Development of cell lines stably expressing human immunodeficiency virus type 1 proteins for studies in encapsidation and gene transfer

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Experiments were done to test cell lines for their capacity to express human immunodeficiency virus type 1 (HIV-1) proteins in a stable manner. Marked differences were seen in the ability to stably express and export viral Gag and Pol proteins. Two cell lines, one suspension (MDS) and one monolayer (SW480), were established which exported these proteins at high level. Two other cell lines, HeLa and THP-1, showed poorer expression and very limited particle release. Single cell cloning was used to select the optimal producing clones from the lines. These produced large quantities of viral core particles pelletable from the supernatants. Cell lines were constructed from these clones which stably expressed in addition either the HIV-1 Envelope or a packageable HIV-based vector. The vector was shown to be packaged within the viral core particles. Transient transfection of envelope expressing constructs into a gag–pol plus vector cell line, or the vector into a gag–pol plus envelope expressing cell line resulted in gene transfer to CD4+ target cells. These cell lines provide useful tools with which to study the assembly and export of viral proteins and RNA, for assay of alternative envelope proteins to pseudotype HIV cores, for assessment of antiviral drugs and as a source of correctly processed proteins for immunological studies.

Introduction

Cell lines stably expressing human immunodeficiency virus type 1 (HIV-1) proteins are desirable for a number of purposes. They have the potential for use in analysis of virus–cell interactions and Gag–Envelope interactions as well as in studies of alternative Envelope usage in the context of a lentiviral core. If retroviruses such as HIV are to be used for gene transfer and gene therapy, stable viral packaging lines from which reproducibly high levels of viral particles can be released will be of considerable value. The stable expression of HIV proteins has many potential difficulties. Cell lines which produce a high level of HIV particles commonly express CD4, the major viral ligand, and cell fusion results when the HIV Envelope is expressed. In addition, viral proteins such as Vpr have been shown to cause cell cycle arrest (Rogel et al., 1995) and might be toxic to a stable packaging cell line. Other groups have found that viral proteins including Protease can be toxic to cells leading to the emergence of cell lines in which mutations have arisen in the toxic gene (Kräusslich, 1992; Kräusslich et al., 1993) and which are then functionally useless for the studies mentioned above. Two previous packaging cell lines based on HIV-1 have been described (Carroll et al., 1994; Yu et al., 1996) but the majority of lentiviral based vector work has been based on transient cotransfection assays (Poznansky et al., 1991; Parolin et al., 1994; Richardson et al., 1993, 1995) for the reasons outlined.

We tested a number of cell lines for their ability to express HIV proteins. We expressed the proteins on separate constructs such that an expressor of all except the envelope gene was initially transduced into the cells and clones expressing the highest level of correctly processed Gag and Pol were selected. These gag–pol expressing cells were then cloned and used as the basis for expression of a second viral construct, either a packageable vector or a viral envelope expressor. A single subsequent transient transfection with the complementary construct produced vector particles infectious for CD4+ cells.

Methods

Cell lines. Two monolayer cell lines with epithelial morphology were assessed; HeLa cells, a human cervical carcinoma, and SW480, a human adenocarcinoma derived cell line (Leibovitz et al., 1976). The latter
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(A) pΔP2GPH (gag-pol expressor)

(B) SVIIIexE7Gpt (env expressor)

(C) Vectors

HVP

LRPLAH5

Fig. 1. (A) The gag-pol expression construct pΔP2GPH was derived from an infectious proviral clone of the HTLV-III isolate HXBc2. It contains a 34 bp deletion (751–785) encompassing the packaging signal «Ψ» and has the 3' LTR replaced with a hygromycin resistance gene under the control of a CMV immediate early promoter and poly(A) signal. A deletion within env renders the protein non-functional. (B) The env expression construct pSVIIIexE7gpt contains the HIV-1 env gene under a truncated LTR promoter and the xanthine–guanine phosphoribosyltransferase gene of E. coli under the control of an SV40 promoter. (C) The previously described vectors (Richardson et al., 1993) were constructed from HXBc2 by removing pol sequences between nucleotide positions 2689 and 5743 and also contain the same env deletion as pΔAP2GPH. The vector LRPLAH5 is a derivative of pHVP with deletions of gag sequences (Coi 830 to BglII 7621). The shaded and hatched boxes indicate the tat and rev genes, respectively. The Rev responsive element (RRE) is underlined.

Plasmids. The plasmids used in these experiments are shown in Fig. 1. The gag-pol expression construct pΔP2GPH was derived from an infectious proviral clone of the HTLV-IIIIB isolate HXBc2. A deletion in the envelope gene (BglII 7041–7621) leads to production of a non-processed truncated Env protein. A 34 bp deletion in the 5' leader sequence renders the RNA packaging defective (Kaye et al., 1995). In addition, the 3' long terminal repeat (LTR) from nt 8740 was replaced by a hygromycin resistance gene under the control of the CMV immediate early promoter and a polyadenylation signal (poly(A)).

The envelope expression construct was pSVIIIexE7Gpt derived from an envelope expressing plasmid (Kowalski et al., 1987) (a kind gift of J. Sodroski). This is under the control of a minimal HIV-1 5' LTR (−167 to +80 with respect to the RNA start site) and polyadenylation signal but contains a gpt selectable marker under the control of an SV40 promoter. The vectors HVP (Richardson et al., 1993) and LRPLAH5 (Kaye et al., 1995) have been described previously. Each contains HIV-1 packaging signals, 5' and 3' long terminal repeats and a puromycin gene as a selectable marker.

The gpt expressor was driven by a CMV immediate early promoter and was a kind gift of Eric Cohen.

Selection and expression of viral proteins. Cells were stably transfected (suspension cells by electroporation, monolayers by a calcium phosphate coprecipitation) (Ausubel et al., 1991) with the gag-pol expressing plasmid and selected with hygromycin B (Sigma) (Ausubel et al., 1991). Cells were cloned by limiting dilution in 96-well plates and the clones screened by reverse transcriptase (RT) assay and Western blot analysis. High expressing clones were selected out and transfected with either the envelope expressing construct or a vector (LRPLAH5) under GPT or puromycin (Ausubel et al., 1991) selection respectively. Expression of gag-pol gene products was confirmed by Western blot analysis using a monoclonal antibody directed against the Gag proteins and detection of the Envelope by use of a monoclonal antibody against gp120 (reagents supplied by the MRC AIDS Reagent Project). Viral particles were purified through sucrose cushions as previously described (Kaye & Lever, 1995) prior to immunoblotting.

RT assay. This was based on the Potts’ microtitre plate RT assay (Potts, 1990) performed with appropriate controls. To compare protein export in different assays at different time points the RT activity was compared to commercial HIV cloned RT enzyme and the results expressed as the proportion of the counts obtained with the commercial enzyme.

Detection of RNA encapsidation. Particles derived from the MDS and SW480 stable expressing cell lines were pelleted and probed for gag-pol and vector RNA using an RNase protection assay as previously described (Kaye et al., 1995). The probe encompassed a region at the 5' end of HIV including the leader sequence, and produced protected species from which it was possible to differentiate between the gag-pol message and the vector which also identified a band corresponding to the 3' LTR.

Vector transfer experiments. The gag-pol plus vector cell lines were transiently transfected with the envelope expressing construct by electroporation or the DEAE-dextran method (Ausubel et al., 1991); 48 h later, supernatants were removed from the cells, filtered (0.45 μm) in order to remove cellular debris and transferred on to HeLa T4 cells. Puromycin selection was commenced 24 h later. After 4 weeks a viable colony count was performed and the cells stained for accurate quantification. Under similar conditions, the gag-pol plus envelope cells were transfected with LRPLAH5 and the supernatant transferred on to HeLa T4 cells and selection undertaken as above. Control transfers were undertaken using the parent cell lines expressing gag-pol alone. Vector transfer was confirmed by antibiotic resistance of the transduced colonies and confirmed by Southern blotting (Ausubel et al., 1991).

PMA stimulation of THP-1 cells. THP-1 cells stably expressing gag-pol were treated with PMA as previously described (Tsuchiya et al., 1980). Western blot and RT assays were performed on the cells and supernatants to observe changes in protein expression.

Results

RT assays and Western blot analyses were performed on cells transfected with the gag-pol expression plasmid (shown in Fig. 1). Assays were performed on both the uncloned lines and from clones derived from the transfected cells. Western blots...
HIV protein expressing cell lines

Fig. 2. Stable HIV-1 gag–pol expression in five different uncloned cell lines and single cell clones. Cells and virion-containing supernatants of stable hygromycin resistant cell populations were harvested and analysed by Western blotting. Uncloned Jurkat-tat cells and uncloned as well as cloned HeLa cells stably expressing the L∆P2GPH construct (A). Stable THP-1 gag–pol expressing cells and single cell clones (B), stable SW480 gag–pol expressors (C) and stable MDS gag–pol expressors (D). The names of the clones (B3 to D9 for HeLa, G3 to G8 for THP-1, C2 to G11 for SW480, E6 to G2 for MDS) are derived from their positions in 96-well titre plates. The proteins were visualized using an anti-Pr55/p24 monoclonal antibody (ARP313) at a 1/5000 dilution. The positions of the Pr55 Gag precursor and p24 Capsid proteins are indicated by arrows.

are shown in Fig. 2. It can be seen that HeLa and THP-1 cells produce readily detectable intracellular Pr55 but with relatively little release of viral protein into the supernatant. RT activity was correspondingly low in these cell lines and in clones derived from these lines, SW480, Jurkat-tat and MDS cells produced high level RT activity and high level protein expression was seen in Western blotting. Individual clones from the cells showed striking variations in their ability to produce and export virus proteins despite all clones being resistant for the selectable marker. Differences of up to a 10-fold order of magnitude were seen in p24 and RT levels in the supernatants from these cells (data not shown). Expression from the retroviral LTR can be enhanced by differentiating THP-1 cells. Treatment with PMA as described previously for this cell type resulted in a 2-fold increase in gag–pol expression as evidenced by RT assays and Western blot (Fig. 3). However, this increase was not a significant improvement in comparison to the SW480 and MDS gag–pol expressors.

The HXBc2 clone is vpu− and this protein is believed to enhance particle production in HeLa cells. Several attempts were made to increase particle export in the HeLa gag–pol expressing cells by transfection of a vpu expressor. However, in our hands, this had no detectable effect on protein processing or particle export (data not shown).
Two clones were chosen for further use, C2 from the SW480 cells and E6 from the MDS cells, as these showed consistently high levels of protein production and normal protein processing with export of a high level of viral core particles. Both of these clones were transfected with the envelope expressor as described and placed under GPT selection for 3 weeks. Significant cell fusion was noted in the SW480 cells. However, SW480 cell lines were generated which demonstrated cellular and supernatant gp120 Envelope glycoprotein expression (Fig. 4). Similar results were obtained for MDS cells (data not shown). The envelope reactive band in the cellular sample from clone C2 is an internally deleted envelope protein product generated from a BglII deletion within the envelope used to inactivate this protein. It is truncated with respect to full-length envelope and not secreted (see supernatant lane of C2 cells). These cells were used for subsequent gene transfer using a packageable vector.

The same clones, C2 and E6, were transfected with the LRPLAHS vector to make an incomplete producer cell line. Puromycin selection was applied. These cells remained positive for RT activity and were puromycin-resistant.

The E6 cell clones were maintained in culture for 6 months and demonstrated protein expression over this period as evidenced by RT assays. However, the level of expression of gag–pol fell by 50% or more both in E6 and E6+ vector cells. A similar fall was seen in SW480 derived cells (data not shown).

**Encapsulation of RNA**

Fig. 5 demonstrates specific encapsidation of the HIV-based vector in the particles derived from the stable cell lines. Detection of full-length vector RNA inside particles derived from gag–pol expressing lines when vector was present demonstrates that encapsidation is independent of any influence on virus assembly from the Envelope glycoprotein. Some encapsidation of unspliced mRNAs derived from the gag–pol expression plasmid was also observed. This effect was more pronounced in the cell lines stably expressing gag–pol alone. When a vector was stably expressed in addition to gag–pol, full-length vector RNA was packaged more efficiently than other RNA species.

**Vector transfer**

A series of vector transfer experiments was set up to test the ability of the gag–pol plus env cell lines to act as packaging cell lines and the gag–pol plus vector cell lines to act as incomplete producer cell lines. A number of attempts were made to create a stable producer cell line containing gag–pol, env and vector plasmids. However, the growth of these cells was always extremely poor and they had a limited life-span.

LRPLAHS and HVP were individually transfected into the SW480 C2 cell clone and the C2 clone expressing HIV-1 env.
Calcium phosphate coprecipitation was used and supernatants harvested 2 days after transfection and put on to HeLa T4 cells. In parallel, the SW480 C2 vector cell line was transfected with the env expressor and supernatants treated identically. The supernatant was checked for RT activity to confirm adequate viral particle production. HeLa T4 cells were incubated with the supernatant for 24 h; the medium was then changed and puromycin selection was begun. Plates were stained after 4 weeks and colonies resistant to puromycin were counted.

Vector transduced HeLa T4 cells were subjected to Southern blot analysis to confirm transfer of the intact vector. Bands corresponding to the appropriate fragments can be seen which are not present in parental cells (Fig. 6).

A similar procedure was used on the MDS E6 cells using either electroporation or DEAE-dextran transfection techniques with LRPLAHS being transfected into the E6 envelope-expressing cells and the env expressor was transfected into the E6 vector cells. Replicate experiments were performed for cell transfer.

Table 1 gives a summary of the vector transfer data. It can be seen that either addition of env gene to an incomplete producer cell line or vector gene into a packaging cell line was able to produce gene transfer, albeit at low titre, into HeLa T4 target cells. Because of the absence of an intact vpu gene from the gag–pol expressor, a vpu-expressing plasmid was cotransfected into the system in another series of experiments in order...
to see whether expression of vpu would lead to enhanced viral particle export and an increased titre. Essentially, the experimental protocol was the same apart from the addition of 5 µg of Vpu expressor per 10 cm plate. However, this did not alter vector titre. An additional control of gag-pol plus LRPLAHS expressing cell lines was tested to confirm that envelope deficient particles were unable to transduce the vector into the target cell line.

**Discussion**

Generation of cells stably expressing HIV protein has led to a number of important findings. In all cell lines tested there was very significant variation in the efficiency of protein expression and export both between different lines and between clones of the same cell line. THP-1 cells were transfected with the gag-pol expressor in the expectation that the cells would require differentiation with PMA in order to induce transcription from the viral long terminal repeat (Moses et al., 1994). To our surprise these cells transcribed and processed the gag-pol proteins adequately in the absence of PMA stimulation. PMA stimulation led to a modest increase in viral protein processing and a small increase in viral protein export. However, this was still low compared to SW480 and MDS cells. Because of poor export, these cells were not further studied. However, they constitute an interesting model in which export of Gag and Gag–Pol proteins could be studied. However, this was still low compared to SW480 and MDS cells.

HeLa cells are reported to export HIV particles less efficiently in the absence of Vpu (Schubert & Strebel, 1994), this function being independent of the CD4 degradation effect (Willey et al., 1992; Yao et al., 1995). This cell line was constructed in the expectation that viral protein would be produced but that Vpu would be required in addition: gag-pol expression was indeed achieved at levels comparable to other cell lines used and yet the cells were unable to export Gag–Pol particles to any extent. However, transfection of a vpu expressor into the HeLa gag-pol expressing cell lines made little difference to core particle export.

The two cell lines which showed the greatest ability to process and export Gag–Pol were SW480 and MDS. Again, significant clonal variation was seen in protein production and high expressor clones were selected for further study. Introduction of the envelope glycoprotein into SW480 initially led to moderate syncytium formation despite this cell line being reported as negative for cell surface CD4. This cell line is reported to express CD4 messenger RNA and hence it is possible that a very low level of CD4 protein, undetectable by conventional staining, is present and is responsible for the fusion. Despite this, with prolonged expression (confirmed by Western blotting), a packaging cell line was generated and used for vector transfer experiments. Expression of the viral proteins continued but with some decline over the course of 6 months and the same cell stock was able to be used for repeated gene transfer experiments.

The MDS cell lines similarly showed clonal variation. There was little syncytium formation with envelope protein expression and the cells again expressed viral proteins stably over a period of several months. Neither MDS or SW480 showed any defect in Gag–Pol processing indicating that the viral protease, which has been shown to be toxic in some other cell lines, could be expressed stably in these cell lines without detriment to cell viability.

Vector RNA encapsidation could be demonstrated in both the MDS and SW480 gag-pol cell lines. Although mRNA derived from the gag-pol expression construct LAP2GPH was also packaged to a lesser extent, full-length vector RNA was encapsidated most efficiently. Low level encapsidation of spliced viral message has been demonstrated before for HIV-1 and may reflect the presence of packaging determinants in the 5’ leader sequence upstream of the splice donor (McBride et al., 1997) which, in the absence of more packageable RNA, are able to interact with Gag and facilitate encapsidation.

Vector transfer experiments were undertaken in two formats; firstly with a cell line expressing gag-pol plus env, a conventional packaging cell line, and secondly with a cell line containing gag-pol plus vector stably expressed, into which an envelope expressor was introduced—a incomplete producer system. The latter cell lines have the advantage that one can assess the ability of a variety of different envelope genes to pseudotype the HIV core using an internally controlled consistently expressing cell population. The level of transfer to HeLa T4 cells was low. The maximum titre achieved was approximately 10^4 c.f.u./ml. This was achieved with both incomplete producer and conventional packaging cell lines as the basis for the transfer. Previous experiments have shown HeLa T4 cells to provide a relatively difficult target to infect and titres on other cells including primary CD4 cells will give more indication as to the utility of the system. Experiments are under way to optimize transfer.

The HIV IIIb construct used in these experiments lacks other accessory genes apart from vpu. The vpr gene is truncated as is the nef gene. Constitutive expression of Vpr might be expected to induce cell cycle arrest (Rogel et al., 1995) and reduce the viability of the cell line and these cell lines were

### Table 1. Vector transfer

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Stably expressed genes</th>
<th>Transiently expressed genes</th>
<th>Titre (c.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480 C2env</td>
<td>gag-pol, env</td>
<td>HVP</td>
<td>10^1</td>
</tr>
<tr>
<td>MDS E6env</td>
<td>gag-pol, env</td>
<td>HVP</td>
<td>10^3−10^4</td>
</tr>
<tr>
<td>MDS E6L</td>
<td>gag-pol, LRPLAHS</td>
<td>envelope</td>
<td>10^8</td>
</tr>
<tr>
<td>THP1</td>
<td>gag-pol, HVP</td>
<td>envelope</td>
<td>10^9</td>
</tr>
</tbody>
</table>
designed such that Vpr could be introduced in trans under transient transfection conditions should gene delivery to cells in G0 be required. The absence of the full-length nef gene may have influenced the efficiency of transfer and experiments are currently in progress to introduce a functional nef gene into the cell line to determine its effects on vector transfer.

In summary, we have demonstrated that it is possible to create cell lines stably expressing a high level of HIV proteins over a prolonged period of time from which vector transfer can be achieved specifically to CD4+ cells. The efficiency of the system on primary cells is, as yet, unknown but the cell lines lend themselves to studies of viral protein export, envelope pseudotyping and analysis of the role of accessory proteins in gene transfer using HIV-based vectors. These cell lines may help in the development of a system by which gene transfer to CD4+ cells may be achieved in vivo in order to deliver antiviral genes into as yet uninfected cells of patients who are HIV-positive. Experiments are under way to address this.

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References


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