THE p40x OF HUMAN T-CELL LEUKEMIA VIRUS TYPE I IS A TRANS-ACTING ACTIVATOR OF VIRAL GENE TRANSCRIPTION

Motoharu Seiki,*1 Jun-ichiro Inoue,*1 Tadayuki Takeda,*2 Atsuko Hikikoshi,*1 Masako Sato*1 and Mitsuaki Yoshida*1

*1Department of Viral Oncology, Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170 and *2Institute of Medical Sciences, The University of Tokyo, Shiroganedai, Minato-ku, Tokyo 108

Human T-cell leukemia virus type I has a unique sequence pX and the product p40x was proposed to be a specific trans-acting transcriptional activator of expression of the viral gene. Recently, a second pX protein p27x-I is in addition to p40x was identified; these two proteins are encoded by overlapping frames III and IV (x-lor). For determination of which product is the trans-acting activator, site-directed mutations were introduced into the pX sequence which was placed under the metallothionein promoter. On cotransfection with pLTR-CAT (a plasmid containing the LTR of HTLV-I and chloramphenicol acetyltransferase gene), only the mutations that affected p40x expression inactivated the transcriptional activation from the LTR.

Key words: HTLV-I — pX gene products — x product — Transcriptional activator — Point mutation

Human T-cell leukemia virus type I (HTLV-I) is an etiological agent of adult T-cell leukemia (ATL) and is directly involved in leukemogenesis. No typical oncogene was found in the HTLV-I genome, and there is no common site for integration of the provirus into leukemic cell DNA in ATL patients. This nonspecific integration could not be explained by a cis-acting function of the integrated provirus activating adjacent cellular genes. Thus, trans-acting viral function was postulated to be involved in the leukemogenesis.

HTLV-I has a unique sequence pX between the env gene and the 3′ LTR, and this pX sequence was found to contain four open reading frames, I–IV, overlapping each other. One of these, open reading frame IV (x-lor), was shown to be expressed by double splicing and to code for a 40 kD protein, p40x. Furthermore, from the finding that trans-activation of the LTR was detected in cells expressing only p40x as a viral protein, this product was proposed to have a trans-acting function of activating transcription from the LTR. The involvement of a viral gene product in the trans-activation was directly shown in cells transfected with plasmids which can express the pX sequences.

Using antibodies against synthetic peptide, however, we recently identified two other pX proteins, phosphoproteins pp27x-III and pp21x-III, encoded by frame III in the pX sequence. Frame III mostly overlaps frame IV for coding for p40x, and these phosphoproteins were detected in all HTLV-I-infected cell lines tested that were previously reported to express p40x. Therefore, the previous experiments do not provide a sufficient basis to conclude which pX product is responsible for trans-activation of the LTR: deletion mutations in the previous experiments could affect both frames, and expression of the genomic fragments of the pX region could splice alternatively, thus expressing different frames. To test the effect of these two proteins, we constructed a plasmid containing the genomic sequences of the env, pX and LTR under the metallothionein promoter and isolated various mutants induced by site-directed mutagenesis using synthetic oligonucleotides.

As reported previously, the cDNA clone of the pX mRNA contains three exons: the
first 118 bp from the LTR, the second 191 bp from the 3' end of pol, and the third from frame IV (x-lor) in the pX sequence. The pX cDNA clone was inserted under the metallothionein promoter and the third exon downstream of the NcoI site in the cDNA sequence was replaced with a provirus DNA fragment containing the env, pX and 3' LTR, thus constructing a plasmid pMTPX (Fig. 1). For site-directed mutagenesis, the pMTPX was cut at the XhoI site, and about 2000 nucleotides were removed from the 3' end of one strand by means of the exonuclease activity of T4 DNA polymerase. A synthetic oligonucleotide containing 11 nucleotides with a single base substitution corresponding to the given position (see Table I) was hybridized to the single-stranded region in the DNA fragment and the single-stranded gaps were repaired with the Klenow fragment of DNA polymerase I. A small DNA fragment containing the mutation was cut out by HpaI and MluI digestions and substituted for the wild type sequence in pMTPX at the same enzyme sites. Using a 32P-labeled oligonucleotide with the same composition as that used for mutagenesis, the mutant was selected and the mutation was confirmed by nucleotide sequencing. The trans-acting activation of the LTR with these mutants was assayed by cotransfection into HOS cells with pLTR-CAT, which has the LTR of HTLV-I and the chloramphenicol acetyl transferase (CATase) gene, followed by assay of CATase activity as described previously.10)

The plasmid pMTPX, which contains the first and second exons of the pX cDNA and the proviral env and pX sequences (Fig. 1), augmented expression of the CAT gene by pLTR-CAT in cotransfection assay. The activity was dependent on the dose of pMTPX, indicating that the viral information is responsible for the trans-activation of the LTR. This trans-acting effect was specific

---

Fig. 1. Construction of the biologically active pX clone pMTPX which can express four possible open reading frames. The third exon of the pXcDNA was substituted with the provirus sequences, which had a deletion (5777–6495) in the env gene, at the NcoI site (5178b) and the recombinant fragment was placed under the metallothionein (MT) promoter. Thus, the constructed clone pMTPX contains the metallothionein promoter, the first and second exons of the cDNA, the 3' half of the env, pX, and 3' LTR. Mutations were introduced into positions 7464b or 7474b, or 5179b from the 5' end of the provirus genome. The solid bars I–IV are open reading frames in the pX sequence. Nco, NcoI; Xho, XhoI; Hpa, HpaI; Cla, ClaI; Mlu, MluI; ATG, initiation codon for env and frame IV. Open arrows with Mu1, Mu3, I1er, III1er, and IV1er indicate mutation sites.

---

Table I. Characteristics of the pX Mutants

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation</th>
<th>Alteration</th>
<th>Position[a]</th>
<th>Amino acid change in frame</th>
<th>Trans-acting activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMTPX</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>pMTPX/Mu1</td>
<td>CATG deletion</td>
<td>5179–5182</td>
<td>(?)</td>
<td>(?)) (?) Met</td>
<td>+</td>
</tr>
<tr>
<td>pMTPX/Mu3</td>
<td>CG deletion</td>
<td>7474–7475</td>
<td>shift</td>
<td>shift</td>
<td>-</td>
</tr>
<tr>
<td>pMTPX/II1er</td>
<td>C→T</td>
<td>7474</td>
<td>Arg→TGA</td>
<td>Ser→Leu</td>
<td>None</td>
</tr>
<tr>
<td>pMTPX/III1er</td>
<td>C→A</td>
<td>7474</td>
<td>None</td>
<td>Ser→TGA</td>
<td>None</td>
</tr>
<tr>
<td>pMTPX/IV1er</td>
<td>G→A</td>
<td>7464</td>
<td>None</td>
<td>Gly→Arg</td>
<td>Trp→TAG</td>
</tr>
</tbody>
</table>

[a] The positions are from the 5' end of the whole provirus genome.

1128 Jpn. J. Cancer Res. (Gann)
to the LTR sequence, because CATase expression of pSV-CAT was not affected by cotransfection with pMTPX.

The pX sequence codes for the two pX proteins, p40x and pp27x-III through reading frames III and IV, which mostly overlap each other, and another frame II also overlaps in this region (Fig. 1). To find a site which affects the activity, a two-base deletion was introduced at the ClaI site constructing pMTPX/Mu2, which induced frame shift in all three frames. The mutant pMTPX/Mu2 was found to be inactive, indicating that this region is important for trans-activation function. To define which frame is responsible for this activity, we introduced mutations into this overlapping region in such a way that each mutation generated a termination codon in each frame, but did not induce drastic alteration of the amino acids of other frames.

The characteristics of the mutants are summarized in Table I. The two mutants pMTPX/IIter and pMTPX/IIIter, which have termination codons in frames II and III, respectively, retained the original trans-acting activity, indicating that neither frame II nor III is responsible for the activity of pMTPX (Fig. 2). On the other hand, the mutant pMTPX/IVter, containing a termination codon in frame IV, was completely inactive (Fig. 2). The mutation in pMTPX/IVter also induced amino acid alteration in frame III. This alteration of amino acid is not significant, since a contribution of frame III is excluded by mutant pMTPX/IIIter. These results indicated that the p40x coded by frame IV is responsible for the activity shown by pMTPX. This conclusion is consistent with the inactivity of the mutant pMTPX/Mul, which has a deletion of the initiation codon ATG for p40x expression.

All these results directly indicate that p40x is required and is sufficient for full trans-activation shown by the plasmid pMTPX. This conclusion was previously reached by other groups based on studies on the expression of the pX sequence of HTLV-I\(^1\) and HTLV-II.\(^1\) However, none of their expression or deletion experiments could identify the product responsible for the trans-activation of the LTR, because at least two products are coded by overlapping frames in the region used for the previous experiments. Thus, deletion can affect both frames, or genomic fragments can splice alternatively, expressing frames II or III. Only the report by Sodroski et al.\(^1\) directly identified the x-lor gene of HTLV-II as the responsible element based on its unambiguous expression. The present results provide the first unambiguous demonstration of the importance of p40x in HTLV-I.

From these mutagenic studies, however, we could not identify the function of the pp27x-III or pp21x-III. The phosphoprotein pp27x-III was detected almost exclusively in the nucleus,\(^1\) like p40x.\(^2\) Thus, this protein probably also has some role in viral gene expression. We detected transient expression of the p40x in these cotransfected cells by blotting analysis (data not shown). However, we could not detect pp27x-III or pp21x-III in these cells. Possibly the level of
expression of pp27\textsubscript{x-III} or pp21\textsubscript{x-III} was undetectably low, or insufficient information was available in pMTPX for expression of pp27\textsubscript{x-III}; the mechanism of its expression is not yet clear. Therefore, it is still possible that pp27\textsubscript{x-III} and/or pp21\textsubscript{x-III} is a cofactor for the expression by p40\textsubscript{x} of maximum trans-activation activity. However, our results clearly show that at least p40\textsubscript{x} is involved in trans-activation of the LTR function.

This work was supported in part by a Grant-in-Aid for Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science and Culture of Japan, and also in part by a grant for Cancer Research from the Princess Takamatsu Cancer Research Fund.

(Received Oct. 11, 1985/Accepted Nov. 11, 1985)

**REFERENCES**


17) Sodroski, J., Rosen, C., Goh, W. C. and Haseltine, W. A transcriptional activator


