SDS at 65°C before autoradiography. Hybridization-positive regions of the nitrocellulose disk were used to localize corresponding regions of the agarose overlay. These regions were carefully excised and vigorously vortexed in Grace medium to release retained virus. The virus-containing medium was used to further infect fresh monolayer cultures. Routinely, five rounds of hybridization screening were performed before the recombinant virus was considered plaque purified.

**Recombinant plasmid constructs.** Standard recombinant DNA techniques were used for construction of recombinant plasmids (23). Oligonucleotides were synthesized with an

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automated DNA synthesizer (model 330A; Applied Biosystems).

To construct plasmid pRSVoligoTthPx, a small synthetic oligonucleotide fragment with the sequence

*Hin*dIII AGGTGGTACC GGGTGAAGGG TCCCAAACCT GTC 5'
5' AGCT TCCACCATGG CCCACTTCCC AGGGTTTGGA CA *Tth*111-I

was inserted into a plasmid construct so that the *Hin*dIII end of the oligonucleotide was fused downstream of the Rous sarcoma virus (RSV) LTR promoter and the *Tth*111-I end is inserted at the unique *Tth*111-I site 16 base pairs (bp) downstream of the beginning of the HTLV-I LOR. The resulting recombinant plasmid (Fig. 1) contained a complete intronless $p40^x$ coding sequence placed under the control of the RSV LTR.

To generate pAcPx, the *Hin*dIII-*Sst*I fragment from pRSVoligoTthPx was excised. The sticky ends were made blunt with T4 DNA polymerase (23). *Bam*HI linkers (New England Biolabs) were ligated to the blunted fragment with T4 DNA ligase (23), and the fragment, after restriction with *Bam*HI, was inserted into the unique *Bam*HI site of plasmid pAc373 (32).

LTR-globin was constructed by fusing sequences from −322 to +264 from the HTLV-I LTR (15) to sequences starting at position +49 in the first exon of the human β-globin gene. The resulting transcriptional unit is driven by the authentic HTLV-I LTR promoter with its regulatory sequences and synthesized a hybrid mRNA.

**Nuclease S1 analysis.** Total cellular RNA was isolated from transiently transfected SF9 cells by guanidinium isothiocyanate-cesium chloride sedimentation (3). Hybridization of RNA to a $^{32}P$-labeled M13 globin probe, S1 nuclease digestion, and PAGE were performed as previously described (21). The M13 globin probe consisted of sequences starting at −128 of the human β-globin gene and included the complete first and second β-globin exon (21). When used in S1 protection, this probe will protect a 240-bp RNA, transcribed from exon 2, and a 120-bp RNA, transcribed from exon 1 of the LTR-globin construct.

**Protein analysis and PAGE.** SF9 cells were solubilized with detergent Nonidet P-40 (NP40) (Calbiochem) into cytoplasmic and nuclear fractions (14). The cytoplasmic fraction was found to be free of DNA, and the nuclear fraction showed no rRNA contamination. To remove adhering cytoplasmic tags, the NP40 nuclear pellet was sonicated (microtip setting 4) for four 1-s pulses with a Branson sonicator. One-dimensional SDS-PAGE (14) and two-dimensional isoelectric focusing (9) gel electrophoresis were performed as previously described. Protein molecular weight markers were myosin (200,000), phosphorylase b (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsin (25,700), β-lactoglobulin (18,400), and lysozyme (14,300) (Bethesda Research Laboratories). Partial proteolysis (4) was performed with *Staphylococcus aureus* V-8 protease, elastase, and chymotrypsin (Sigma). Enzyme concentrations were first titrated individually to optimize partial protein digestion conditions before use. Immunoblotting (34) with $^{125}I$-protein A was performed as described by Giam et al. (10).

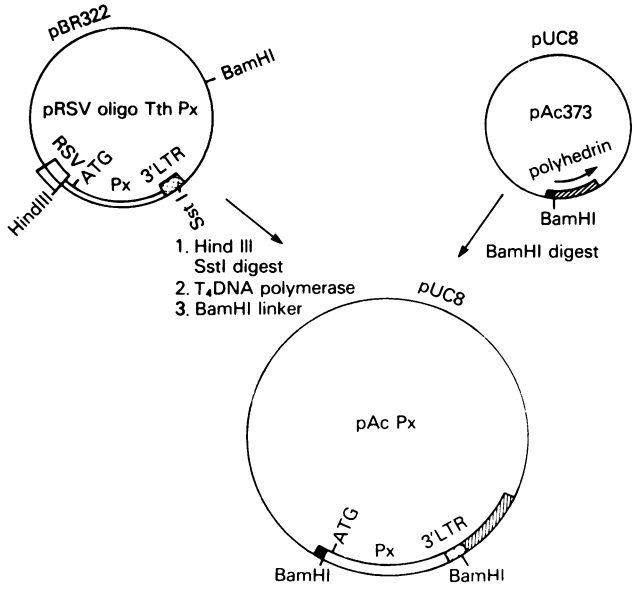


FIG. 1. Schematic representation of recombinant manipulations used to generate plasmid pAcPx. The complete intact $p40^x$ coding region (Px) contained within plasmid pRSVoligoTthPx was removed by *Hin*dIII-*Sst*I cleavage. The protruding ends were blunted with T4 DNA polymerase, and *Bam*HI linkers were added. The resulting fragment was inserted downstream of the polyhedrin promoter in plasmid pAc373 (32) to create plasmid pAcPx. In pAcPx the $p40^x$ coding region (Px) is driven by the baculovirus polyhedrin promoter, but the authentic HTLV-I $p40^x$ AUG is provided for translational start.

## RESULTS

**Expression of HTLV-I $p40^x$ protein in invertebrate cells.** Since the baculovirus expression vector system has not yet been shown to be capable of intron removal, we removed the $p40^x$ intron (27, 35) by the synthetic reconstruction of necessary sequences upstream of the LOR. A small synthetic oligonucleotide embodying a consensus translational initiation sequence (19) and $p40^x$ coding sequences upstream of the single *Tth*111-I site (positioned 16 bp downstream of the beginning of the LOR) were ligated in a correct orientation into this unique restriction site (see Materials and Methods). The resulting construction was an intronless but complete $p40^x$ coding sequence which was initially placed under the control of an RSV LTR (pRSVoligoTthPx) and subsequently transferred into the pAc373 plasmid (32) containing the strong AcNPV polyhedrin promoter (Fig. 1). The final product, pAcPx (Fig. 1), was used in cotransfection with wild-type AcNPV DNA to generate through in vivo recombination a recombinant baculovirus (vAcPx) carrying the $p40^x$ sequences in place of the viral polyhedrin gene. vAcPx was plaque purified as described in Materials and Methods.

Plaque-purified vAcPx virus was used in lytic infection of invertebrate eucaryotic SF9 cells. The protein profile of vAcPx-infected cells (Fig. 2, lane 4) was compared with that from mock-infected cells (Fig. 2, lane 2) and SF9 cells infected with wild-type AcNPV (Fig. 2, lane 3) in SDS-PAGE analysis. A strongly stained novel band of 40 kilodaltons (kDa) was present in cells infected with vAcPx baculovirus (Fig. 2, lane 4) but absent in the mock-infected and wild-type baculovirus-infected cells. This 40-kDa pro-

tein comigrated with the p40$^x$ protein synthesized in *Escherichia coli* (10) (Fig. 2, lane 6) that had been purified approximately 100-fold (Giam, unpublished data). Western immunoblot analysis (34) confirmed the identity of the baculovirus p40$^x$ (Fig. 2, lanes 10 and 13), revealing a similar antigenic reactivity with a monospecific anti-p40$^x$ serum (gift of S. Oroszlan) as seen for bacterial p40$^x$ (Fig. 2, lanes 12 and 15) and weakly reacting lymphocyte p40$^x$ (Fig. 2, lanes 11 and 14). Other weakly reacting bands in the lymphocyte sample (Fig. 2, lane 14) were nonspecifically reactive proteins that were not observed in other experiments. We estimate from comparison with known amounts of molecular weight marker proteins that approximately 1 ng of p40$^x$ protein was produced per single vAcPx-infected SF9 cells.

The role of the HTLV-I p40$^x$ protein as a transcriptional *trans*-activator is commensurate with its location in the nucleus of HTLV-I-infected T cells (17). We examined the localization of the baculovirus p40$^x$ protein by detergent fractionation with NP40. The results demonstrated that despite the tremendous overproduction of p40$^x$ in infected SF9 cells, all of the protein was quantitatively found in the nucleus (results not shown). This represents one piece of evidence that p40$^x$ behaves in a biologically relevant manner in invertebrate cells.

**p40$^x$ biologically functional in SF9 cells.** The biological activity of the baculovirus p40$^x$ in SF9 cells was assessed by examining the *trans*-activation ability in vAcPx-infected cells transfected with an HTLV-I LTR target plasmid construct. A fusion gene consisting of the HTLV-I LTR linked to human β-globin coding sequences (LTR-globin) was constructed (see Materials and Methods) as a reporter sequence for evaluating transactivation by baculovirus p40$^x$. This construct was transfected into SF9 monolayer cultures by calcium phosphate precipitation (12), followed 12 h later by infection with either wild-type AcNPV or vAcPx. At 24 h after this infection, transcription of β-globin mRNA was assayed by quantitative S1 nuclease analysis (21). Although there was a low level of β-globin transcription in cells transfected with the LTR-globin plasmid alone (Fig. 3, lane 5), this transcription increased approximately 50-fold after superinfection with vAcPx (Fig. 3, lane 4). As expected, there were no detectable globin-specific transcripts in mock-infected cells (Fig. 3, lane 2); infection with wild-type AcNPV (Fig. 3, lane 3) actually abolished the basal activity of the transfected LTR-globin construct. This latter observation probably relates to the fact that AcNPV lytic infection decreases host cell macromolecular synthesis. Accordingly, we observed that the *trans*-activation phenomenon from superinfection of vAcPx occurred best during a relatively brief time after infection.

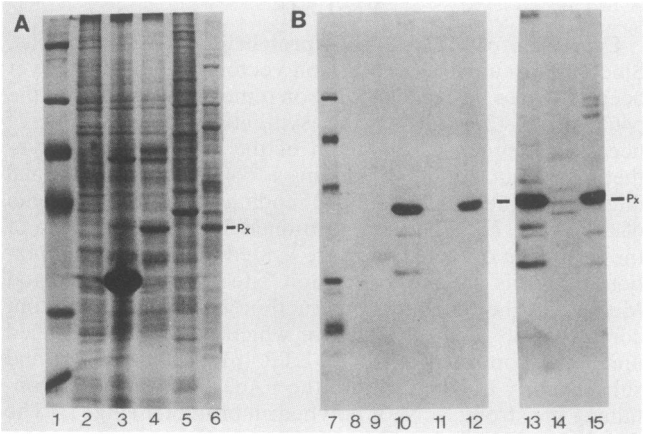


FIG. 2. Synthesis of p40$^x$ in insect cells. SF9 cells were infected with vAcPx; total cellular proteins were solubilized in SDS-β-mercaptoethanol (14) and resolved in a 10% SDS-polyacrylamide gel. (A) Gel stained with Coomassie blue. (B) The protein profile from an identical gel was transferred to nitrocellulose (34) and treated first with hyperimmune serum to p40$^x$ and then reacted with $^{125}$I-protein A and visualized by autoradiography. Lanes: 1 and 7, protein molecular weight markers (200,000, 97,400, 68,000, 43,000, 25,700, and 18,400 from top to bottom); lanes 2 and 8, uninfected SF9 cells; lanes 3 and 9, SF9 cells infected with wild-type AcNPV; lanes 4 and 10, SF9 cells infected with vAcPx; lanes 5 and 11, protein extracts from the HTLV-I-transformed cell line C81-66-45 (22); lanes 6 and 12, cell lysates from a p40$^x$-producing bacterial expression system enriched approximately 100-fold (10); lanes 13, 14, and 15, longer exposures of lanes 10, 11, and 12, respectively. The location of p40$^x$ (Px) is shown.

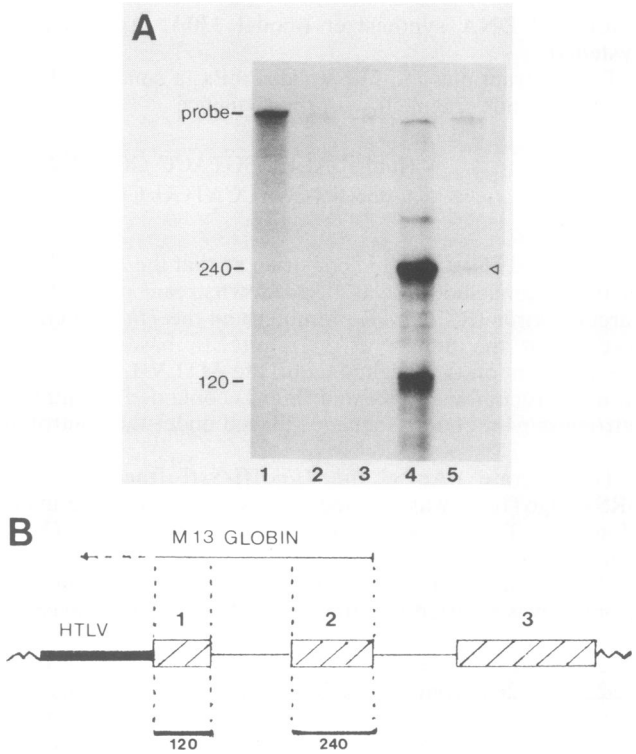


FIG. 3. Transcriptional *trans*-activation by p40$^x$ in SF9 cells. An HTLV-I LTR-human β-globin (LTR-globin) plasmid was transiently transfected into SF9 cells, followed by superinfection with either wild-type AcNPV or recombinant vAcPx. S1 analysis of globin mRNA production was carried out as previously described (21) with a single-stranded M13 probe. (A) Lane 1, Intact M13 $^{32}$P-globin single-stranded probe used for the S1 analysis. Lanes 2 to 5, Hybridization reactions of the probe with RNA from (lane 2) mock-transfected SF9 cells, (lane 3) LTR-globin-transfected SF9 cells superinfected with wild-type AcNPV, (lane 4) LTR-globin-transfected cells superinfected with vAcPx, or (lane 5) SF9 cells transfected with the LTR-globin plasmid. The two protected bands in lane 4 are 240 and 120 bp in size and represent globin-specific RNA transcribed from exon 2 and exon 1 in the LTR-globin plasmid, respectively. (B) Diagrammatic representation of the LTR-globin plasmid and the M13 probe used for S1 analysis. The LTR-globin plasmid contains sequences from −322 to +264 from the HTLV-1 LTR fused to +49 of the first human β-globin exon. Exons 2 and 3 are contained in their entirety. The M13 probe used has been described in detail elsewhere (21). The expected protected pieces are 120 and 240 bp in size.

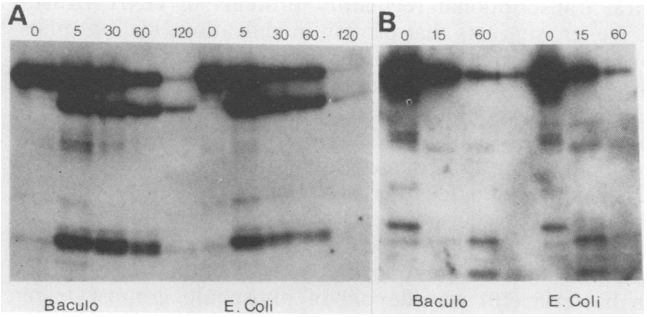


FIG. 4. Comparisons of p40$^x$ produced in eucaryotic and procaryotic cells by partial proteolysis. p40$^x$ protein was isolated as a gel band from infected SF9 cells (Baculo) or a bacterial producer strain (*E. coli*) (10). (A) Eucaryotic and procaryotic forms of the protein were digested with *S. aureus* V-8 protease (200 μg/ml) for 0, 5, 30, 60 or 120 min. (B) Similar digestions (0, 15, or 60 min) were carried out with elastase (200 μg/ml). The partial digestion profiles were visualized by transfer of proteins from SDS-PAGE to nitrocellulose, which was reacted with hyperimmune p40$^x$ serum followed by incubation with $^{125}$I-protein A. An autoradiographic exposure is shown.

**Modification of the p40$^x$ protein.** The potential importance of the biochemical structure of the p40$^x$ protein in its role as a *trans*-activating protein led us to further physically characterize the polypeptide. Since we expressed the p40$^x$ protein in abundance in both procaryotic (10) and eucaryotic cells, a comparative analysis of the two substrates could provide important information on posttranslational modifications of the p40$^x$ protein. In a one-dimensional SDS-PAGE analysis, the migration of procaryotic p40$^x$ (Fig. 2, lane 6) was indistinguishable from that of the protein synthesized in eucaryotic cells (Fig. 2, lane 4). This suggested that certain eucaryotic modifications (e.g., glycosylation) that often cause major changes in apparent molecular weight are probably not involved in the posttranslational processing of p40$^x$. Since it is difficult to assess small apparent changes in a molecular size of 40 kDa, we cleaved the intact protein into subfragments to accentuate small changes (Fig. 4). Partial proteolytic mapping (4) was performed with *S. aureus* V-8 protease (Fig. 4A), elastase (Fig. 4B), and chymotrypsin (not shown). In all three cases, no significant divergence in the digestion pattern was seen between the baculovirus p40$^x$ and the *E. coli* p40$^x$. These results suggest that even minor molecular weight differences between procaryotic and eucaryotic p40$^x$ probably do not exist.

A second category of frequent posttranslational modifications of proteins is altered charge of the molecules. Protein phosphorylation is one of the most commonly observed forms of posttranslational charge modification. Since phosphorylated and dephosphorylated forms of the same molecule are often impossible to resolve by one-dimensional SDS-PAGE, two-dimensional isoelectric focusing analysis was performed on eucaryotic SF9 p40$^x$ (Fig. 5B) and procaryotic *E. coli* p40$^x$ (Fig. 5C) as well as proteins from uninfected SF9 cells (Fig. 5A). Based on marker proteins, the baculovirus p40$^x$ (arrow, panel B) migrated to a pI of 4.4, in contrast to the pI of 6.6 predicted from the primary amino acid composition of the polypeptide. Isoelectric focusing of the procaryotic p40$^x$ was unsuccessful, probably due to the formation of insoluble large aggregates by the *E. coli* p40$^x$ protein which prevented migration into the isoelectric focusing gel and became trapped at the top (arrow, Fig. 5C) of the gel matrix.

The differences between the experimental and predicted pI for p40$^x$ can be accounted for by a posttranslational charge modification. We found that the p40$^x$ protein synthesized in vAcPx-infected cells and also in a lymphoid cell line (22) were both phosphoproteins. Preliminary partial proteolysis analysis shows that the phosphorylation pattern

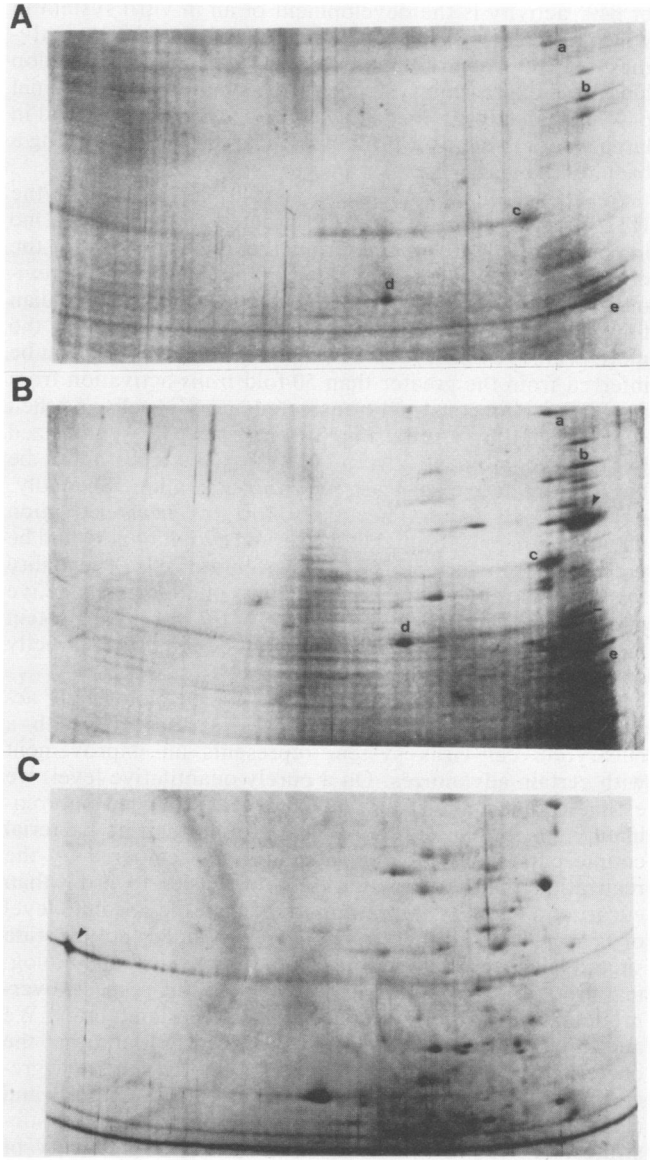


FIG. 5. Two-dimensional isoelectric focusing of eucaryotic and procaryotic p40$^x$. Protein lysates were resolved in the first dimension by isoelectric focusing in microcapillary tubes with a 3 to 10 ampholyte gradient (9). The second dimension was obtained in 10% SDS-PAGE. Protein spots were visualized by silver staining with a Biorad silver stain kit as described by the manufacturer. (A) Uninfected SF9 cells; (B) SF9 cells infected with vAcPx; (C) protein lysate purification enriched for p40$^x$ by ammonium sulfate precipitation from a producer bacterial strain. Lowercase letters refer to reference polypeptides. Arrows refer to p40$^x$, which was identified on the basis of molecular size and comigration with purified p40$^x$ protein. Positions d and e had pIs of 6.6 and 4.4, respectively. p40$^x$ in panel C was subsequently also found by other criteria to be a precipitated aggregate.

of $p40^x$ in both cells is virtually identical (Jeang, unpublished observations).

## DISCUSSION

The HTLV-I $p40^x$ protein is a strong *trans*-activator of transcription from the viral LTR (2, 5, 8, 28, 33) and is apparently essential for the replication of the HTLV-I genome (2). One approach to understanding the mechanism of $p40^x$ activity is the development of an in vitro system in which purified proteins can be added and appropriate activities tested. Toward that end, we attempted to develop high-level expression systems for the synthesis of functional $p40^x$ protein (10). In this study we successfully expressed in large amounts the $p40^x$ protein in eucaryotic cells by using a baculovirus vector.

At present it is not clear how $p40^x$ interacts with the HTLV-I LTR to increase LTR-directed transcription. One important point to emerge from the present study is the observation that the *trans*-activation abilities of $p40^x$ previously described for lymphoid cells (33) and mammalian fibroblasts (5, 8, 10, 28) can apparently also occur in the more primitive eucaryotic SF9 cells. Two corollaries can be inferred from the greater than 50-fold *trans*-activation from the LTR-globin construct transfected into SF9 cells and then superinfected by vAcPx (Fig. 3). First, the $p40^x$ synthesized by the recombinant vAcPx virus in SF9 cells must be biologically active, at least for *trans*-activation. Secondly, any host cell factors necessary for this *trans*-activation phenomenon must be highly conserved evolutionarily and be present in both vertebrate and invertebrate cells. The ability to synthesize and isolate large amounts of biologically active $p40^x$ protein should eventually allow the assay of protein factors and DNA sequences that may be important directly or indirectly in *trans*-activation by $p40^x$.

Although we had previously synthesized biologically active $p40^x$ in *E. coli* (10), the present approach with a eucaryotic cell-virus system represents an improvement with certain advantages. On a purely quantitative level, we estimate that each vAcPx-infected cell produced approximately 50- to 100-fold more $p40^x$ protein than its bacterial counterpart. This apparent advantage is tempered by the realization that procaryotic cells are easier to grow than eucaryotic cultures. More important than the absolute level of synthesis, the heterologously produced polypeptide should as closely as possible retain the physical and biological properties of its natural homolog. In this respect, overproducing procaryotic systems show some limitations. We and others (1, 11) (Fig. 5C) have frequently found that the bacterially synthesized protein forms poorly soluble aggregations. This potentially complicates purification efforts and can adversely affect the biological usefulness of these substrates. A second significant drawback is the inability of bacterial cells to perform eucaryotic posttranslational modifications. Thus, it is not known whether the observed biological activity of procaryotic adenovirus EIA (6, 20) and HTLV-I $p40^x$ (10) after introduction into eucaryotic cells is predicated on intracellular protein modifications by host cell factors. Indeed, Rosenberg and colleagues have observed processing of bacterial E1A 12S protein after mixing with a eucaryotic cell extract (7). Since one of our goals was to study functional $p40^x$ in vitro, the baculovirus system with its ability for posttranslational processing (24, 31, 32) may be highly useful.

To date there have been no published reports on posttranslational processing of $p40^x$ protein. Experience with viral transcriptional regulatory proteins in DNA viruses, however, suggests that protein modifications such as phosphorylation can be very important to the biological activities of regulatory polypeptides (26, 36). Relevant to this suggestion is the observation that the experimentally determined pI of baculovirus $p40^x$ was considerably more acidic than that predicted from the primary amino acid sequence (Fig. 5). One explanation for this discrepancy is our recent data demonstrating that $p40^x$ exists in a phosphorylated form in both infected SF9 cells and an HTLV-I nonproducer lymphoid cell line. In comparison, we found that $p40^x$ produced in bacteria (10) was devoid of phosphate groups (Jeang, unpublished observation). Thus, the existence of HTLV-I $p27^x$ and $p21^x$ as phosphorylated moieties (18) suggests that phosphoproteins may not be an uncommon form for HTLV-I nucleoproteins and that the activities of these proteins could potentially be regulated at the level of posttranscriptional modification.

Since SF9 cells can be grown at room temperature to high density ($2.5 \times 10^8$ cells per liter) in suspension culture, the production and purification of $p40^x$ should be relatively simple. In a good infection of SF9 cells by vAcPx, the synthesis of $p40^x$ accounted for roughly 10 to 20% of the total cellular protein mass. Currently, we estimate that 200 mg of $p40^x$ can be produced per liter of infected cells. The purification of this protein will contribute to an understanding of its physical and biological properties.

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