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Kinetics of viral RNA synthesis following cell-to-cell transmission of human immunodeficiency virus type 1

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The temporal appearance and levels of human immunodeficiency virus type 1 (HIV-1) *tat*, *rev*, *nef*, *env* and *gag* mRNA species were examined using a synchronized, one-step, cell-to-cell HIV-1 infection model involving HUT-78 cells and HIV-1 persistently infected H3B cells. Individual mRNAs were quantified by RT–PCR using RNA standards transcribed *in vitro* from cDNA clones. Consistent with an infection that produces high yields of virus, significant levels of *env* and *gag* mRNAs were detected in the cytoplasm of infected cells late in the infection cycle. However, at no time after infection did levels of *tat*, *rev* and *nef* mRNA, which encode the regulatory proteins of HIV-1, exceed their levels present in the persistently infected virus donor H3B cells. The absence of early phase induction of these mRNAs is in contrast to what is observed in cell-free HIV-1 infections or in PMA-stimulated HIV-1 chronically infected cell lines. Our results suggest that *tat* and *rev* mRNAs are already present in the cytoplasm of the persistently infected virus donor cells at levels sufficient for initiation and establishment of a highly productive infection in HIV-1 fusion-mediated infected cells. Thus, lack of sufficient Tat and Rev proteins is not likely to be the limiting factor for virus production in H3B cells, nor is increased production of these proteins likely to be the cause of the increased virus production seen following cell-to-cell transmission.

Introduction

Human immunodeficiency virus type 1 (HIV-1) can infect T lymphocytes either as free virus particles or by direct transmission between infected and uninfected cells. HIV-1 spreads extensively during infection *in vivo* and is continually replicating and reinfecting new cells. Cytopathic, syncytium-inducing HIV-1 strains tend to predominate in patients progressing from lymphadenopathy to acquired immunodeficiency syndrome (Fenyo *et al.*, 1988). These virus strains efficiently spread through the fusion of infected and uninfected T cells and have been implicated in the rapid decline of $CD4^+$ T cells in AIDS patients (Gupta *et al.*, 1989; Li & Burrell, 1992; Lifson *et al.*, 1986; Yoffe *et al.*, 1987). Hence, cell-to-cell infection may represent a major mechanism of virus dissemination and disease progression *in vivo*.

During infection of T cells with cell-free virus a temporal shift in RNA transcripts is detected, from predominantly multiply spliced 2 kb mRNAs encoding the viral regulatory proteins early in infection, to viral genomic RNA and unspliced 9 kb and singly spliced 4 kb mRNAs, encoding the viral structural proteins, late in infection (Kim *et al.*, 1989*a*). A similar pattern of viral gene expression has also been observed in macrophages acutely infected with HIV and in chronically infected T cells and promonocytes following activation by phorbol esters (Michael *et al.*, 1991; Munis *et al.*, 1992).

Regulation of HIV-1 gene expression is complex and involves cellular- and virus-encoded factors. The viral Tat and Rev proteins, encoded by the multiply spliced mRNAs, are essential for HIV-1 replication and exert profound effects at the transcriptional and post-transcriptional levels of viral gene expression (reviewed by Cullen, 1994). Tat binds to the TAR element present on all nascent transcripts and upregulates transcription from the HIV-1 LTR by increasing transcription initiation and enhancing transcriptional elongation. Rev associates with the RRE element of unspliced and singly spliced HIV-1 transcripts and favours the accumulation of these transcripts in the cytoplasm. Rev appears to affect this through stabilizing transcripts in the nucleus, facilitating their transport into the cytoplasm and enhancing their translation. Nef, also encoded by multiply spliced viral mRNAs, is not essential for

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virus growth in T cell lines. Initial experiments suggested that Nef inhibited viral LTR-mediated gene expression (Maitra *et al.*, 1991; Terwilliger *et al.*, 1986), but these conclusions have not been supported using other model systems (Hammes *et al.*, 1989; Kim *et al.*, 1989*b*). Recent studies have shown that Nef protein enhances virion infectivity (Chowers *et al.*, 1994) and is required for optimal reverse transcription (Aiken & Trono, 1995; Schwartz *et al.*, 1995). After acute infection with cell-free virus in T lymphocytes and monocytes/macrophages, *tat*, *rev* and *nef* transcripts appear early and simultaneously and then increase, although at different levels (Guatelli *et al.*, 1990; Klotman *et al.*, 1991; Munis *et al.*, 1992).

We have previously established a cell-to-cell transmission model for one-step, synchronous HIV infection using cells persistently infected with HIV as virus donor cells and HIV-susceptible cells as recipient cells (Li & Burrell, 1992). In this model rapid *de novo* reverse transcription occurs and is mandatory for the new round of HIV replication that proceeds following cell-to-cell transmission (Li *et al.*, 1992). We also showed that possible contributions made by small amounts of cell-free virus in the system are insignificant and that donor cells, rather than free virions, were responsible for initiation of infection (Li *et al.*, 1992, 1994).

In the current study we employed this system as a model for cell-to-cell HIV infection and examined, by quantitative RT–PCR, the levels and distribution of *tat*, *rev*, *nef* mRNAs of the 2 kb class, *env* mRNA of the 4 kb class and *gag* mRNA of the 9 kb class following infection. We report that despite the sharp induction of HIV-1 *env* mRNA and genomic RNA and significant production of virus, levels of *tat*, *rev* and *nef* mRNA at no time exceeded the levels present in the chronically infected H3B donor cells that originally transmitted the virus.

Methods

■ **Cells and virus.** The virus donor H3B cells are a laboratory clone of H9 cells persistently infected with the HTLV-IIIB strain of HIV-1. The H3B cells contain an average of two proviral copies per cell and are > 95% HIV p24 antigen-positive as judged by immunofluorescence (Li & Burrell, 1992). HuT-78 cells are an uninfected $CD4^+$ lymphoblastoid cell line obtained from the NIH AIDS Research and Reference Reagent Programme. The cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum, L-glutamine and standard antibiotics at 37 °C and 5% CO_2. To ensure that cells were always at a similar stage of growth they were routinely subcultured at a density of 1×10^6/ml 18 h before each experiment.

■ **Virus infection.** Cell-to-cell HIV infection was initiated by coculturing H3B and HuT-78 cells at a ratio of 1:4 at a total cell density of 5×10^5/ml (Li & Burrell, 1992). Immediately after cell mixing (0 h) and at 2, 4, 8, 12, 16 and 24 h after mixing, cells were harvested and RNA extracted. The virus inoculum used for cell-free virus infection consisted of H3B cell culture medium after clarification to remove cells and debris. On average the virus titre of this inoculum was 10^5–10^6 $TCID_{50}$/ml. HuT-78 cells were infected with cell-free virus at a nominal multiplicity of 1 $TCID_{50}$ virus per cell using a centrifugal enhancement technique (Li & Burrell, 1992; Pietroboni *et al.*, 1989). RNA was extracted from cells immediately after exposure to the virus (0 h) and 4, 8, 12, 16 and 24 h later.

■ **RNA extraction and purification.** Cytoplasmic RNA was extracted from cells using the method of Gough (1988). Cell pellets containing $2{\cdot}5 \times 10^6$ cells were resuspended for 30 s in ice-cold lysis solution containing 0·65% NP40, 0·15 M NaCl, 0·01 M Tris–HCl pH 7·5 and 1·5 mM $MgCl_2$. Nuclei were pelleted in a microcentrifuge at 6500 r.p.m. for 1 min. Under these conditions, the pelleted nuclei appeared perfectly intact, without any indication of damage, when examined by phase-contrast microscopy after trypan blue staining. RNA was extracted from the cytoplasmic fraction in 7 M urea, 1% SDS solution and phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with ethanol. The RNA (equivalent to $2{\cdot}5 \times 10^6$ cells) was treated for 15 min at 37 °C with 1 unit/ml DNase 1 (RNase-free; Boehringer Mannheim) in the presence of 400 units/ml RNasin. Treated RNA was ethanol-precipitated and stored in aliquots of 1 μg/μl at −70 °C.

■ **Primers and probe oligonucleotides.** Oligonucleotides were synthesized by Bresatec or by the Division of Haematology, IMVS. The sequences and nucleotide positions of the oligonucleotides were based on the sequence of HIV-HXB2R (Myers *et al.*, 1990). β-Actin primers ba1 and ba2 were designed from the human cytoplasmic β-actin gene sequence, HUMACCYBB (Nakajima-Iijima *et al.*, 1985). The nucleotide sequences of the primers and probes used in this study are summarized in Table 1. The positions of these primers relative to the HIV proviral genome and the positions and lengths of the cDNA products and the probes used to detect these cDNAs are shown in Fig. 1.

■ **Generation of RNA copy number standards.** RNA standards were transcribed *in vitro* from pBluescript (pBS) clones containing each of the major H3B HIV RNA-derived cDNAs using the protocol specified by the manufacturer (Stratagene). The *in vitro*-transcribed RNAs were > 95% undegraded RNA product, as assessed by Northern blot hybridization probed with a cDNA-specific oligonucleotide. The RNA was also measured spectrophotometrically ($OD_{260}/OD_{280} > 1{\cdot}7$).

■ **Quantitative HIV-1 RT–PCR.** RNA (2 μg) was reverse transcribed to cDNA using 100 ng of each HIV-specific 3′ primer and 100 ng of human β-actin-specific 3′ primer and 1·8 Units of RAV 2 reverse transcriptase (Amersham) for 1 h at 37 °C per 20 μl reaction. Five μl of the reaction product was PCR-amplified in a 50 μl reaction containing 1·25 Units AmpliTaq (Perkin Elmer) under the following cycling conditions, depending on the HIV-specific primer pair used: adp1/adx2 (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min; repeat for 25 cycles then 72 °C, 5 min; 4 °C hold); adp1/ex2 or adg1/adg2 (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; repeat for 20 cycles then 72 °C, 5 min; 4 °C hold). In order to quantify each HIV-1-specific RNA transcript, different quantities of *in vitro*-transcribed RNA standards corresponding to the mRNA species being analysed were reverse transcribed and amplified in the presence of 2 μg of uninfected HuT-78 cellular RNA, using identical reagents and reaction conditions as with sample RNA, but in separate tubes. Four-fold dilutions of RNA standards were used. The cDNA products and ^{32}P-labelled *Hpa*II-digested pUC-19 size markers were resolved on 8% non-denaturing PAGE gels. The DNA was transferred electrophoretically (Bio-Rad Trans-Blot) onto Hybond-N + membrane at 50 mA for 1·5 h in 0·3 × TBE buffer and alkaline-fixed. For Southern blot hybridization, membranes were incubated at 55 °C for probes p1.4, p1.4a, p1.5 and pba, or 42 °C for probe pG, in prehybridization solution (25 × Denhardts, 6 × SSC, 0·5% SDS and 100 μg/ml denatured ssDNA) overnight, and then hybridized in the same solution containing 2·5 × Denhardts and the appropriate oligonucleotide probe, labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Pharmacia). The filters were washed (15 min per

Table 1. Oligonucleotide sequences of primers and probes used in this study

Name	Sequence	Sequence coordinates*
adp1 (+) primer	5′ tagtactgcagtctcgacgcaggactcggc 3′	nt 230–248[a]
adx2 (−) primer	5′ atgaatctagattccttcgggcctgtcggg 3′	nt 7967–7949[a]
ex2 (−) primer	5′ gaatctagatcccaaggagcatggtgcc 3′	nt 5840–5822[a]
adg1 (+) primer	5′ ccacctatcccagtaggag 3′	nt 1095–1113[a]
adg2 (−) primer	5′ tcttgccttatggccgggt 3′	nt 1418–1400[a]
p1.4 (−) probe	5′ tgtcgacacccaatt/cagtcgccgcccctc 3′	nt 5309–5324/289–274[a]
p1.4a (−) probe	5′ aggagatgcctaagg/cagtcgccggcccctc 3′	nt 5486–5501/289–274[a]
p1.5 (−) probe	5′ cgctgtctccgcttcttc/cagtcgccgcccctc 3′	nt 5505–5523/289–274[a]
pG (−) probe	5′ cttatgtccagaatgc 3′	nt 1191–1176[a]
ba1 (+) primer	5′ caactccatcatgaagtgtgac 3′	nt 2597–2618[b]
ba2 (−) primer	5′ ccacacggagtacttgcgctc 3′	nt 2892–2872[b]
pba (−) probe	5′ ccacacggagtacttgcgctc 3′	nt 2892–2872[b]

*a, HXB2R (Myers *et al.*, 1990); b, HUMACCYBB (Nakajima-Iijima *et al.*, 1985).

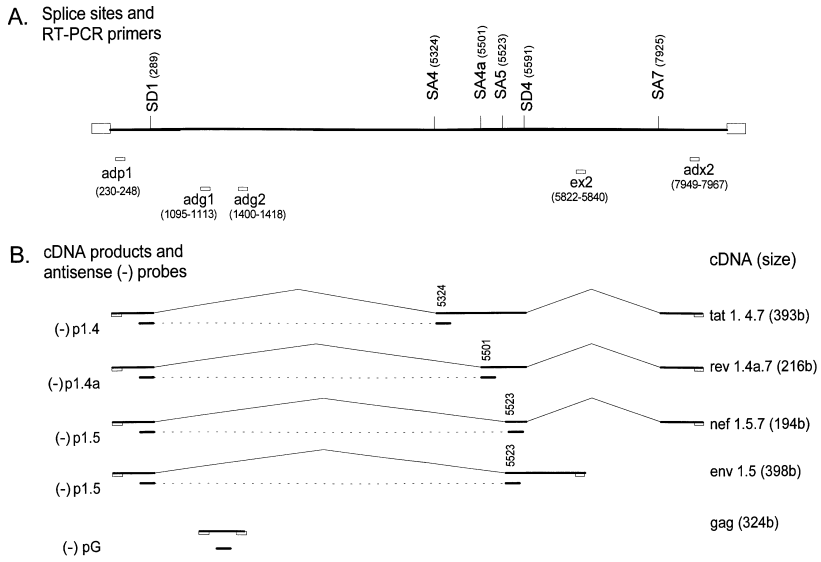

Fig. 1. Primers and probes used for the detection of alternatively spliced HIV-1 mRNAs by RT–PCR. (A) Organization of the genome. Solid line depicts the HIV-HXB2R genome, not drawn to scale, showing (above the line) the coordinates of splice donor (SD) and splice acceptor (SA) sites relevant to this study and (below the line) the oligonucleotide primers used in the RT–PCR reactions. Splice sites are numbered according to Schwartz *et al.* (1990). Primers with suffix 1 are sense and suffix 2 are antisense with respect to the RNA sequence. adp1 and adx2 amplify cDNAs of the 2 kb class of mRNAs *tat*, *rev* and *nef*. adp1 and ex2 amplify cDNAs of the 4 kb class of mRNAs which include *env* mRNA. adg1 and adg2 amplify unspliced RNA in the *gag* region. (B) cDNA products and antisense probes. Solid lines with open box ends depict cDNAs (aligned with the SD and SA sites which they utilize) and dotted lines depict excised introns. Numbers in brackets indicate the length of the respective cDNA products. Open boxes indicate the specific sense or antisense RT–PCR primer used. Lines under each cDNA represent the antisense probe used to detect the cDNA molecule by Southern blot hybridization. P1.4, p1.4a and p1.5 span the splice junction characterizing each mRNA variant. pG hybridizes to the exon of *gag*.

wash) at 65 °C, twice with 2 × SSC/0·1% SDS, three times with 0·2 × SSC/0·1% SDS (for probes p1.4, p1.4a, p1.5 and pba) or washed at 42 °C five times with 5 × SSC/0·5% SDS (for probe pG). Blots were analysed using PhosphorImage technology (Molecular Dynamics). From each blot a precise standard curve was generated, derived from the copy number standards. The copy numbers of corresponding HIV RNA species were then calculated from the linear range of the curve after each virus-specific signal had been normalized against the signal from β-actin mRNA which had been co-PCR-amplified in the same tube. The final copy numbers of HIV RNA species were based on an average of three separate gel experiments using the same RNA preparation.

■ **Nuclear run-off assay.** Samples of 5×10^7 cells harvested at different times after cell-to-cell infection, or uninfected HuT-78 cells, were centrifuged at 300 ***g*** for 4 min at 4 °C and washed twice with ice-cold PBS. The cells were resuspended in 5 ml lysis solution (10 mM Tris–HCl pH 7·4, 3 mM $MgCl_2$, 1 mM KCl and 0·1% NP40) for 2 min

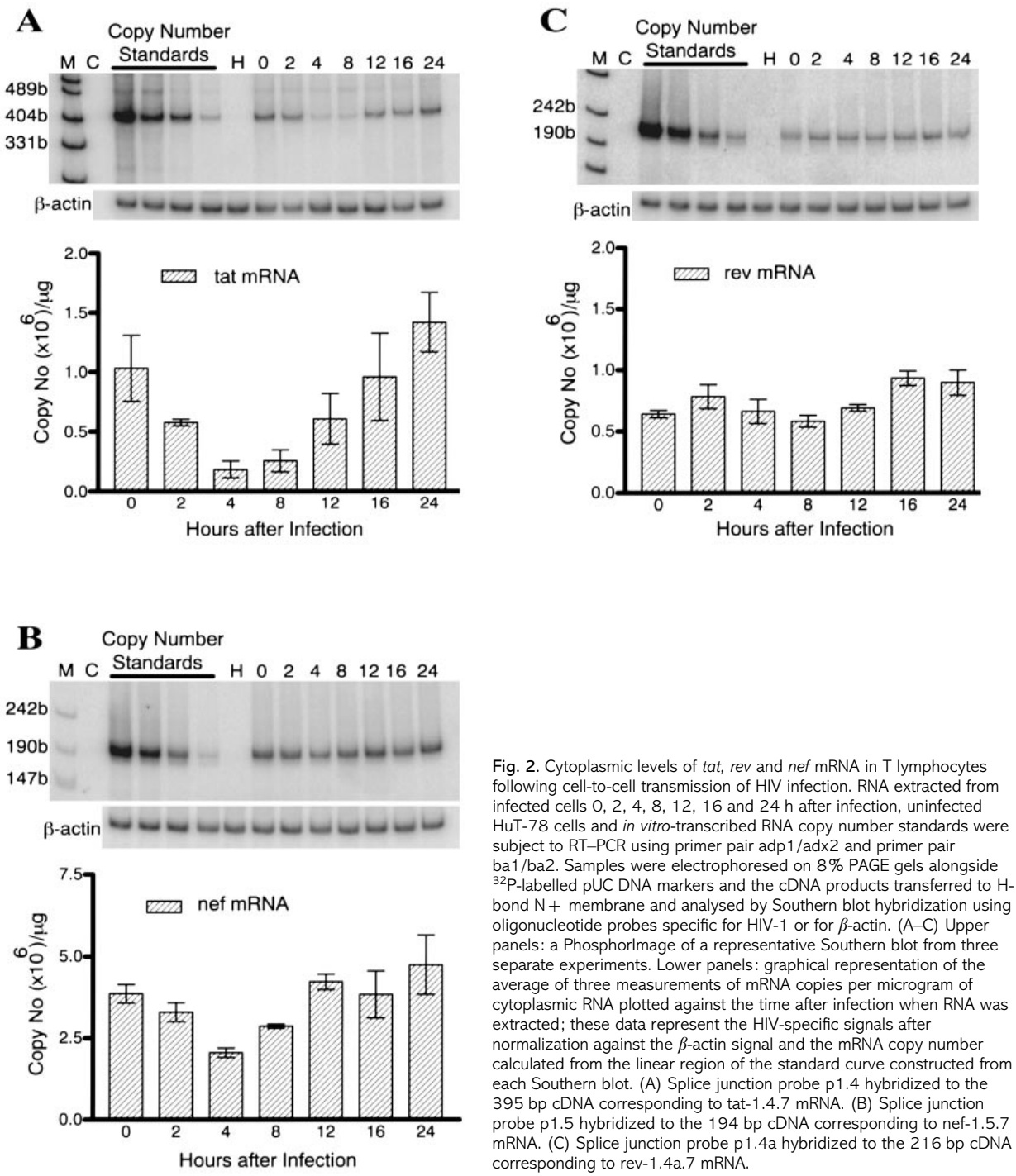

Fig. 2. Cytoplasmic levels of *tat*, *rev* and *nef* mRNA in T lymphocytes following cell-to-cell transmission of HIV infection. RNA extracted from infected cells 0, 2, 4, 8, 12, 16 and 24 h after infection, uninfected HuT-78 cells and *in vitro*-transcribed RNA copy number standards were subject to RT–PCR using primer pair adp1/adx2 and primer pair ba1/ba2. Samples were electrophoresed on 8% PAGE gels alongside ^{32}P-labelled pUC DNA markers and the cDNA products transferred to H-bond N+ membrane and analysed by Southern blot hybridization using oligonucleotide probes specific for HIV-1 or for *β*-actin. (A–C) Upper panels: a PhosphorImage of a representative Southern blot from three separate experiments. Lower panels: graphical representation of the average of three measurements of mRNA copies per microgram of cytoplasmic RNA plotted against the time after infection when RNA was extracted; these data represent the HIV-specific signals after normalization against the *β*-actin signal and the mRNA copy number calculated from the linear region of the standard curve constructed from each Southern blot. (A) Splice junction probe p1.4 hybridized to the 395 bp cDNA corresponding to tat-1.4.7 mRNA. (B) Splice junction probe p1.5 hybridized to the 194 bp cDNA corresponding to nef-1.5.7 mRNA. (C) Splice junction probe p1.4a hybridized to the 216 bp cDNA corresponding to rev-1.4a.7 mRNA.

and pelleted through a 1·5 M sucrose solution in the same buffer without NP40 at 1800 ***g*** for 10 min at 4 °C to remove cellular debris. The nuclei pellet was washed once in nuclear storage buffer [40% glycerol (v/v), 50 mM Tris–HCl pH 7·4, 5 mM $MgCl_2$ and 0·1 mM EDTA] and then stored in 220 μl aliquots of the same buffer in liquid nitrogen. Transcription reactions with [^{32}P]UTP were performed as described by Linial *et al.* (1985) using a 30 min incubation. Next, HSB buffer (0·5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM Tris–HCl pH 7·4) containing 100 units of DNase-1 (RNase-free; Boehringer Mannheim) was added, the sample was incubated at 30 °C for 1 h and then adjusted to 1% SDS, 5 mM EDTA, 10 mM Tris–HCl pH 7·4 and Proteinase K (200 μg/ml), and incubated at 37 °C for a further 1 h. RNA was extracted with phenol (water-buffered):chloroform:isoamylalcohol (50:49:1), precipitated and resuspended in an equal volume of prehybridization solution (5 × Denhardts, 5 × SSC, 0·5% SDS, 50% deionized formamide, 200 μg/ml denatured ssDNA and 50 μg/ml denatured yeast tRNA).

Typically 1–2 × 10^7 c.p.m. of total ^{32}P-labelled RNA was recovered from each reaction.

The hybridization targets were pBS plasmids containing either a 9·7 kb complete HIV-HXB2 cDNA sequence or a 2 kb human β-actin cDNA sequence. The plasmids were linearized and then 5 μg of each applied, after boiling, onto a strip of Hybond N+ membrane using dot-blot apparatus and the DNA fixed with 0·4 M NaOH. Each strip was placed in the abovementioned prehybridization solution without labelled RNA for 4 h at 45 °C. All of the radioactivity from each transcription reaction – equivalent to 5 × 10^7 nuclei – was denatured and added, and the filters hybridized in a total volume of 1 ml for 36 h at 45 °C. The filters were washed sequentially (each for 30 min) in 2 × SSC/0·1% SDS at 45 °C; 0·1 × SSC/0·1% SDS at 45 °C; 2 × SSC/RNase A (5 μg/ml) at 37 °C; and 2 × SSC at 37 °C. Hybridized labelled viral transcripts were measured by signal intensity using PhosphorImage technology, and transcription levels calculated at different times after cell-to-cell infection by normalizing the virus-specific signals with respect to the β-actin signal.

Results

Quantification of HIV-1 mRNAs

Within 4–6 h following cocultivation of H3B cells with uninfected HuT-78 cells, massive cell fusion and development of syncytia are observed. By 12 h, more than 90% of cells are incorporated into multinucleated giant cells. At 4 h post-infection (p.i.) unintegrated linear DNA is detected by Southern blotting. Maximum levels were observed by 8 h p.i. This is followed by the sequential appearance of circular DNA forms (Li & Burrell, 1992). The new round of HIV-1 replication begins and proceeds in a synchronous fashion.

To examine the levels of different mRNA species, cytoplasmic RNA was extracted from the cells at various times after cocultivation and subjected to RT–PCR using specific oligonucleotide primer pairs. Cytoplasmic RNA was used since the levels of viral mRNAs in this compartment would more closely reflect the temporal appearance of viral proteins and their functional significance during infection. Unique primer pairs and cycling conditions were chosen to detect mRNA species of each HIV-1 RNA class.

The copy number of each mRNA species was determined using a quantitative RT–PCR technique, similar to that of Seshamma *et al.* (1992). To quantify each species of HIV mRNA, 2 μg of total cytoplasmic RNA from each sample, and copy number standards spiked in 2 μg of cytoplasmic RNA from uninfected HuT-78 cells, were reverse transcribed and amplified in separate tubes using the same pair of primers and identical reverse transcription and PCR reagents and conditions. The RT–PCR products were thereafter analysed on the same gel. This approach determined copy number (per microgram of cytoplasmic RNA) for each RNA species, taking into account inherent differences in reverse transcription and PCR efficiency as well as blot hybridization conditions. In addition, since the same cytoplasmic RNA preparation at each time-point was used for the quantification of all viral RNA species, relative levels of mRNA species of all size classes could be compared. Finally, as a control for sample-to-sample variation within a single experiment, β-actin mRNA was co-amplified by RT–PCR and signal intensities of virus-specific bands then normalized against those of the β-actin mRNA. Estimates of copy numbers of each mRNA species were expressed as the mean value (±SD) of three independent RT–PCR/Southern blotting experiments.

The particular *tat*, *rev*, *nef* and *env* mRNAs measured (see below) have been shown in several studies to be the most abundant transcripts that encode those proteins (Purcell & Martin, 1993; Robert-Guroff *et al.*, 1990). This was confirmed in our study by the fact that their cDNA products tat-1.4.7, rev-1.4a.7, nef-1.5.7 and env-1.5, named according to the exons that they contain (Muesing *et al.*, 1985), were preferentially cloned at the exclusion of other minor species and preferentially hybridized to exon probes specific for all mRNA kinds. The predominance of these mRNAs was maintained 24 h after cell-to-cell infection (data not shown).

Levels of 2 kb RNAs following infection

Primers adp1 and adx2 flank all characterized splice sites along the HIV-HXB2 genome (Fig. 1A), but by using a one-minute extension period per PCR cycle only the shortest mRNAs (< 4 kb; unpublished data, also see Delidow *et al.*, 1993) – those contained within the 2 kb class – were amplified (Fig. 1 B). After gel electrophoresis of the RT–PCR products, the major species, tat-1.4.7, rev-1.4a.7 and nef-1.5.7, were detected separately by Southern blot hybridization with probes p1.4, p1.4a and p1.5, respectively, which span their unique splice junction sites (Fig. 1B). Results from triplicate experiments showed that levels of *tat* and *nef* mRNA (represented by a 393 bp and 194 bp cDNA product, respectively) decreased in the first 4 h after infection and then increased (Fig. 2A, B). The level of the major *tat* mRNA continued to increase up to 24 h p.i., while the level of the major *nef* mRNA increased from 4–12 h p.i. and then plateaued (Fig. 2A, B). In contrast, the major *rev* mRNA, detected by the 216 bp cDNA product, remained at a relatively constant level throughout the infection period (Fig. 2C). In each case it was striking that major *tat*, *rev* and *nef* mRNAs did not exceed H3B levels during the new round of virus replication, despite dramatic *de novo* synthesis of viral DNA (Li & Burrell, 1992; Li *et al.*, 1992) and sharp increases of 4 kb and 9 kb viral RNAs (see below).

Levels of 4 kb and genomic length RNAs

A second primer pair, adp1 and ex2, was designed to specifically co-amplify RNAs from the 4 kb class (Fig. 1A). The position of ex2 falls within the 3′ intron commonly spliced from all 2 kb transcripts thereby excluding their amplification, and in addition the one-minute PCR extension period prevented competing amplification of the longer unspliced 9 kb HIV-1 RNA. The most abundant Env-encoding mRNA transcript, env-1.5, was detected by hybridization with splice

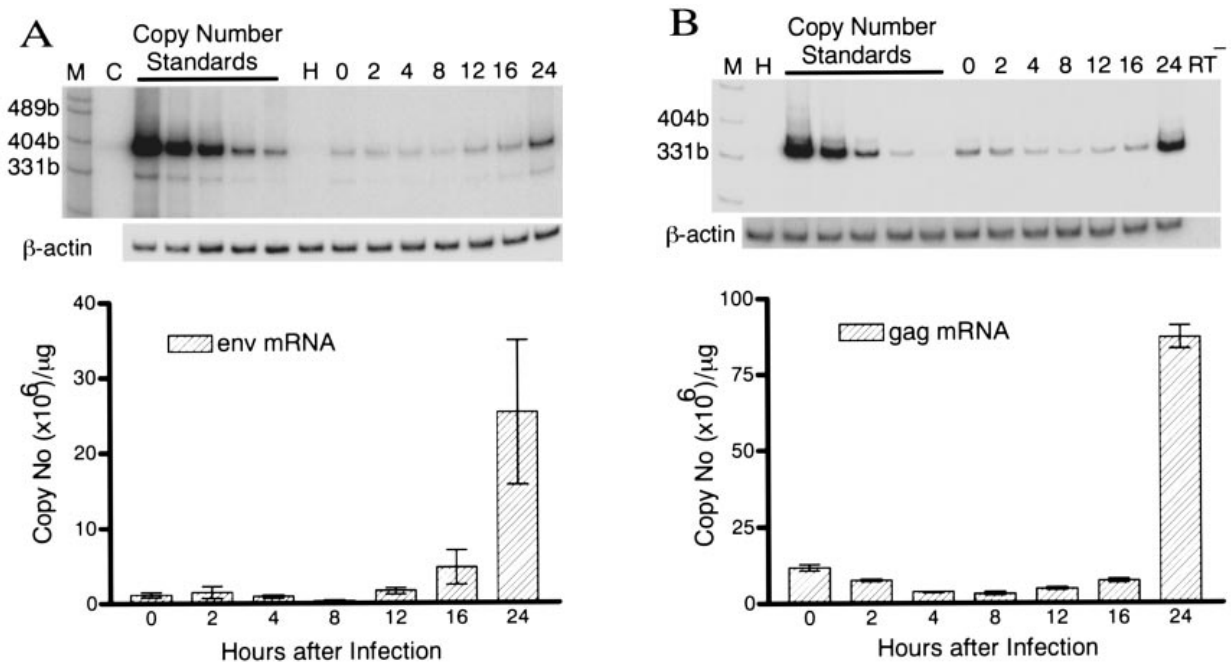

Fig. 3. Cytoplasmic levels of *env* and *gag* mRNA in T lymphocytes following cell-to-cell transmission of HIV infection. (A) Splice junction probe p1.5 hybridized to the 398 bp cDNA corresponding to *env* 1.5 mRNA. (B) Exon probe pG hybridized to the 324 bp cDNA corresponding to *gag* mRNA. For other details see legend to Fig. 2.

junction probe p1.5 (Fig. 1B). A third primer pair, adg1 and adg2, located within the *gag* gene of the HIV-1 genome (Fig. 1A), amplified unspliced 9 kb RNA only and this product was detected by hybridizing to a *gag* exon probe, pG. To check for contaminating DNA that could also be amplified by adg1 and adg2, an identical RNA extract harvested at 12 h p.i. when maximum levels of viral DNA are detected (Li & Burrell, 1992) was subjected to the same RT–PCR reaction without reverse transcriptase. No signal was detected in this track by Southern blot hybridization (Fig. 3B) indicating that the signals measured were specific for mRNA. Within 4–8 h after cell mixing, levels of both the *env* and *gag* mRNA decreased and then returned to original levels 12–16 h after infection (Fig. 3A, B). However, by 24 h dramatic increases of both *env* and *gag* mRNAs were observed. The increase was first detected at 16 h in the case of *env* mRNA (Fig. 3A, B). The levels of *env* and *gag* transcripts in the cytoplasm at 24 h p.i. corresponded to a 20-fold and 7-fold increase, respectively, compared with 0 h.

In comparing the relative copy number of the various transcripts, *gag* mRNA was the most abundant species in both steady-state chronically infected H3B cells and at 24 h after cell-to-cell infection (Fig. 4). Furthermore, the ratio of *gag* mRNA to *tat*, *rev* and *nef* mRNA was significantly greater at 24 h (20–80-fold) than that seen in H3B virus donor cells (3–12-fold). Within the 2 kb class, *tat* and *rev* mRNA consisted of $0{\cdot}5–1 \times 10^6$ copies/μg total cytoplasmic RNA at all times tested, while *nef* mRNA, which varied from $2–4 \times 10^6$ copies/μg total cytoplasmic RNA, exceeded *tat* and *rev* mRNA levels by approximately 3-fold. The greater abundance of *nef* compared to *tat* and *rev* transcripts is consistent with other reports (Purcell & Martin, 1993; Robert-Guroff *et al.*, 1990), although the higher levels of *rev* than *tat* mRNAs, also reported in those studies, was not seen in the current study.

mRNA levels after cell-free HIV infection

For comparison with the above, a similar study was set up using infection of HuT-78 cells with cell-free virus at a multiplicity of 1 $TCID_{50}$ virus/cell. The use of centrifugally enhanced inoculation would have resulted in an effective multiplicity of 10 $TCID_{50}$ virus/cell (Pietroboni *et al.*, 1989). During the adsorption period, cells and virus were initially incubated at 4 °C for 2 h, before being incubated at 37 °C to promote synchronous virus entry into cells. Very low levels of *tat*, *rev* and *nef* mRNA were detectable at 30 min p.i., with a significant increase between 12 h and 16 h after infection. Other investigators have also detected low levels of the 2 kb class mRNA very early after HIV infection (i.e. before reverse transcription and integration; Guatelli *et al.*, 1990; Klotman *et al.*, 1991). This may reflect splicing of incoming 9 kb RNA immediately targeted to the cell nucleus by p17 (Bukrinskaya *et al.*, 1992) or trace amounts of 2 kb mRNA in the virus inoculum. The first detection of *env* mRNA occurred later, at 16 h p.i., followed by a significant increase after 24 h during which time levels of *gag* mRNA had also increased dramatically (data not shown). This temporal shift in RNA expression after

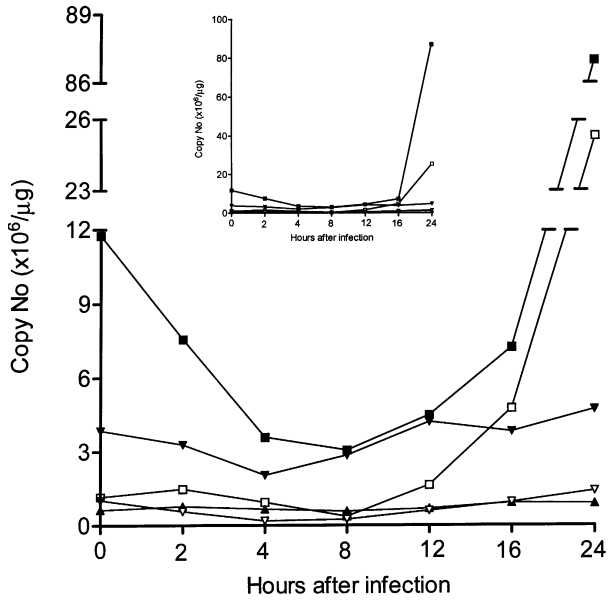

Fig. 4. Temporal expression of viral mRNAs following cell-to-cell transmission of HIV infection. Graph of the accumulated data of Fig. 2 and Fig. 3 representing copy numbers of *gag* (■), *env* (□), *nef* (▼), *tat* (▽) and *rev* (▲) mRNAs at various times following cell-to-cell infection. Graph inset is the large graph without adjustment of the *y*-axis.

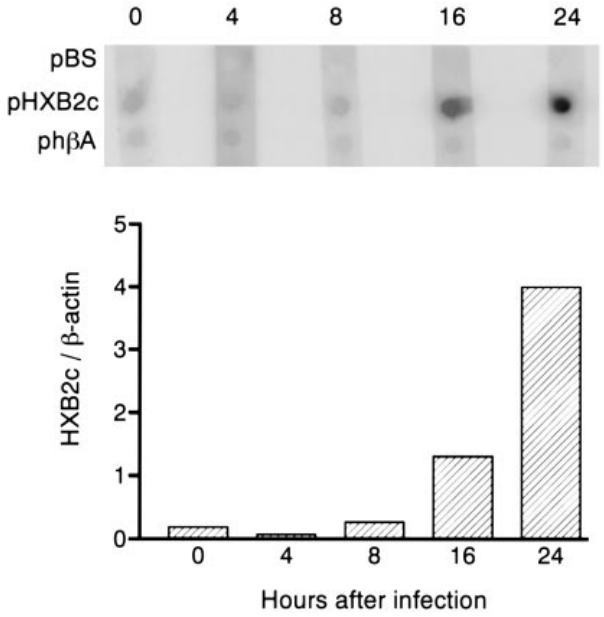

Fig. 5. Nuclear run-off analysis of virus transcription after cell-to-cell transmission of HIV infection. Upper panel: a PhosphorImage exposure of five identical filters hybridized to RNA extracted from infected nuclei isolated and labelled at the time-points shown. Transcripts of HIV and human *β*-actin were measured by reverse hybridization to three different plasmid DNA preparations spotted onto a single filter. Plasmid pHXB2c, pBS containing the complete DNA sequence of HIV; ph*β*A, pBS containing human *β*-actin cDNA; and pBS plasmid DNA used as a background control. Lower panel: corresponding graphical representation showing total HIV transcription normalized with respect to the *β*-actin signal at different times after infection. The data reflect results from two independent experiments.

cell-free HIV infection is in agreement with previous studies (Guatelli *et al.*, 1990; Kim *et al.*, 1989*a*).

Total HIV transcription in infected nuclei

Levels of cytoplasmic viral transcripts during an infection may be affected by a variety of factors including rates of RNA synthesis and degradation and nuclear–cytoplasmic transport. Therefore we endeavoured, using a 30 min pulse nuclear run-off assay from nuclei harvested at different times after infection, to estimate viral RNA transcription rates and correlate these with changes in cytoplasmic viral RNA levels following cell-to-cell infection. In the first 4 h after infection the rate of *in vitro* HIV-1 transcription from infected nuclei decreased 2·5-fold (Fig. 5). This coincided with a decrease in mRNA levels of *tat*, *nef*, and *gag* observed in the same period. In contrast there was no significant change in *β*-actin transcription, which was used as a control in the same experiment, consistent with the steady level of *β*-actin mRNA seen by RT–PCR (Figs 2 and 3). Between 4 h and 8 h the rate of virus transcription returned to initial levels, and then increased by 5-fold between 8 h and 16 h after infection, and a further 3-fold 16–24 h after infection. The total increase in primary HIV-1 transcription observed 8–24 h after infection corresponded closely with the cytoplasmic increase in *env* and *gag* mRNA levels between 16 h and 24 h.

Discussion

Following HIV-1 infection with cell-free virus, two phases of expression of viral RNA have been described. The first phase involves the induction of mRNAs of the 2 kb class, followed by a second phase characterized by induction of 4 kb and 9 kb classes of viral RNA. This pattern has been observed in acutely infected cells as well as in chronically infected cells after stimulation by PMA (Kim *et al.*, 1989*a*; Michael *et al.*, 1991). Different RNA expression patterns in other chronically infected cell lines have also been described (Butera *et al.*, 1994). We confirmed the paradigm in a high multiplicity cell-free HIV-IIIB virus infection of HuT-78 cells and observed the appearance of *tat*, *rev* and *nef* mRNAs in the cytoplasm 12–16 h after infection, and the marked increase in *env* and *gag* mRNA 4–8 h later. In contrast, following HIV-1 infection by cell-to-cell transmission, the new round of virus replication involved a similar late marked increase of *env* and *gag* viral RNA species, but without a preceding increase in 2 kb viral RNA species.

An almost constant level of *rev* mRNA throughout cell-to-cell HIV infection was unexpected. Rev promotes cytoplasmic transportation of *gag* and *env* mRNAs which contain the RRE element and it has been suggested that this function is dependent on Rev protein levels reaching a certain concentration threshold in the infected cell (Pomerantz *et al.*, 1992). Following cell-to-cell infection the appearance of *gag*

and *env* mRNA in the cytoplasm 16–24 h after infection is characteristic of Rev activity. Therefore, if the relatively constant level of *rev* mRNA seen above reflects the corresponding protein level, one would assume that the 'threshold' level of Rev may have already been present in the virus donor (H3B) cells in the beginning of cell-to-cell infection. Cytoplasmic transport of *env* and *gag* mRNAs may depend not only on Rev levels but also on other viral and cellular factors. For example, ablation of splice acceptor site 4b, utilized by some *rev* mRNA variants, resulted in reduced Rev protein levels but an increase in *env* mRNA and in Env (gp160/gp120) (Purcell & Martin, 1993). We propose that levels of *rev* mRNA and Rev protein in H3B cells may be functionally sufficient to sustain the increased levels of cytoplasmic *env* and *gag* mRNA during the first 24 h of cell-to-cell infection. In order to investigate this further, however, Rev protein levels will need to be analysed.

Nef is not essential for virus replication in T cell lines. In H3B cells, which are infected with the HXB2 strain, the *nef* gene encodes a premature translation stop codon preventing synthesis of functional Nef. Levels of *nef* mRNA remained more abundant than either *tat* or *rev* mRNA during the course of cell-to-cell infection, as has been reported in other kinetic studies. This may reflect the necessity for HIV-1 to conserve splice acceptor 5, utilized by both nef-1.5.7 and the most abundant env-1.5 mRNA variant.

Next, we demonstrated using a nuclear run-off assay that the rate of transcription from the HIV-1 promoter increased at 8–16 h and again at 16–24 h after cell-to-cell infection. Between 16 h and 24 h cytoplasmic levels of *env* mRNA and *gag* mRNA also increased significantly. We propose that an increase in transcription and the ability of Rev to shuttle between the nuclear and cytoplasmic compartments (Meyer & Malim, 1994; Richard *et al.*, 1994) contribute to the significant increase of cytoplasmic *env* mRNA and *gag* mRNA observed. Within the first 4 h after cell-to-cell infection the rate of transcription from the HIV-1 proviral DNA decreased (2·5-fold) while transcription of β-actin mRNA was unchanged (Fig. 5). The reasons for this are not known. Being so early, it would seem that it may have been triggered in direct response to cell-to-cell contact. A similar effect on HIV transcription has also been observed in numerous T cell lines transfected with HIV LTR-driven reporter gene constructs when exposed to defective HTLV-IIIB virus particles (Berube *et al.*, 1996).

HIV-1 transcription is regulated by both cellular and viral regulatory and auxiliary proteins. Low levels of Tat are required for processive transcription through its interaction with transcription factors such as TFIID (Kao *et al.*, 1987; Kashanchi *et al.*, 1994; Peng *et al.*, 1995). Among the cellular transcription factors, NFκB plays an important role in enhancing HIV-1 transcription (Folks *et al.*, 1987; Nabel & Baltimore, 1987; Osborn *et al.*, 1989). Reduced levels of NFκB-binding activity have been reported in the H3B virus donor cells used in this study. This led to the suggestion that reduced levels of NFκB activity in H3B cells may play a role in the relatively low level of viral gene expression (Zhang *et al.*, 1994). Similarly, in both monocytes (Raziuddin *et al.*, 1991) and unstimulated U1 cells(Pomerantz *et al.*, 1990), reduced levels of NFκB and restricted HIV replication are associated and may be causally related. It is therefore possible that the virus donor (H3B) cells contain *tat* mRNA levels (and Tat protein levels) sufficient for the new round of virus replication but transcription is suboptimal. Upon cell fusion and an influx of the active form of NFκB present in HuT-78 cells, increased viral transcription may then proceed without further requirements for further increases of *tat* mRNA beyond those levels already present in H3B cells (Zhang *et al.*, 1994). A similar situation may occur within HIV-1-induced syncytia formed between dendritic cells and primary T cells where NFκB and SP1 donated from the dendritic cell and T cell, respectively, could be responsible for upregulation of virus transcription (Granelli-Piperno *et al.*, 1995). In ACH-2 cells, which like H3B cell are chronically infected with HIV-1, it has also been found that levels of Tat are sufficient for increased HIV-1 production, and that the block to optimal proviral transcription may be mediated by other conditions such as chromatin condensation or methylation at the site of integration (Cannon *et al.*, 1994; Duan *et al.*, 1994). The increase of integrated proviral DNA, as a result of the productive infection following cell-to-cell virus transmission, may also contribute to the increase in HIV transcription.

In summary, we have found no evidence following cell-to-cell transmission of HIV infection for the 'classical' first phase of increased 2 kb class mRNA synthesis that is characteristic in cell-free HIV-1 infection and in chronically infected T cell lines following PMA stimulation. In contrast to a cell-free HIV-1 infection, *tat* and *rev* mRNAs were already available in the cytoplasm of the virus donor cells and in the resulting syncytia at sufficient levels to support the ensuing productive virus replication. Considering the potential role for cell-to-cell spread in transmission of HIV *in vivo* it is important for proper understanding of HIV pathogenesis that details of mechanisms involved be further clarified.

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