Ordered appearance of human immunodeficiency virus type 1 nucleic acids following high multiplicity infection of macrophages

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The order of appearance of human immunodeficiency virus type 1 (HIV-1) nucleic acids was examined in monocyte-derived macrophages following a high multiplicity infection with macrophage-tropic virus. Using the polymerase chain reaction, viral DNA was first detected 2 h after infection and continued to accumulate over the next 24 h. Transcripts representing tat, rev and nef splicing were detected by 24 h, and transcripts representing env splicing were detected by 48 h after infection. Coincident with the appearance of env transcripts, new synthesis of cellular and extracellular p24 antigen began, multinucleated giant cells formed and progeny infectious virus emerged. This analytical system provides a foundation for further studies on the effects of antiviral agents and cellular factors on the replication cycle of HIV-1 in non-transformed, primary monocyte-derived macrophages.

Introduction

An important part of the study of any virus is the analysis of the order of expression of its genetic programme in a representative host cell. For the human immunodeficiency virus (HIV), this type of analysis has not been completed. In the first report on the kinetics of HIV replication, Kim et al. (1989a) studied the growth of a lymphotropic strain of HIV-1 in the H9 T lymphocytoid cell line and described the sequential appearance of viral nucleic acids. Beginning at 4 h post-infection, Southern blotting began to detect the presence of viral DNA. By 16 h, Northern blotting detected the approximately 2 kb mRNAs encoding the regulatory genes, along with traces of singly spliced 4-3 kb mRNAs. At 24 h, full-length 9-2 kb genomic mRNA began to appear, and virions began to be released by 28 h (as indicated by the appearance of reverse transcriptase in the culture supernatants).

More recently, we and others described the use of the polymerase chain reaction (PCR) as a sensitive tool for the detection of HIV DNA (Ou et al., 1988; Zack et al., 1990) and RNA transcripts (Arrigo et al., 1989; Schwartz et al., 1990a; Robert-Guroff et al., 1990; Guatelli et al., 1990). The PCR technique permits the detection of minute amounts of specific nucleic acid sequences. When used in conjunction with a reverse transcription step, it can also differentiate between spliced transcripts that are too similar in size to be resolved by Northern blotting. Using this approach, Guatelli et al. (1990) reported the kinetics of HIV-1 LAI (lymphadenopathy-associated virus type 1 (LAV-1)) replication in the CEM T lymphoblastoid cell line. In those studies, the signal produced from nef mRNA was the principal PCR product at early time points, with signals from tat and rev mRNAs present in much lower amounts. In this study, we have extended this same type of analysis to a macrophage-tropic strain of HIV-1, HIV-1 BaL [formerly human T cell lymphotrophic virus type III (HTLV-III) BaL85], and determined the order of appearance of HIV DNA and mRNAs in monocyte-derived macrophages. Using a virus stock of high titre, the majority of cells were exposed to HIV during a brief inoculation period and subsequent events were evaluated during the first cycle of viral replication. As for the lymphotropic isolate, we found that the mRNA for nef was a prominent early transcript in macrophages and that tat mRNA (whose protein product trans-activates the viral promoter) appeared in trace amounts at about the same time.

Methods

Macrophage culture. Peripheral blood mononuclear cells were obtained by centrifugation of the heparinized venous blood of healthy, seronegative donors over Ficoll-Hypaque. Monocytes were then isolated by sequential fibronectin and plastic adherence and allowed to
mature into macrophages (Freundlich & Avdalovic, 1983; Kornbluth et al., 1989). Macrophages were cultured at 37 °C in 5% CO2 and 5% O2, at an initial density of either 4 x 10^5/well in 48-well plates or 3 x 10^6/flask in 25 cm^2 flasks (Costar) in RPMI 1640 medium (Irvine Scientific) supplemented with 10% (v/v) autologous or human AB serum and 50 μg/ml gentamicin. All media and sera were monitored for endotoxin contamination by Limulus lysate assay as well as by a sensitive monocyte procoagulant activity assay (Kornbluth & Edgington, 1986).

Virus stock. High titre stocks of the macrophage-tropic HIV-1 strain HTLV-III_Lun_L (the gift of Drs S. Gartner and M. Popovic (Gartner et al., 1986) were prepared by passage of the virus in macrophage cultures. Endotoxin contamination was avoided (as described above), and no exogenous cytokines or growth factors were added to virus stock cultures. Mycoplasmas were undetectable in these cultures using a sensitive rRNA hybridization assay (Mycoplasma T.C.I.; Genprobe). At 10 to 14 days following infection, supernatants from the HIV-1_Lun_L-infected macrophage cultures were clarified by centrifugation (1200 g for 10 min) and stored in aliquots at −70 °C. Virus stocks were checked for contaminating HIV-1 DNA by PCR amplification (as described below) and titrated by quadruplicate endpoint dilution in macrophage cultures (without subsequent washing), using p24 antigen production as measured by ELISA (Abbott) as the indicator of infection. The virus stock used for these experiments had an infectious titre of 10^6 TCID_50/ml, calculated by the method of Kärber (1931).

Infection. Five days after plating, the macrophage cultures were infected with HIV-1_Lun_L at an m.o.i. of 6 (unless otherwise noted). Prior to infection, the tissue culture plates or flasks were chilled to 4 °C for 30 min. Chilled virus stock was placed over the culture monolayers, and the cultures were returned to 4 °C for 60 min to allow virus attachment to occur. The cultures were then incubated at 37 °C for 60 min to allow for virus penetration, washed four times with serum-free medium to remove the unadsorbed inoculum, and re-incubated at 37 °C. Culture supernatants were harvested at multiple time points after infection and stored at −70 °C prior to assays for p24 antigen and infectivity. Cells were harvested by washing the macrophage monolayers with serum-free medium, dislodged with a cell scraper into fresh serum-free medium, counted and stored in freezing medium (90% FBS/10% DMSO) at −70 °C prior to analysis for nucleic acid and cell-associated p24 antigen. Cultures were also observed by phase contrast microscopy for the presence of multinucleated giant cells with > six nuclei/cell, which is the principal cytopathic effect in these cultures. Uninfected cultures were treated in an identical fashion except that medium alone was used in place of the virus inoculum.

In separate experiments, monocyte-derived macrophages infected by this protocol were cultured for 8 days post-infection and evaluated for HIV-1 infection by immunocytochemistry and in situ hybridization. HIV-1 antigens were detected using biotinylated immunoglobulins (Richman et al., 1985) from an HIV antibody-positive human serum, followed by streptavidin–alkaline phosphatase binding and colour development with naphthol phosphate (Fast-Red; BioGenex). In the presence of normal human serum (to block Fc receptors), >98% of HIV-infected macrophages were positive; uninfected macrophage cultures served as negative controls. Similarly, HIV-1 mRNAs were detected using in situ hybridization (Insite; Applied BioTechnology). Again, >98% of the macrophages in infected cultures (but none in the uninfected cultures) were positive for HIV-1 mRNAs.

p24 antigen determinations. Cell-associated p24 antigen was isolated from washed infected macrophages by pipetting them up and down (20 times) in the presence of 0.5% Triton X-100/1% gelatin in Dulbecco’s PBS (without calcium or magnesium). The ratio of cell number to buffer volume was equivalent to the ratio of cell number to medium volume in the original infected flasks, and the Triton/gelatin buffer solution used to isolate cell-associated protein was similar to that used as a solubilizing buffer in the p24 ELISA for serum samples (Abbott).

 Supernatant infectivity assays. Infectivity was assayed by endpoint dilution as for the virus stocks except that duplicate rather than quadruplicate macrophage cultures were used at each dilution.

Nucleic acid preparation for PCR. For DNA analysis, total cellular nucleic acids were prepared from macrophages by resuspending the cells in a lysis buffer containing 0.2% SDS, 10 mM-EDTA, 150 mM-NaCl, 200 μg/ml proteinase K, 20 mM-Tris–HCl pH 7-5, and incubating at 50 °C for 45 min. The nucleic acids were then extracted twice with phenol–chloroform, once with chloroform, adjusted to 0.8 M LiCl, and precipitated in ethanol. For assay of RNA transcripts, total cellular RNA was prepared using a modification of the single-step acid guanidinium thiocyanate–phenol–chloroform procedure (Chomczynski & Saachi, 1987) (RNAzo1, Cinna/Biotech).

Detection of viral DNA by PCR. The precipitated total nucleic acid pellets were resuspended in 5 mM-Tris–HCl pH 8.3 before being made up into 100 μl aliquots containing the amplification mixture (0.2 mM each dNTP, 0.25 μg of each oligonucleotide primer and 0.1 mg/ml BSA in a buffer containing 10 mM-Tris–HCl pH 8.3, 50 mM-KCl and 1.5 mM-MgCl2). The samples were then denatured by boiling for 2 min and cooled at 42 °C for 1 min before the addition of 2.5 units of thermostable DNA polymerase (Thermus aquaticus) and amplification in an automated thermal cycler (both from Perkin-Elmer Cetus) for 30 cycles. The cycle profile included primer extension at 72 °C for 3 min, denaturation at 94 °C for 1 min and annealing at 55 °C for 1 min. All synthetic oligonucleotide primers were prepared and gel-purified by Genosys Biotechnologies. Because the nucleic acid sequence of HIV-1_Lun_L had not yet been reported at the time these experiments were performed, oligonucleotide primers for the amplification of a 202 bp fragment from env region DNA were designed using the sequence of HXB2 (Myers et al., 1991) (showing their respective nucleotide positions in HXB2 in parentheses): DNA sense primer, 5’ AAAGG-TATCCTTTGAGCACAATCCCTCA 3’ (6841 to 6968); DNA antisense primer, 5’ TCTAATTACTACCTCTTCTCTAG- ACT 3’ (7042 to 7013). When compared to a recent report on the partial sequencing of HIV-1_Lun_L (M. Reitz et al., in Myers et al., 1991), the sense primer is mismatched at the fifth nucleotide, which should not significantly affect its usefulness.

Amplification products were extracted with chloroform and analysed by electrophoresis in 2% agarose/ethidium bromide gels using HoeIII-digested φX174 DNA as a size standard. For detection with radiolabelled probes, the amplification products were capillary blotted onto nylon filter membrane (Zeta-Probe; Bio-Rad) in 0.4 M NaOH. The filters were neutralized with 1 M-Tris–HCl pH 7.4 and hybridized for 1 h at 55 °C in 1% SDS, 5 × saline–sodium phosphate–EDTA (SSPE), 0.5% polyvinylpyrrolidone and 0.5% BSA in the presence of 107 c.p.m. 32p end-labelled oligonucleotide probe (internal to the gene or HIV env region). The HIV signal was compared to the co-attaching 0.1 ng/ml of ssDNA of the SacIII-digested pBR322 DNA. The filters were then washed at 55 °C with 1% SDS in 1 × SSPE, dried and autoradiographed using an intensifying screen at −70 °C for 3 h.

Slot blot analysis was performed in order to quantify PCR amplification products and to correct for variations in sample-to-sample amplification efficiency. In this modification of differential PCR (Frye et al., 1989), the HIV signal was compared to the co-amplified β-actin signal, where β-actin served as the reference gene. Equivalent volumes of each sample were vacuum-transferred to niny filters (Zeta-Probe) pre-soaked in 10 × SSC. The filters were then denatured in 0.4 M-NaOH, neutralized in 1 M-Tris–HCl pH 8.0, and probed with 32P end-labelled oligonucleotides from either the β-actin gene or HIV env. After autoradiography, individual slots were excised from the filters and radioactivity was counted in a liquid scintillation counter. Corrected HIV counts represent the products of HIV counts...
Table 1. Oligonucleotides used for the PCR analysis of HIV-1 cDNA

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>C.p.m.</th>
</tr>
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<tbody>
<tr>
<td>A (sense primer)</td>
<td>5'ACGGCAAGGGCCAGGGGACTG3'</td>
<td>716-742</td>
<td></td>
</tr>
<tr>
<td>B (antisense primer)</td>
<td>5'CTTTGATAGAATACTGATGCTG3'</td>
<td>6042-6016</td>
<td></td>
</tr>
<tr>
<td>C (sense probe)</td>
<td>5'CGGAGAGGGCCAGGGAGACCTTCTCAAGGC3'</td>
<td>5983-6012</td>
<td></td>
</tr>
<tr>
<td>D (antisense primer)</td>
<td>5'CCTCATGCTGCGACGTGTCTGC3'</td>
<td>6235-6208</td>
<td></td>
</tr>
<tr>
<td>E (antisense primer)</td>
<td>5'CGAAGAAAGGCAAGCTTTTACAATA3'</td>
<td>5931-5905</td>
<td></td>
</tr>
<tr>
<td>F (sense probe)</td>
<td>5'AGAGCCTTGAAGCATCCAGGAATGTCG3'</td>
<td>5853-5881</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers listed in parentheses indicate the nucleotide positions of the sequences in HXB2.

**Amplification of cDNA from HIV-1 transcripts.** Total cellular RNA pellets were resuspended in the same amplification mixture described above except that the buffer contained 10 mM-KCl and 2.5 mM-MgCl₂ in 50 mM-Tris–HCl pH 8.3. Secondary RNA structure was denatured by heating to 65 °C for 90 s, followed by cooling to 42 °C before the addition of 100 units of Moloney murine leukaemia virus reverse transcriptase (Gibco/BRL) and incubation at 42 °C for 30 min. Following reverse transcription, the cDNA target was amplified by PCR using a protocol identical to that above. In experiments using primers A and D (Table 1), the amplification products were boiled for 2 min and run on a 6% polyacrylamide gel before being electrotransferred in Tris-borate EDTA (TBE) to a nylon filter and heat-fixed at 80 °C for 50 min prior to hybridization and autoradiography.

As before (Guatelli et al., 1990), primers for spliced HIV mRNAs were chosen to span the differential splicing events in the 5' half of the viral genome (Fig. 2). In experiments using primers A and B, the amplification products were resolved on a 6% non-denaturing acrylamide gel, capillary blotted to a nylon membrane in 1 x TBE, then denatured on the membrane in 0.4 M-NaOH, and neutralized in 1 M-Tris–HCl pH 8-0. In experiments using primers A and E, amplification products were resolved in 2% agarose gels and blotted as described for DNA PCR above. Probes for Southern blots of the PCR reactions were designed to be internal to their respective primer-pairs. These primer-pairs (A and B, A and D, and A and E) bracket discrete splicing events in the formation of different HIV-1 mRNAs. From the size of the amplification products, the presence of spliced mRNAs coding for different HIV-1 gene products was deduced. In the case of tat and rev mRNAs, the identity of the corresponding cDNA PCR fragments was confirmed by direct sequencing of the appropriately sized band following extraction from the gel and reamplification using asymmetrical PCR (Guatelli et al., 1990; Gyllensten & Erlich, 1988).

The oligonucleotide sequences used (Table 1) were originally designed to amplify cDNAs from HIV-1Bl (LAV-1). When compared to the report cited above on the sequence of HIV-1Bl, primer B has a mismatched base at its 3' terminus; probe C is mismatched at bases 16 and 26; primer D is mismatched at base 2; and primer E and probe F are perfectly homologous. Since the sequence of the 5' end of HIV-1Bl has not yet been reported, primer A cannot be compared with it. However, primer A matches the sequence of another macrophage-tropic HIV-1 isolate, HIV-1Pyr2, except at base 19 (Li et al., 1991; X. Li et al., personal communication). Of all these differences, the greatest concern was that the mismatch at the 3' end of primer B would impair its function in PCR. However, in over 20 amplifications of HIV-1Bl, we consistently observed successful amplifications using primers A and B as primer-pairs. In any case, since the mismatch in primer B is the same for the cDNAs from nef, env, rev and tat, the relative proportions of the PCR products from these genes should not be affected.

**Results**

**High multiplicity infection of macrophages with HIV-1**

Monocyte-derived macrophages were infected with HIV-1Bl by exposing them to virus at 4 °C for 60 min at an m.o.i. of 6. By the Poisson distribution, an m.o.i. of 6 should result in infection of > 99% of the cells, assuming that the majority of viruses bind to the macrophages during this time period. This high m.o.i. was chosen so that most or all of the cells would be exposed to HIV during a short, defined time period. To show that all of the macrophages were susceptible to HIV infection, other cultures were infected in an identical manner and cultured for 8 days. In these cultures, essentially 100% of the macrophages were positive for HIV-1 antigens by immunohistochemistry and contained HIV-1 mRNAs detected by in situ hybridization (Meylan et al., 1992).

**Accumulation of HIV-1 DNA following infection**

Preparations of total cellular nucleic acid were made from monolayers of infected macrophages at various time points following infection. Samples were subjected to PCR amplification using primers specific for a 202 bp conserved region within env. Southern blots of the products were probed with a 32P-labelled internal oligonucleotide. A representative experiment (one of three) is shown in Fig. 1. A small amount of HIV-1 DNA was detectable by PCR in the inoculum (Fig. 1a), but the post-inoculation washings were sufficient to remove this input DNA from the infected cultures (Fig. 1a; the '0 h' time point was devoid of signal). The first detectable HIV-1 DNA appeared 2 h after infection.

To control for varying nucleic acid isolation and amplification efficiencies, each sample was co-amplified for β-actin and analysed by slot blot analysis (Fig. 1b). Because the β-actin genomic DNA signal should be invariant between identical numbers of cells, the HIV-1 viral DNA was normalized against the β-actin signal and plotted on a logarithmic scale (Fig. 1c). This analysis showed that the HIV-1 viral DNA signal increased markedly during the course of this first cycle of infection.
Because no progeny virions were produced during the first 24 h (see below), this increase could not be attributed to secondary rounds of viral infection. Consistent with this interpretation, α-amanitin (1 μg/ml), an inhibitor of transcription which prevented HIV-1 replication in macrophages, did not significantly alter the pattern of viral DNA accumulation (data not shown). Thus, the progressive increase in viral DNA signal during this period indicated a delay in the formation of viral DNA in some of the cells. Although the infection of the macrophages was designed to be uniform and synchronized, evidently variations in the events following the pulse of exposure to virus resulted in asynchronous reverse transcription.

Sequence of formation of HIV-1 transcripts

To analyse the appearance of specific HIV-1 mRNAs, the PCR strategy of Guatelli et al. (1990) was applied. As shown in Fig. 2(a), this approach exploited the fact that although transcripts encoding the regulatory genes tat, rev and nef have identical 5' and 3' ends, they differ in their utilization of splice acceptors used during the excision of the 5' intron. PCR amplification spanning these upstream splice sites using primers A and B (Fig. 2) resulted in a series of differently sized products, each of which represents a specific mRNA. tat mRNA was represented by a 290 bp amplification product, rev mRNA by a 113 bp product and nef mRNA by a 96 bp product. However, amplifications using primers A and B do not distinguish between the doubly spliced nef mRNA and the singly spliced env mRNA. Therefore, to detect specifically the latter, amplifications were also performed using primers A and D.

Total cellular RNA was isolated from aliquots of infected cultures taken from the same representative experiment as shown in Fig. 1 (m.o.i. of 6). Complementary DNA was made from this RNA with antisense primer B and amplified with primers A and B. As shown in the Southern blot in Fig. 2(b), the first spliced transcript detected using these primers generated the 96 bp product representing nef mRNA (see below), and it appeared 24 h after infection. At the same time, a faint signal was detected at 113 bp which represented the rev spliced transcript. Because the 290 bp amplification product representing the tat spliced transcript was not detected at 24 h using primers A and B, tat cDNA was specifically sought using primers A and E for the amplification. As shown in Fig. 2(c), this approach detected a weak signal at 24 h of a 181 bp product representing the tat spliced transcript. As discussed above, singly spliced env mRNA was not distinguishable...
Fig. 2. Kinetics of HIV transcription events following high multiplicity infection of macrophages. (a) Splicing events leading to the formation of transcripts appropriate for tat, rev, nef and env expression. PCR primers and probes (A to F) are shown at their respective annealing sites, and initiation codons are indicated with arrows. Splice donors are designated by closed circles, and acceptors by open circles. To the right, the expected lengths of amplification products are shown for each transcript and for each of the two pairs of PCR primers used. (b) Southern blot transfer from a 6% non-denaturing polyacrylamide gel demonstrating the products that resulted from the amplification of samples with primers A and B followed by probing the filter with oligonucleotide C. The sizes of the expected products for tat (290 bp), rev (113 bp) and nef or env (96 bp) spliced transcripts are indicated. The representative infection analysed here is the same as that depicted in Fig. 1 and 3 (m.o.i. of 6). The first spliced transcripts appeared at 24 h and produced a 96 bp product representing nef. A faint 113 bp band was also seen at this time point, representing rev. U, uninfected; C/L, CEM/LAV. (c) To detect tat RNA at its earliest time point, amplification with primers A and E followed by probing with oligonucleotide F was required. This demonstrated the appearance of the tat spliced RNA at 24 h. (d) Samples from the same infection amplified with primers A and D in order to detect env (285 bp)-specific transcripts which appeared at 48 h. By a process of elimination, the absence of a signal at 24 h indicated that the 96 bp signal seen in (b) was generated by nef mRNA.

From nef mRNA using the A and B primers. Consequently, PCR amplification was also carried out using primers A and D. As shown in Fig. 2(d), a 285 bp amplification product representing the env-specific spliced transcript appeared by 48 h, which indicated that the major 96 bp product detected at 24 h using the A and B primers represented nef mRNA.

To confirm the identities of the 290 bp (tat) and 113 bp (rev) amplification products, these bands were excised from a polyacrylamide gel and directly sequenced after reamplification by asymmetrical PCR. The nucleotide sequences which resulted were: tat, 5'-GGGGAGGCGACCTGAAATTGGGTGCCGACATAGCAGAATAGGTATTACTCCACAGAGGAGAGCAAGAAATGGAGCCAGTAGATCCTA 3' and rev, 5'ACTGCGATCTCCTATGGCAGAGGAAGCCGGAG-ACAG 3'. ['V' indicates splice junctions immediately downstream of the 5' splice donor-site primer (A); initiation codons are underlined.] The splice acceptor sites for these two transcripts were identical to those of published lymphotropic strain sequences (Schwartz et al., 1990a; Guatelli et al., 1990). The rev splice acceptor found corresponded to the more 3' of the two splice acceptors previously mapped by cDNA PCR in lymphotropic strains of HIV-1 (Schwartz et al., 1990a; Guatelli et al., 1990).

Time course of p24 antigen and infectious virus production

Newly produced p24 antigen and infectious virus appeared at about 48 h after infection (Fig. 3), and multinucleated giant cells (evidence of c.p.e.) began to appear.
the first replicative cycle of HIV-1 in this system was complete between 24 and 48 h after infection. During that interval, p24 antigen was produced in the cell and secreted into the supernatant, env mRNA appeared in the cells and there was a 1000-fold increase in infectious virus in the supernatant.

Discussion

The two primary host cells for HIV replication appear to be CD4+ T lymphocytes and antigen-presenting cells such as macrophages. Both probably play critical roles in the pathogenesis of HIV-related diseases. To facilitate the analysis of HIV infection in the macrophage, we have examined the sequence of expression of HIV genes following the synchronized infection of primary human macrophages.

Using PCR (Saiki et al., 1988), we found, as expected, that viral DNA was the first viral component to be synthesized following infection. In this study, viral DNA was first detected 2 h after infection and increased more than 10-fold during the first 24 h. The increase was not due to additional rounds of infection caused by progeny virus, because the production of env mRNA, p24 antigen, and the release of infectious virus did not occur until after this time point. This unsynchronized increase in viral DNA was unexpected, and it may reflect delayed entry, uncoating or reverse transcription (Zack et al., 1990). Further studies will be required to differentiate between these possible explanations.

Subsequent to the formation of the viral DNA template, HIV-1 transcripts appeared in macrophages by 24 h after infection. The most obvious product of the PCR analysis represented nef mRNA. This was surprising in view of the putative role of nef as a factor that negatively regulates viral transcription in some (Fisher et al., 1986; Luciw et al., 1987; Ahmad & Venkatesan, 1988; Cheng-Mayer et al., 1989; Niederman et al., 1989) but not all studies (Kim, 1989b). Using a similar PCR system for detecting spliced HIV-1Bal mRNAs in macrophages (but without conducting a time-course analysis), Robert-Guroff et al. (1990) also found a strong nef mRNA signal in macrophages that had been infected with HIV-1Bal for 12 to 14 days. In addition, a similar early prominence of nef mRNA was detected during high-multiplicity infection of lymphoid cell lines with HIV-1 (GuateUi et al., 1990; Klotman et al., 1991). However, the significance of nef mRNA as an early transcript in macrophages is tempered by an absence of information on the formation, stability and post-translational modification of nef protein in these cells. Despite this caveat, the prominence of nef mRNA in

Fig. 3 summarizes the events in the replicative cycle of HIV-1 in this culture system for detecting spliced HIV-1BaL mRNAs in macrophages (but without conducting a time-course analysis). Robert-Guroff et al. (1990) also found a strong nef mRNA signal in macrophages that had been infected with HIV-1Bal for 12 to 14 days. In addition, a similar early prominence of nef mRNA was detected during high-multiplicity infection of lymphoid cell lines with HIV-1 (GuateUi et al., 1990; Klotman et al., 1991). However, the significance of nef mRNA as an early transcript in macrophages is tempered by an absence of information on the formation, stability and post-translational modification of nef protein in these cells. Despite this caveat, the prominence of nef mRNA in
these primary cells is consistent with some positive role for nef in HIV infection, perhaps analogous to the importance of nef in maintaining high virus loads of simian immunodeficiency virus in rhesus monkeys (Kestler et al., 1991).

A second early HIV-1 transcript in macrophages was rev mRNA. In some cells rev alters the balance of transcript processing away from the doubly spliced tat, rev and nef regulatory mRNAs in favour of singly spliced env mRNA and unspliced gag/pol and genomic mRNAs (Vaishnav & Wong-Staal, 1991). In other cells, rev acts on the translational apparatus to permit the accumulation of gag and env proteins (Arrigo & Chen, 1991). In this study, rev mRNA appeared more than 12 h prior to the appearance of either singly spliced env mRNA or p24 core antigen (which is formed from the gag/pol polyprotein). The timing of the appearance of rev mRNA does not indicate what mode of action this gene has in macrophages, but it does indicate that rev mRNA appeared prior to the structural proteins of the virus.

Two other studies have used PCR to study the order of HIV regulatory gene expression. Guatelli et al. (1990) employed the same cDNA PCR system as used here to study the transcription of HIV-1w13 (LAV-1) in the CEM lymphoblastoid cell line. Klotman et al. (1991) used a similar cDNA PCR system to study the expression of HIV-1w13 in the H9 lymphoblastoid cell line and the U937 monoblastoid cell line. As in this study, both these reports described nef mRNA as the most prominent early mRNA in macrophages, with smaller amounts of rev and tat mRNAs detected simultaneously.

It should also be noted that the splicing events characterized here for HIV-1w13 correspond to those described previously (Schwartz et al., 1990a; Robert-Guroff, 1990; Guatelli et al., 1990; Arrigo et al., 1990; Schwartz et al., 1990b). Although we found only one of the two rev splice acceptor sites, this result may reflect the limitations of asymmetrical PCR for sequence analysis. However, it is interesting to note that the result of amplification for singly spliced mRNAs (using primers A and D) generated a single product (Fig. 2d), whose size suggests the use of the splice acceptor 5’ of the vpu start codon. The use of this acceptor site to generate singly spliced mRNAs encoding vpu and env has recently been reported for lymphotropic strains of HIV-1 (Guatelli et al., 1990; Arrigo et al., 1990; Schwartz et al., 1990b). In addition, Robert-Guroff et al. (1990) also described in macrophages a nef mRNA formed by a single splicing event using the 5’-most splice donor and the 3’-most splice acceptor in the HIV-1 genome. Since we cannot detect such an mRNA with the primers used in this study, our signals for nef mRNA may in fact underrepresent the mRNAs capable of expressing this gene.

There are several potential limitations to the experimental methods used in our studies. First, the PCR of HIV-1 transcripts might have radically different amplification efficiencies for each different mRNA. This possibility seems unlikely because we used the same primers in the same PCR reactions to amplify tat, rev and nef mRNAs, so that the hybridization of these primers to their target sequences should be virtually identical in each case. However, it is still possible that secondary structures or post-transcriptional modifications of the mRNAs differentially impair the reverse transcription step or the annealing step of PCR. Although we have not attempted to use PCR to quantitate precisely the levels of these regulatory transcripts, if the amplification efficiencies of these transcripts were radically different, then it might affect our assessment of the order of appearance of these different mRNAs. A second limitation is that the PCR methods that we used did not guarantee that the mRNAs detected were intact in regions not bounded by the primers. A third limitation of this analysis is that each of the regulatory genes acts as a protein, and PCR cannot assess the efficiency of translation of the mRNAs that were detected. Consequently, it will be useful to perform further studies using other criteria for the expression of the tat, rev and nef genes in the infection system described here.

In spite of these limitations, PCR is a promising tool in the analysis of the HIV-1 replicative cycle in primary host cells. From this type of analysis, the unique replicative characteristics of HIV in primary human cells and the effectiveness and sites of action of various antiviral drugs can be pinpointed with greater accuracy. For example, we have shown that 3'-azido-3'-deoxythymidine acts to prevent the formation of proviral DNA (data not shown), as expected. Similarly, it will be interesting to examine the effects of inhibitors of regulatory genes such as tat, rev and nef in this system as they become developed.

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