Expression of a Provirus of Human T Cell Leukaemia Virus Type I by DNA Transfection

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SUMMARY

We isolated the full length provirus of human T cell leukaemia virus type I (HTLV-I) from MT-2, a lymphoid cell line producing HTLV-I. In three non-lymphoid cell lines (COS7, human osteosarcoma HOS cells, and HeLa) this provirus expressed a trans-acting activity after co-transfection with a recombinant plasmid carrying a bacterial chloramphenicol acetyltransferase gene under the control of a long terminal repeat of HTLV-I provirus. The trans-acting protein p40 was detected by immunoprecipitation in transfected HOS cells. Structural proteins of HTLV-I, the gag and env products, were also formed and processed in the same manner as observed in MT-2 cells. In transfected HeLa cells, the p40 protein was mainly localized in the nucleus, while other structural proteins were detected in the cytoplasm and/or the membrane by indirect immunofluorescence. Syncytium formation was observed in HeLa cells after transfection. These results demonstrated that non-lymphoid cells could produce the major proteins of HTLV-I after DNA transfection of the cloned provirus.

INTRODUCTION

Human T cell leukaemia virus type I (HTLV-I) is an exogenous virus associated with an endemic human T cell malignancy termed adult T cell leukaemia (ATL) (Poiesz et al., 1980; Hinuma et al., 1981; Yoshida et al., 1982; Kalyanaraman et al., 1982; Popovic et al., 1983). Experimentally, human T cells can be productively infected, transformed and immortalized by co-cultivation with HTLV-I-producing cells derived from ATL patients (Miyoshi et al., 1981; Yamamoto et al., 1982). HTLV-I can infect human cells other than T lymphocytes, such as osteosarcoma cells, B lymphocytes and endothelial cells (Clapham et al., 1983; Okada et al., 1984; Ho et al., 1984; Hoxie et al., 1984) but there is no evidence for transformation of these cells. Although the mechanism causing transformation of T cells by HTLV-I remains unknown, HTLV-I is unique in other respects. It has the unique pX sequence located between the env gene and the 3' long terminal repeat (LTR) (Seiki et al., 1983). One of the pX open reading frames, termed tat, encodes a 40000 mol. wt. protein (p40) which is responsible for transcriptional trans-activation from the LTR (Sodroski et al., 1984, 1985; Kiyokawa et al., 1984; Slamon et al., 1984, 1985; Miwa et al., 1984; Lee et al., 1984; Hatanaka & Kobayashi, 1985; Felber et al., 1985). The trans-acting activity of p40 has been suggested to be related to HTLV-I leukaemogenesis, possibly by inducing the expression of some cellular genes in the infected cells (Sugamura et al., 1984; Sodroski et al., 1985; Chen et al., 1985; Hatanaka & Kobayashi, 1985).

In the present study we isolated a full-length provirus of HTLV-I and examined its expression in cell lines by DNA transfection.
(DMEM) containing 10% FCS. HOS cells (Clapham et al., 1983) were maintained in Eagle's medium (MEM) containing 20% FCS.

**Transfections.** The calcium phosphate precipitation method (Wigler et al., 1979) was used; generally, recipient cells were seeded into 100 mm culture dishes 24 h prior to transfection at a density of 3 × 10^5 cells per dish. A total of 20 μg DNA was used for each transfection.

**Chloramphenicol acetyltransferase (CAT) assay.** At 48 h after transfection, cells were harvested, washed twice with phosphate-buffered saline (PBS), and then suspended in 100 μl of 250 mM-Tris-HCl pH 8.0. Cell extracts were prepared by three cycles of freezing (~70 °C) and thawing (37 °C) followed by centrifugation to remove cellular debris. CAT activities were assayed according to Gorman et al. (1982). To allow comparison of CAT activities between several transfections, the quantity of DNA transfected was standardized at 10 μg of pSV2CAT or pHLC1, or 2 to 10 μg HTLV-I provirus and 10 μg of pHLC1.

**Antisera.** Sera from Japanese ATL patients which reacted with HTLV-I env and gag products (gp61, gp46 and gp21, and p53, p24 and p19) were used (Koyanagi et al., 1984; Hattori et al., 1984). A monoclonal antibody, M73, was used which reacted with p40 and gp68, the latter being a product of a defective type provirus in MT-2 cells (Takeuchi et al., 1985). A second monoclonal antibody, GIN-7, reacted with p19 and p28, the latter being a product of another defective type provirus in MT-2 cells (Kobayashi et al., 1984b; Iino et al., 1986), and also with p53 (Tanaka et al., 1983).

**Labelling cells with [3H]leucine and immunoprecipitation.** At 48 h after transfection, 10^6 to 2 × 10^6 cells were incubated in leucine-free RPMI 1640 medium containing 10% FCS and 1 mCi [3H]leucine (58 Ci/mmol, ICN Radiochemicals) for 8 h. Cell extracts were prepared by lysis in low salt extraction buffer [1 × 10^7 cells/ml; 10 mM-Tris-HCl pH 8-0, 0.14 M-NaC1, 3 mM-MgCl2, 0.5% (w/v) NP40 (Nakarai Chemicals, Kyoto, Japan), 1 mM-dithiothreitol, 2 mM-PMSF] for 1 h at 4 °C. Cell lysates were clarified by centrifugation, then incubated overnight at 4 °C with the appropriate antiserum. Immune complexes were recovered by using Affi-Gel–Protein A (Bio-Rad) and analysed on 12% polyacrylamide gels containing 0.1% SDS as described previously (Iino et al., 1986).

**Indirect immunofluorescence assays.** At 48 h after transfection, cells were fixed and then stained as follows. p40: cells were fixed in 3%-7% formaldehyde, made permeable with 0.1% NP40 in PBS, treated with MI73 and then with FITC-conjugated anti-mouse IgG goat serum (Felber et al., 1985). gag products and ATLA (ATL-associated antigens, mainly env and gag products; Kobayashi et al., 1984b): cells were fixed in acetone and then treated with GIN-7 or an ATL patient's serum.

**RESULTS**

**Isolation of a full length HTLV-I provirus DNA**

We reported previously that MT-2 cells contain various HTLV-I proviruses, including defective and non-defective types (Kobayashi et al., 1984a). We have cloned a non-defective provirus using a pol fragment as a probe (Kobayashi et al., 1984a). First, a genomic library was constructed from SacI-digested MT-2 genomic DNA using the λgtWES3.B vector. One isolated clone, λKM4-3, contained a full size HTLV-I provirus (Fig. 1a). We then reconstructed the provirus by adding LTR DNA fragments, which had been digested with EcoRI and SacI, to the cloned insert in the following order: U3R, insert DNA (gag-pol-env-tat), RU5. The reconstructed provirus DNA was re-ligated to the λgtWES3.B vector at the EcoRI site (λHTLV1C) as shown in Fig. 1(b). For the DNA transfection experiments, the DNA of this recombinant phage was digested with EcoRI and its insert DNA was isolated by agarose gel electrophoresis.

**Trans-acting activity in cells transfected with HTLV-I provirus DNA**

We examined the expression of the trans-acting activity of the cloned HTLV-I provirus by DNA transfection. Two recombinant plasmids (Fig. 1c) carrying the CAT gene were used to measure the CAT activity in the transfected cells. The CAT gene is regulated by the simian virus 40 (SV40) early promoter and transcription termination regions in pSV2CAT, and by the promoter region of the HTLV-I LTR in pHLC1. The latter plasmid was constructed by substituting the LTR for the SV40 early promoter region (Fig. 1c). The CAT activity in cells transfected by pSV2CAT or pHLC1 is expressed at a basal level depending on the promoter. These activities were used as references for calculating the trans-acting activity of the HTLV-I provirus introduced into cells (Table 1), by normalizing against the CAT activity of pSV2CAT or pHLC1 (Table 1). CAT activity was demonstrable in non-lymphoid cells (COS7, HOS and
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(a) Clone 2KM4-3, with the HTLV-I genome missing portions of LTR at both ends, and the pol probe used for isolating 2KM4-3 are shown. (b) Reconstruction of the recombinant phage AHTLV1C containing the complete HTLV-I provirus from 2KM4-3 and pMT-2-15E containing only the LTR sequence of HTLV-I isolated from the MT-2 genome (H. Siomi et al., unpublished). Dashed lines indicate λgtWES2B vector. (c) Plasmid pSV2CAT contains a bacterial CAT gene under transcriptional control of the SV40 early promoter (Gorman et al., 1982). In pHLC1, the HTLV-I provirus LTR which is truncated at +328 (cap +1) is substituted for the SV40 early promoter and enhancer region (H. Siomi et al., unpublished). Arrows indicate the orientation of transcription and the site of transcription initiation. The restriction sites for AccI, SacI, EcoRI, and HindIII are shown as A, S, E and H respectively. Amp', ampicillin resistance.

Table 1. *Enhancement of CAT activity in cells transfected with CAT plasmid and HTLV-I provirus*

<table>
<thead>
<tr>
<th>Cells</th>
<th>pSV2CAT</th>
<th>pHLC1</th>
<th>pHLC1 + HTLV-I</th>
<th>Ratio of enhancement of CAT activity†</th>
<th>pHLC1 + HTLV-I</th>
<th>pHLC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS7</td>
<td>6:12</td>
<td>1:42</td>
<td>36:2</td>
<td>5:9</td>
<td>25:5</td>
<td></td>
</tr>
<tr>
<td>HOS</td>
<td>0:38</td>
<td>0:96</td>
<td>55:5</td>
<td>145:3</td>
<td>57:5</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>0:19</td>
<td>0:41</td>
<td>21:3</td>
<td>112:1</td>
<td>520</td>
<td></td>
</tr>
</tbody>
</table>

* The values indicate the percentage conversion of chloramphenicol to its products in 10 min. Results represent the average of at least two independent transfections.

† The values indicate the ratio of the CAT activity of cells transfected with pHLC1 and HTLV-I provirus to that of cells transfected with pSV2CAT or pHLC1.

HeLa) transfected by the calcium phosphate precipitation method (Fig. 2, Table 1). While pSV2CAT expressed a high CAT activity in COS7 cells, the co-transfection of HTLV-I provirus and pHLC1 enhanced the activity in all the cell lines examined (Table 1). The trans-acting activity in HTLV-I-transfected non-lymphoid cells was comparable to levels in the HTLV-I-producing human T lymphoid cell line, MT-2 (data not shown).
Fig. 2. CAT activity in cells. (a) Transfection of COS7 cells. The following DNAs were transfected: 1, pHLCl 10 μg; 2, pSV2CAT 4 μg; 3, pHLCl 10 μg and HTLV-I (provirus) 2 μg. CAT assays were performed 48 h after transfection. Two incubation times, 10 min (left) and 60 min (right), were used in each assay. The unreacted substrate (Cm) and acetylated reaction product (Ac-Cm) are shown. (b) Transfection of HOS cells. DNAs: 1, pHLCl 4 μg; 2, pSV2CAT 5 μg; 3, pHLCl 4 μg and HTLV-I 1 μg. (c) Transfection of HeLa cells. DNAs: 1, pHLCl 10 μg; 2, pSV2CAT 10 μg; 3, pHLCl 10 μg and HTLV-I 0.2 μg; 4, pHLCl 10 μg and HTLV-I 5 μg. (d) Plots of data in (c). Symbols correspond to numbers in (c): 1, O; 2, △; 3, ●; 4, ▲.

Immunoprecipitation analysis of HOS cells transfected with HTLV-I provirus DNA

Next, we tried to identify the trans-acting protein p40 in non-lymphoid cells transfected with HTLV-I provirus DNA. The transiently expressed gene products of HTLV-I in HOS cells were analysed by immunoprecipitation using various antibodies; immune complexes were analysed by SDS–PAGE. A band corresponding to p40 was detected in HOS cells transfected with the provirus and in MT-2 cells, but not in HOS cells transfected with pBR322 (Fig. 3a). Therefore, these non-lymphoid cells produce p40.

Other gene products of HTLV-I were also analysed. The gag precursor protein p53 and the processed gag protein p19 were identified by an anti-p19 monoclonal antibody, though the ratios of p53 to p19 were different in transfected HOS cells and MT-2 cells (Fig. 3b). Another gag protein, p24, was detected using an ATL patient's serum (Fig. 3c). The env precursor protein gp61 and its processed proteins gp46 and gp21 were also identified (Fig. 3c). Thus structural
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Fig. 3. Immunoprecipitation of HOS cells transfected with HTLV-I provirus DNA. 1 x 10⁶ HOS cells transfected with 5 µg HTLV-I provirus DNA were labelled with 1 mCi [³H]leucine for 8 h. Cell extracts were precipitated with (a) anti-p40 monoclonal antibody, (b) anti-p19 monoclonal antibody and (c) an ATL patient's serum. The immunoprecipitates of transfected cells were analysed by SDS–PAGE and are shown in lanes 2 and 2'. As a control pBR322 was introduced instead of HTLV-I provirus DNA (lane 3). An MT-2 cell extract (0-2 mCi [³H]leucine, 7 h labelling, 2 x 10⁶ cells) was analysed and is shown as a standard in lanes 1 and 1'. Periods of fluorography were (a, b) lane 1, 2 days; lanes 2 and 3, 12 days; (c) lanes 1, 2' and 3, 6 days; lane 2, 12 days; lane 1', 2 days.

Microscopical and immunofluorescence observations on expression of HTLV-I provirus DNA in HeLa cells

We detected viral antigens in HeLa cells transfected with the HTLV-I provirus DNA by indirect immunofluorescence. The p40 was identified in the nuclear region as previously reported (Slamon et al., 1985; Felber et al., 1985) (Fig. 4a). Less than 1% of the transfected HeLa cells were stained, whereas all MT-2 cells were stained (data not shown). The gag products were visualized using a monoclonal antibody (Fig. 4b). Transfected HeLa cells were also stained with an ATL patient's serum that recognized mainly env and gag gene products (Fig. 4c). No stained cells were detected in mock-transfected cells or in HTLV-I-transfected cells treated with myeloma culture fluid as controls (data not shown). Furthermore, we observed syncytia containing more than 10 nuclei 7 days after transfection of HeLa cells (Fig. 4d). Addition of ATL patient serum to the transfected cells strongly inhibited syncytium formation (data not shown).

DISCUSSION

An HTLV-I provirus has been isolated from the leukaemic cells of an ATL patient and its sequence has been determined (Seiki et al., 1983). However, there has been no report of a complete provirus capable of producing its own viral products or virus particles. Therefore, we isolated the full length provirus from the HTLV-I-producing cell line MT-2 and investigated its ability to form gene products in non-lymphoid cells. The results showed no significant difference in the production of tat, gag and env gene products between non-lymphoid cell lines and MT-2 cells. In addition, the trans-acting activity in non-lymphoid cells was significantly high.
Processing of \textit{gag} protein occurred in HOS cells, suggesting the expression of a protease gene like other retroviruses. In HeLa cells, we observed syncytium formation. Since this is known to be caused by the \textit{env} gene product (Nagy \textit{et al.}, 1983; Hoshino \textit{et al.}, 1983; Hayami \textit{et al.}, 1984), the result implies the transmission of the virus or the interaction of \textit{env} gene products with neighbouring cells.

\textit{T} lymphocyte-specific transformation by HTLV-I is an important phenomenon for understanding the relationship between HTLV-I and the T cell malignancy, ATL. There are several reports concerning the tissue specificity of HTLV-I infection or expression. First, no apparent tropism of HTLV-I for the cell surface receptors on T lymphocytes was observed using pseudotypes (Weiss \textit{et al.}, 1985). Next, no tissue specificity of the enhancer element of the HTLV-I LTR was reported (Rosen \textit{et al.}, 1986). Third, HTLV-I is known to form the trans-acting protein p40 responsible for its replication in HTLV-I-producing T lymphocytes (Chen \textit{et al.}, 1985). We have demonstrated that HTLV-I can express the \textit{tat} gene in non-lymphoid cell lines following DNA transfection; the \textit{gag} and \textit{env} genes were also expressed simultaneously in these cell lines. Our results suggest that the expression of HTLV-I in non-lymphoid cells may occur in the same manner as in naturally transformed T lymphocytes. Although no difference was apparent between T lymphocytes and other cells in the expression of HTLV-I, there might be a specific function(s) of HTLV-I in T lymphocytes, for example the induction of a cellular...


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