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Expression of the complete human T-cell leukemia virus type I pX coding sequence as a functional protein in *Escherichia coli*

(adult T-cell leukemia/transcriptional control/human T-cell leukemia virus type I long terminal repeat)

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ABSTRACT **Human T-cell leukemia virus type I (HTLV-I), a virus associated with adult T-cell leukemia, contains a long open reading frame (LOR) in the 3′ end of its genome between the *env* region and the 3′ long terminal repeat (LTR). This open reading frame encodes a 40-kDa protein (designated p40^x) that has been implicated as a positive control element for transcription from the HTLV-I LTR in a phenomenon known as trans-activation. We now report the expression of the complete p40^x coding sequence as a 40-kDa protein in *Escherichia coli*. The p40^x protein produced in bacteria is shown, using the protoplast fusion technique, to possess biological activity by its ability to trans-activate a HTLV-I LTR–chloramphenicol acetyltransferase plasmid that is stably integrated into the genome of mouse L cells. This stimulatory activity could be detected within 2 hr after fusion, suggesting the possibility of a direct role for p40^x in trans-activation of the HTLV-I LTR. The production of p40^x in large quantities in *E. coli*, together with the rapid protoplast fusion assay for its biological activity, should facilitate the analysis of p40^x mutants and the elucidation of the molecular mechanism of trans-activation.**

Viral-encoded proteins that positively stimulate transcription from the promoters of viral or cellular genes have been recognized as a common feature among DNA viruses such as simian virus 40, adenovirus, and the herpesviruses (for review, see ref. 1). This phenomenon, known as trans-activation, has also recently been demonstrated in a group of retroviruses, including human T-cell leukemia viruses type I and type II (HTLV-I and -II), bovine leukemia virus, and the acquired immunodeficiency syndrome (AIDS) retrovirus (2–9).

HTLV-I is associated with a form of adult T-cell leukemia found mainly in southwestern Japan, the Caribbean, and Africa (10–13). Nucleotide sequence analysis has revealed that in addition to the *gag*, *pol*, and *env* genes, which are common among retroviruses, the HTLV-I genome contained a long open reading frame (LOR) in the 3′ region between the *env* gene and the 3′ long terminal repeat (LTR) (14). This LOR has the capacity to encode a 40-kDa protein designated p40^x or TAT (15). A similar LOR has also been found in HTLV-II and in bovine leukemia virus (15, 16). The LOR region appears not to share any sequence homology with other known oncogenes or cellular genes (14).

Transfection of a DNA construct containing the HTLV-I LTR placed upstream of an indicator gene, chloramphenicol acetyltransferase (CAT), into human T cells showed a marked increase of CAT activity in HTLV-I-infected or transformed cells as compared to uninfected cells (2). This observation prompted the proposal that the LOR product is involved in trans-activation (2).

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A 40-kDa nuclear protein specific to HTLV-I-infected T cells was suggested to be derived from the HTLV-I LOR region (4, 13–20). Similarly, a 37-kDa protein was also found in HTLV-II-infected cells (18). Recently, it has been demonstrated both for HTLV-I and HTLV-II that through RNA splicing, the initiating methionine codon of the *env* gene together with one additional guanosine nucleotide are joined in-frame to the LOR. This produces a 2.1-kilobase (kb) transcript that can encode the HTLV-I 40-kDa or the HTLV-II 37-kDa proteins (21, 22).

Our interest in the molecular mechanism of trans-activation has motivated us to produce the p40^x protein of HTLV-I in *E. coli* in large quantity. This paper describes the expression of the complete HTLV-I p40^x protein in *Echerichia coli*. We also demonstrate that the bacterially derived HTLV-I p40^x protein exhibits biological function in trans-activating the HTLV-I LTR after its introduction into mouse L cells by protoplast fusion.

MATERIALS AND METHODS

Cell Lines and Media. Cell line C81-66-45 is an HTLV-I-transformed human T-cell line that constitutively expresses p40^x protein. LHC cells are a stable mouse L-cell line that contains the integrated pU3RCAT plasmid construct (2). C81-66-45 was propagated in RPMI medium containing 10% fetal calf serum and LHC cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Bacterial strain HB101 (23) was used as the host cell for all the plasmids described in this communication.

Recombinant DNA Techniques. Recombinant DNA experiments were conducted as described in ref. 24. Oligonucleotides were synthesized by using an automated DNA synthesizer (model 380A) from Applied Biosystems, purified from a 10% nondenaturing acrylamide gel, and cloned into the recipient vector, pRSV-PX. The sequences of the synthetic oligonucleotides were confirmed by the Maxam and Gilbert sequencing method (25).

Protoplast Fusion and CAT Assay. Bacterial protoplasts were prepared as follows. One hundred A_{600} unit equivalents of an overnight culture ($\approx 2 \times 10^{10}$ cells) grown in tetracycline (3 μg/ml) was harvested and resuspended in 2.5 ml of a buffer containing 20% sucrose, 50 mM Tris·HCl (pH 7.5). Lysozyme was added to a final concentration of 200 μg/ml, and the suspension was incubated on ice for 2–3 min, followed by the addition of 10 μl of 0.25 M EDTA solution (pH 7.5). The mixture was gently swirled and placed on ice for 3 min. Subsequently, 2.5 ml of 50 mM Tris·HCl (pH 7.5) was added dropwise into the bacterial suspension, and the mixture was incubated at 37°C for 5 min. At the end of the 5-min incubation, 20 ml of 10% sucrose in DMEM was added slowly

Abbreviations: HTLV-I and -II, human T-cell leukemia viruses types I and II; LTR, long terminal repeat; LOR, long open reading frame; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); Tc^r, tetracycline resistance; IFN-γ, γ-interferon.

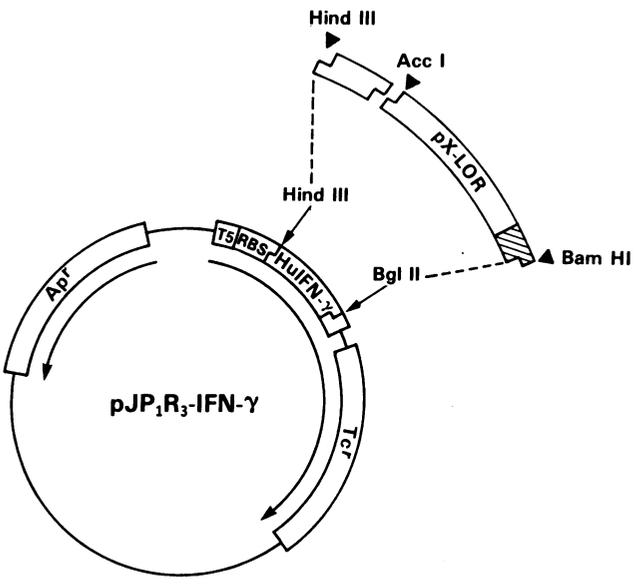

FIG. 1. Construction of the complete p40^x coding sequence and its insertion into a bacterial expression vector. The p40^x-LOR region depicted in this figure derives from a plasmid pRSV-PX that contains the entire HTLV-I LOR region, flanked by a *Hin*dIII site on the 5′ side and a *Bam*HI site on the 3′ side. The *Acc* I site located within the LOR at the codon for amino acid 17 is unique in the pRSV-PX DNA. Two complementary oligonucleotides with the sequences

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5′ AGCTTATGGCCCACTTCCCAGGGTTTGG
3′     ATACCGGGTGAAGGGTCCCAAACC
HindIII                    Acc I
                ACAGAGTCTTCTTTTCGGATACCCAGT  3′
                TGTCTCAGAAGAAAAGCCTATGGGTCAGA 5′
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were synthesized by the phosphoamidite method using an automatic DNA synthesizer. The two oligonucleotides were mixed in equimolar amounts, heated to 90°C, and reannealed at 37°C overnight. The reannealed product was then ligated to the *Hin*dIII- and *Acc* I-digested pRSV-PX DNA. Transfer of the ligation mixture into *E. coli* strain HB101 gave rise to clones that contained the complete p40^x coding sequence, including the methionine initiation codon. Digestion with *Hin*dIII and *Bam*HI yielded a 1.6-kb fragment, which was purified and inserted into the pJP$_1$R$_3$-IFN-γ vector DNA that had previously been digested with *Hin*dIII and *Bgl* II to remove the IFN-γ insert. The symbols T5, RBS, HuIFN-γ, Tc^r, and Ap^r represent bacteriophage T5 promoter, synthetic ribosome binding site, HuIFN-γ sequence, Tc^r gene, and ampicillin-resistant gene, respectively.

with swirling. Conversion of the bacteria to spheroplasts or protoplasts was >95% as judged by phase-contrast microscopy. About 3×10^9 protoplasts were routinely used for each fusion experiment. The protoplasts were centrifuged at 1500 rpm in a bench top centrifuge at 4°C for 20 min and after removal of the supernatant by aspiration, the protoplast pellet was resuspended in 120 μl of DMEM with 10% sucrose. Tissue culture cells were prepared by trypsin treatment followed by centrifugation at 1000 rpm in a bench top centrifuge and 10^7 cells were resuspended in 50 μl of DMEM for each fusion. After combining the tissue culture cells and the protoplasts (final vol, ≈70 μl), 0.35 ml of 57% polyethylene glycol 1000 (PEG 1000) in DMEM was added, and the suspension was gently mixed by hand. Two minutes after the addition of PEG 1000, 10 ml of DMEM with 10% fetal calf serum, and three antibiotics—kanamycin, streptomycin, and penicillin (each at 200 μg/ml)—was mixed in gently with the cell suspension and let stand at room temperature for 15 min. At this point, the cells were plated on a 10-cm dish and incubated in a CO_2 incubator for various lengths of time.

After protoplast fusion, tissue culture cells were collected by scraping the tissue culture dishes and harvested by low-speed centrifugation at 1000 rpm in a bench top centrifuge. The cell pellets were washed once with 1 ml of phosphate-buffered saline (50 mM sodium phosphate, pH 7.5/0.8% NaCl) and resuspended in 100 μl of 0.25 M Tris·HCl (pH 7.8). After brief sonication and centrifugation in a Microfuge for 5 min, the supernatant solutions containing the CAT enzyme were assayed (26).

RESULTS AND DISCUSSION

Expression of HTLV-I pX in *E. coli*. Since the p40^x coding region is not contiguous in the HTLV-I genome, we have constructed an intact coding sequence by making use of the *Acc* I recognition site, which is found only once within the LOR at the codon for amino acid 17. The missing upstream sequences that encode amino acids 1–17 were provided by a

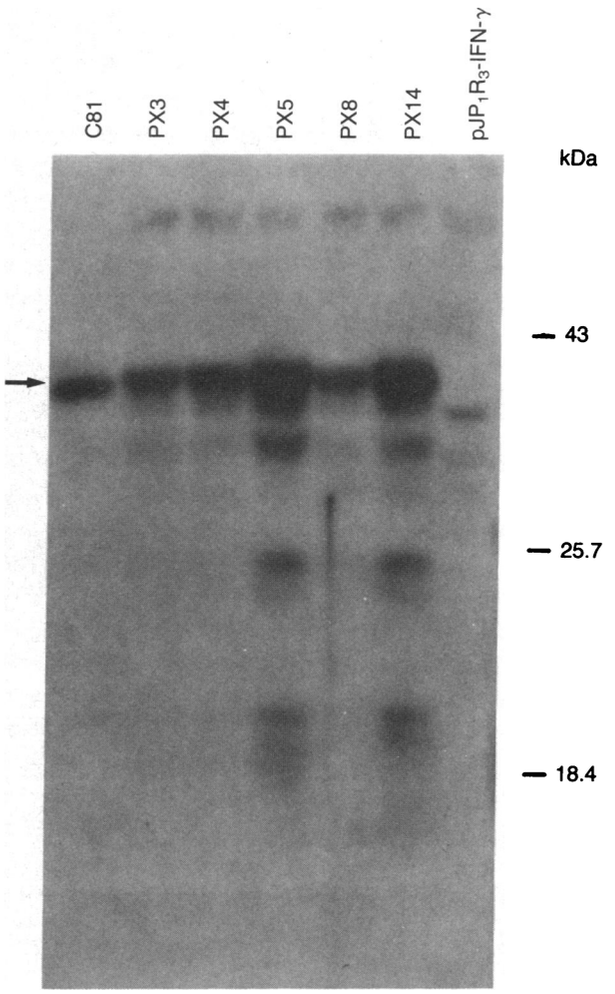

FIG. 2. Immunoblot analysis of the p40^x protein synthesized in *E. coli*. Total bacteria cell proteins were prepared by resuspending 1.5 ml of an overnight bacterial culture grown in the presence of tetracycline (3 μg/ml), in 200 μl of 1% NaDodSO$_4$, followed by brief sonification and boiling for 5 min. Cell lysates from the HTLV-I-transformed T-cell line C81-66-45 were similarly prepared. Proteins from ≈2 × 10^7 bacteria or 10^6 C81-66-45 cells were dissolved in Laemmli gel sample buffer and electrophoresed in a 10% NaDodSO$_4$/polyacrylamide gel (29). The proteins separated in the gel were transferred by electrophoresis onto a piece of nitrocellulose filter paper and subjected to immunoblot analysis as described (30) using an anti-p40^x serum (provided by Stephen Oroszlan). Lane C81 represents proteins from the C81-66-45 cell line (17), the positive control. Proteins from p40^x-producing *E. coli* strains PX3, PX4, PX5, PX8, and PX14 are designated. Lane pJR$_1$R$_3$-IFN-γ contains proteins from *E. coli* containing the pJP$_1$R$_3$-IFN-γ plasmid as the negative control. Arrow indicates the position of p40^x.

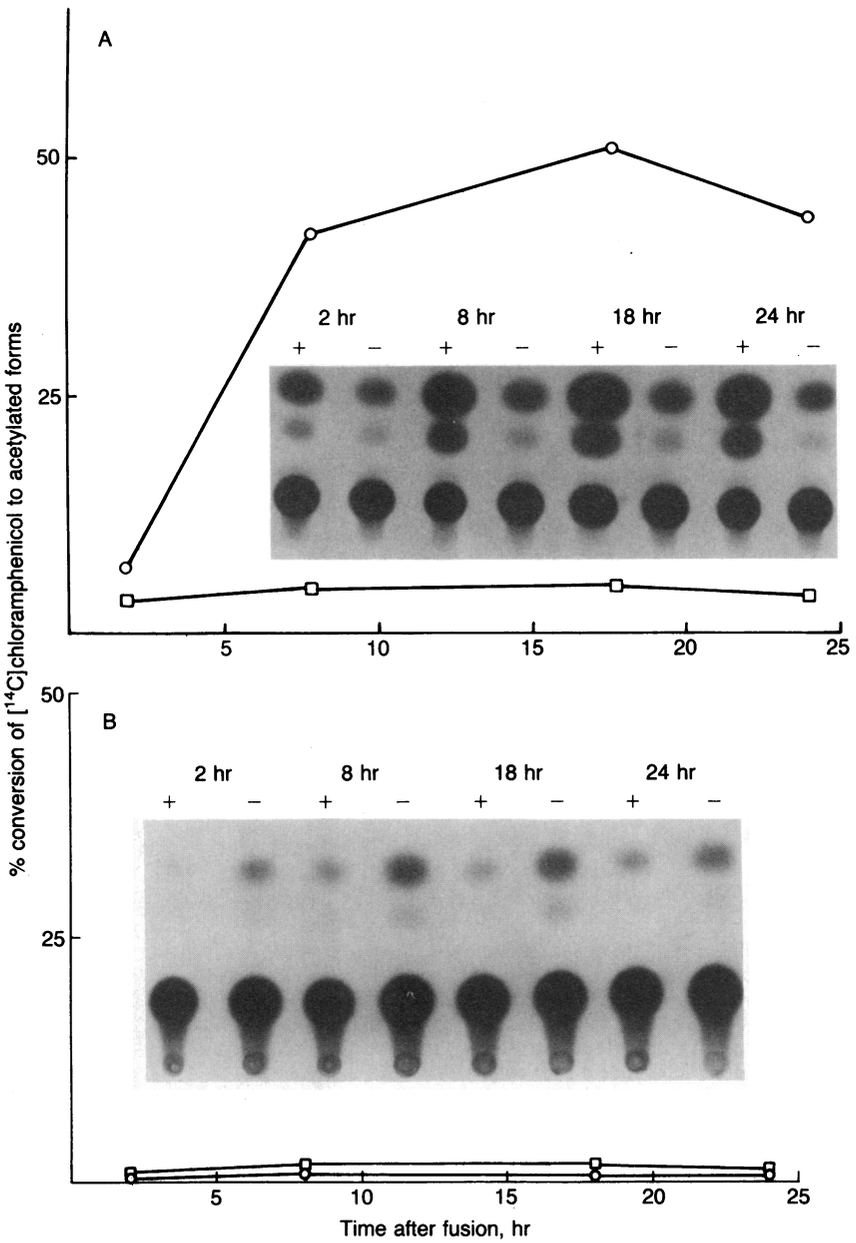

FIG. 3. Trans-activation by polyethylene glycol-mediated protoplast fusion. (*A*) LHC cells are established cell lines derived from cotransfection of the pU3RCAT DNA (2) and a plasmid carrying the neomycin-resistant gene into mouse L cells. One such cell line, LHC-5, was used for the experiment presented here. Protoplast fusion was carried out as described. The kinetics of trans-activation are shown in this figure. CAT assays were done as described, by incubating the reaction mixtures at 37°C for 30 min (26). Plus (+) and minus (−) signs indicate fusion with $p40^x$-producing *E. coli* (○) or *E. coli* containing the pJP_1R_3 plasmid (□). Results are expressed graphically as the percent conversion of [^{14}C]chloramphenicol at various times after fusion. (*B*) An experiment similar to that in *A* was performed by using a mouse NIH 3T3 cell line, SV-1, which contains an integrated copy of pSV2cat. Symbols are analogous to those in *A*.

51-base-pair chemically synthesized DNA duplex, with a *Hin*dIII end on the 5′ side and an *Acc* I end on the 3′ side. Ligation of the synthetic fragment (amino acids 1–17) and the genomic fragment (amino acids 18–353) provide a complete $p40^x$ coding sequence (Fig. 1).

Expression of this sequence was carried out by using a constitutive bacterial expression vector, pJP_1R_3 (27, 28). The salient features of this expression vector are noted: First, it contains a synthetic bacteriophage T5 promoter and a synthetic ribosome binding site located at a precise distance 5′ to the *Hin*dIII cloning site that is optimal for translation. Second, it has the pBR322 tetracycline-resistant gene (*Tc*^{r}) without its promoter positioned downstream of the *Hin*dIII cloning site. When the heterologous DNA fragment is inserted, a dicistronic mRNA spanning the insert and the *Tc*^{r} gene is transcribed. By selecting for the expression of the distal *Tc*^{r} gene, the transcription of the 5′ insert is assured.

In previous experiments, a pJP_1R_3 derivative carrying the human γ-interferon (HuIFN-γ) sequence was shown to accumulate HuIFN-γ at a level >20% of the total *E. coli* cell protein (27, 28). The $p40^x$ coding sequences were inserted into the pJP_1R_3-IFN-γ parental vector by introducing a 1.6-kb *Hin*dIII to *Bam*HI fragment that encodes the $p40^x$ product (see Fig. 1). This construction also removes a significant portion of the IFN-γ coding sequence. After ligation of the 1.6-kb fragment to the expression vector, DNA was transferred into a bacterial host, HB101. All bacterial clones that contained the $p40^x$ sequence showed resistance to tetracycline, indicating that the inserted $p40^x$ sequence was being expressed. When total protein extracts from several of these

clones were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, a protein band of ≈40 kDa was revealed by Coomassie blue staining (data not shown). That this 40-kDa protein is the authentic $p40^x$ is evidenced by an immunoblot analysis using an antiserum against $p40^x$ (Fig. 2). The *E. coli* 40-kDa protein reacted with the anti-$p40^x$ antiserum and showed the same electrophoretic mobility in a $NaDodSO_4$/polyacrylamide gel as the authentic $p40^x$ protein from an HTLV-I infected nonproducer cell line, C81-66-45. The minor protein bands that cross-reacted with the anti-$p40^x$ serum were probably degradation products of the overproduced $p40^x$ protein, since they are absent from the negative control, pJP_1R_3-IFN-γ, and their amounts were proportional to that of $p40^x$ seen in the same preparations. Since *E. coli* cells are not likely to contain activities that modify eukaryotic proteins, the similar electrophoretic behavior of the *E. coli*-derived $p40^x$ protein and the authentic $p40^x$ protein from HTLV-I-infected human T lymphocytes suggests that the eukaryotic $p40^x$ does not undergo extensive post-translational modification.

Bacterially Derived $p40^x$ Trans-Activates the HTLV-I LTR. To test whether $p40^x$ synthesized in *E. coli* possesses trans-activating activity, bacterial cells producing $p40^x$ were fused to mammalian cells by the PEG-mediated protoplast fusion technique (31, 32). The recipient mammalian cells used for fusion with $p40^x$-producing *E. coli* protoplasts were mouse L cells that harbored integrated copies of pU3RCAT, a plasmid that contains the HTLV-I LTR linked to the CAT gene (2). These cell lines were obtained by cotransfection of the pU3RCAT DNA with a plasmid carrying the neomycin-resistant gene using the calcium phosphate transfection technique (33). Four independent cell lines (LHC-1, 3, 5, 8) were isolated by selection with the antibiotic G-418 (400 μg/ml) (34, 35).

About 3×10^9 protoplasts, prepared from either the $p40^x$-producing *E. coli* strains ($p40^{x+}$) or an isogenic strain that carried the constitutive bacterial expression vector ($p40^{x-}$), were fused to 10^7 LHC cells. A marked increase in the level of CAT activity was observed with the LHC cells that were fused with the $p40^x$-producing bacteria (Fig. 3). Trans-activation was observed in all four LHC cell lines, although the levels varied from one cell line to another (data not shown). One such experiment is shown in Fig. 3*A*, in which LHC-5 cells were fused with protoplasts prepared from either the $p40^x$-producing *E. coli* strain PX5 or a pJP_1R_3-containing control strain that did not produce $p40^x$. The fusion was carried out in the presence of 48% PEG 1000 for 2 min. The LHC-5 cells were harvested 2, 8, 18, and 24 hr after fusion and the levels of CAT activity from the cell extracts were measured (26, 36). By comparing the CAT activities from several experiments, we established the level of trans-activation is 10- to 20-fold 8 hr after fusion. The increase in the level of CAT mRNA in cells fused with $p40^x$-producing *E. coli* also reached a maximum ≈8 hr after protoplast fusion, followed by a sharp decrease 16 hr and 24 hr after fusion, as analyzed by the quantitative S1 nuclease protection assay (data not presented). The increase in CAT mRNA in cells fused with $p40^x$-producing *E. coli* is consistent with a transcriptional stimulation of the LTR CAT by the $p40^x$ protein. The rapid decline in CAT mRNA may be due to the intrinsic instability of the CAT transcripts in eukaryotic cells (unpublished results).

Similar protoplast fusion experiments were also carried out using a mouse NIH 3T3 cell line, SV-1 (37), harboring a construct, pSV2cat, that contained the simian virus 40 early regulatory region instead of the HTLV-I LTR upstream of the bacterial *cat* gene (26). No trans-activation could be detected when SV-1 was fused with $p40^x$-producing bacteria in the presence of PEG 1000, suggesting that $p40^x$-mediated trans-activation is likely to be specific for HTLV-I LTR sequences (Fig. 3*B*). Trans-activation mediated by the *E. coli* $p40^x$ could also be detected by transiently transfecting mouse L cells with calcium phosphate-precipitated pU3RCAT DNA, followed 24 hr later by protoplast fusion as described above. The extent of trans-activation, however, was not as dramatic as with LHC cells, most likely because of the low efficiency of DNA uptake by mouse L cells (data not presented). These data suggest that the *E. coli* $p40^x$ possesses the ability to trans-activate an indicator gene under the control of the HTLV-I LTR. Although the prokaryotic and eukaryotic proteins have the same mobility on $NaDodSO_4$ gels, it is not yet clear whether this activity of the bacterial $p40^x$ requires protein modification after introduction into eukaryotic cells.

Since the trans-activation can be detected in mouse L cells, it appears that other human T-cell-specific transcription factors are not absolutely required for this activation. The kinetics of trans-activation by the *E. coli* $p40^x$ was found to be very rapid. The increase in CAT activity when LHC cells were fused with PX5 protoplasts could be detected within 2 hr and persisted up to 24 hr after fusion (Fig. 3*A*). While we cannot yet exclude the possibility that the bacterial $p40^x$ protein induces the synthesis of, or activates preexisting cellular transcriptional factors, the rapid kinetics suggest that trans-activation of the LTR sequence may be mediated by $p40^x$ directly.

The expression of a biologically active $p40^x$ product in *E. coli*, together with a rapid assay for trans-activation as described above, should readily allow purification of biologically active $p40^x$ in large quantity, the analysis of $p40^x$ mutants, and, hopefully, the eventual elucidation of the molecular mechanism of trans-activation.

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