Initiation of reverse transcription during cell-to-cell transmission of human immunodeficiency virus infection uses pre-existing reverse transcriptase

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H3B cells, a laboratory clone of H9 cells persistently infected with the HTLV-IIIB strain of human immunodeficiency virus (HIV), contained significant levels of cell-associated reverse transcriptase (RT) activity measured by *in vitro* assays using either exogenous or endogenous templates. The cell-associated RT activity detected using exogenous template was almost wholly in a soluble (non-sedimentable) form whereas endogenous activity sedimented as a particulate structure associated with viral RNA. Despite this, H3B cells did not contain episomal HIV DNA detectable by Southern blot, indicating that *in vivo* reverse transcription was not occurring to any significant extent in these cells. However, when susceptible HUT 78 cells were infected by co-cultivation with H3B cells, dramatic synthesis of episomal HIV DNA occurred. Concurrently with this *de novo* initiation of reverse transcription, however, we found no detectable change in intracellular levels or cleavage profiles of immunoprecipitable RT polypeptides. Finally, actinomycin D pre-treatment of H3B cells to prevent *de novo* transcription from donor cell proviral DNA after co-cultivation did not affect the initiation of *in vivo* reverse transcription following cell-to-cell HIV infection. These results demonstrated that cells persistently infected with HIV contained significant fully cleaved cell-associated RT in a form that was active *in vitro* but not *in vivo* and that following cell-to-cell transmission of HIV infection to susceptible cells, *de novo* reverse transcription was initiated without detectable evidence of further synthesis or proteolytic processing of HIV RT. The nature of this initiation process requires further study.

Introduction

Cell-to-cell transmission of human immunodeficiency virus (HIV) infection is a potentially important mode of virus spread that may play a significant role in HIV pathogenesis (Gupta *et al.*, 1989; Kok *et al.*, 1993; Levy, 1993). We have previously established a one-step, single-cycle model for synchronous cell-to-cell transmission of 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 we have demonstrated rapid *de novo* reverse transcription, which is mandatory for the new round of HIV replication that follows cell-to-cell virus transmission. We also demonstrated that possible contributions made by small amounts of cell-free virus in the system were insignificant and that the donor cells, rather than free virions, were responsible for initiation of infection (Li *et al.*, 1992).

We next wished to define the origin and nature of the reverse transcriptase (RT) involved. It is generally believed that maturation of retroviral RT occurs at or after budding of the virus from infected cells (Witte & Baltimore, 1978; Eisenman *et al.*, 1980). Structural studies have suggested that HIV maturation may occur after virion assembly and budding (Gelderblom, 1991; Arnold & Arnold, 1991). The mature HIV RT found in the virion is a heterodimer p66/p51, in which the p66 chain contains both the polymerase and the RNase H activities whereas the p51 chain constitutes the N-terminal part of the p66 chain lacking the RNase H domain (Veronese *et al.*, 1986; Lightfoote *et al.*, 1986; Johnson *et al.*, 1986). A recent structural study by X-ray crystallography has established that the p66 subunit possesses the active polymerization site, whereas the p51 subunit with amino acid sequences identical to the N terminus of p66 is arranged into a different conformation which does not form the polymerization site (Kohlstaedt *et al.*, 1992). The p66 chain of the HIV RT is a proteolytic cleavage product of the HIV Gag–Pol precursor polyprotein p160 which is expressed as a minor product from the *gag–pol* open reading frame (Jacks *et al.*, 1988; Wilson *et al.*, 1988). Apart from RT, the *pol*

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gene of HIV also encodes the viral integrase and the viral protease that is responsible for the cleavage of the Gag and Gag–Pol precursor polyproteins and production of mature RT, integrase, protease and mature viral core proteins (Johnson *et al.*, 1986; Farmerie *et al.*, 1987; Debouck *et al.*, 1987). Despite all the detailed molecular characterization of the genes and gene products involved, the biological processing and maturation of HIV RT in relation to virion maturation remains poorly understood.

There are three possible mechanisms for the activation of *de novo* reverse transcription following cell-to-cell virus transmission. Firstly, the cell fusion that occurs upon co-cultivation might induce *de novo* transcription and translation of RT from donor cell proviral DNA. Secondly, as suggested for type C oncoviruses, pre-existing HIV RT precursors might be present in donor cells prior to budding, but not fully cleaved to the active form until cell fusion occurs. Thirdly, a mature form of RT might be present in donor cells but rendered inactive by some other mechanism prior to transmission of HIV infection to susceptible cells. In this study we show that persistently infected cells contained cell-associated RT that was active in *in vitro* assays despite the lack of detectable reverse transcription products within the cells *in vivo*. Following transmission of viral infection to uninfected susceptible cells, dramatic *de novo* reverse transcription was initiated with no detectable evidence of further synthesis or proteolytic processing of HIV RT. Thus it seemed likely that the initiation of reverse transcription following cell-to-cell transmission of HIV infection involved activation of pre-existing, fully cleaved enzyme by a mechanism that is at present unknown.

Methods

Cells and virus. HUT 78 cells were obtained from the NIH AIDS Research and Reference Reagent Programme. The H3B cells are a laboratory clone of H9 cells persistently infected with the HTLV-IIIB strain of HIV. H3B cells are $CD4^-$ and > 95% HIV p24-positive, as shown by immunofluorescence. H3B cells contain an average of two copies of integrated proviral HIV DNA per cell and secrete to the culture supernatant approximately 0·01 $TCID_{50}$ of virus/cell/h (Li & Burrell, 1992).

RT assays. Routine exogenous RT assays measuring [^{3}H]thymidine incorporation using a synthetic template [poly(A).$(dT)_{10}$] were carried out as described (Hoffman *et al.*, 1985). Endogenous RT assays were performed using a method essentially as described (Yong *et al.*, 1990) except that the final reaction mix contained 0·0125% Triton X-100 and 15 μg/ml melittin (Sigma).

Electron microscopy. Untreated H3B cells, H3B cells which had been vortexed for 3 × 10 s and washed, and H3B cells which had been further vortexed for 3 × 1 min and washed (see text) were fixed in 2·5% glutaraldehyde (0·05 M-cacodylate buffer pH 7·4) for 24 h at room temperature, post-fixed in 2% OsO_4 for 1 h, dehydrated in ethanol and embedded in TAAB resin. Thin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

Immunoprecipitation/Western blotting. RT-containing preparations (see text) were lysed in 1% Triton X-100 and 0·1% SDS and precipitated with anti-HIV RT monoclonal antibody (MAb) RTMAb6, kindly provided by Drs M. Tisdale and G. Darby of the Wellcome Research Laboratory (Tisdale *et al.*, 1988). The precipitated RT was collected by Protein A–Sepharose CL-4B beads, boiled in gel loading buffer and run on 10% or 10 to 20% gradient polyacrylamide gels. Pre-stained protein M_r standards (Bio-Rad) were included as markers. After Western blot transfer, the resulting nitrocellulose filter was sequentially probed with HIV-positive patients' sera, followed by biotinylated goat anti-human secondary antibody (Sigma), and then streptavidin-conjugated horseradish peroxidase (Dakopatts). Specific protein bands were visualized by using a commercial colour development kit (Bio-Rad).

Cell-to-cell infection and analysis of unintegrated viral DNA. A synchronous, one-step HIV infection model was used in which 5×10^5 H3B cells and 2×10^6 HUT 78 cells were washed and then co-cultured at a density of 5×10^5 cells/ml. At appropriate time points after infection, cells were lysed for analysis of associated RT by immunoprecipitation/Western blotting as described above, or for analysis of the synthesis of unintegrated HIV DNA by Southern blotting as described previously (Li & Burrell, 1992; Li *et al.*, 1992). Pre-treatment of H3B cells with actinomycin D and removal of budding-associated RT from H3B cells are described in the text.

Sucrose gradient sedimentation and RNA detection. Cell-associated RT preparations were adjusted to 0·5% Triton X-100 and 6% sucrose, then layered onto a 10 ml gradient of 15 to 30% sucrose in buffer A

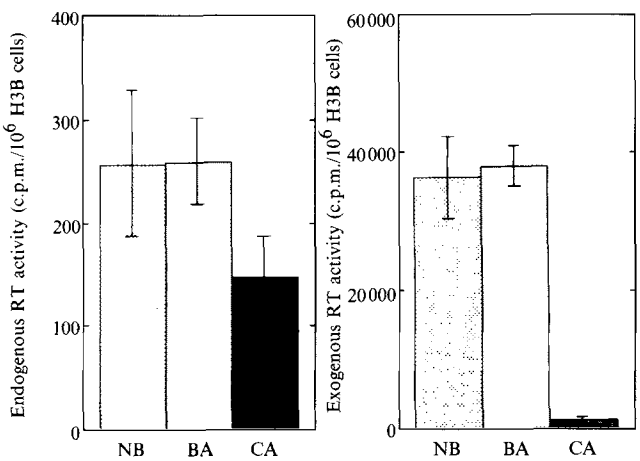

Fig. 1. Budding-associated and cell-associated RT in HIV persistently infected cells. H3B cells (2×10^7) were washed and incubated in 1 ml RPMI 1640 supplemented with 10% fetal calf serum at 37 °C for 1 h. RT released into the culture supernatant was referred to as naturally budded (NB). Cells were collected and resuspended in 0·5 ml medium and vortexed 3 × 10 s (see text). RT thus released into the culture supernatant was removed. Cells were collected and resuspended in 0·5 ml medium and further vortexed 3 × 1 min (see text). RT thus released into the culture supernatant was removed and combined with the previously released RT and referred to as budding-associated (BA). Cells were collected, resuspended in 1 ml medium and frozen and thawed three times. Cell debris was centrifuged down at 8500 *g* for 10 min. RT released into the clear lysate was referred to as cell-associated (CA). Exogenous and endogenous RT assays were carried out as described in Methods. RT activities, expressed as c.p.m. per 10^6 cells (±S.D.), are average values of three independent measurements.

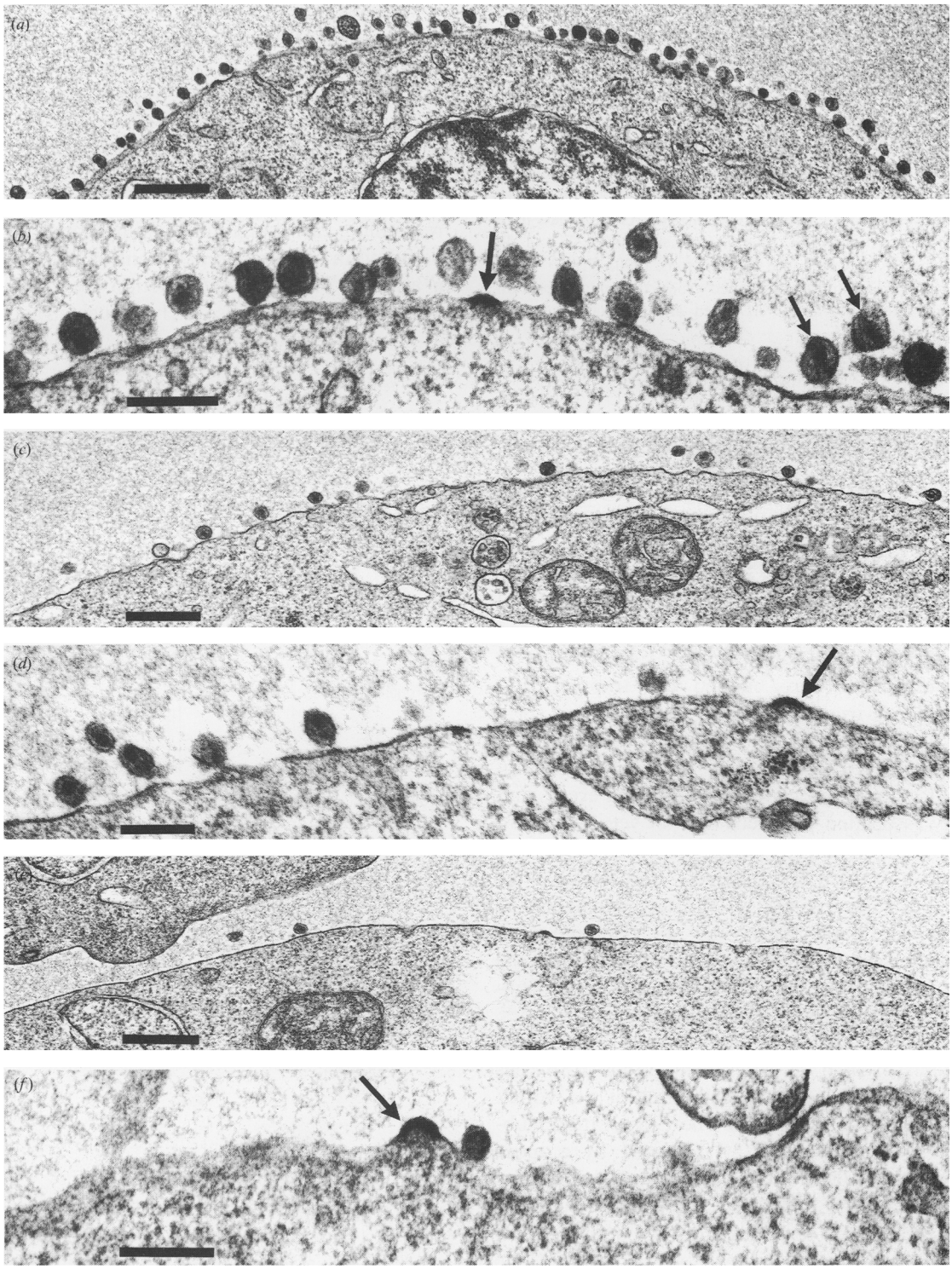

Fig. 2. Electron microscopy of H3B cells prior to or after removal of budding-associated virus particles. (*a* and *b*) Undisturbed H3B cells; (*c* and *d*) post-BA1 H3B cells (after vortexing 3 × 10 s); (*e* and *f*) post-BA1 and -BA2 H3B cells (after vortexing 3 × 10 s and 3 × 1 min). For details of sample preparation see text and Methods. Bars in (*a*), (*c*) and (*e*) represent 0·5 μm; bars in (*b*), (*d*) and (*f*) represent 0·2 μm. Small arrows in (*b*) indicate mature virions; large arrows in (*b*), (*d*) and (*f*) indicate an early budding structure which is considered to be part of the cell-associated RT preparation (see text).

(10 mM-Tris–HCl pH 7·4, 150 mM-KCl, 1 mM-DTT, 20 μg/ml aprotinin; Ellison *et al.*, 1990) containing 0·5% Triton X-100 and centrifuged for 90 min at 35000 r.p.m. at 4 °C using a Beckman SW41 rotor. Fractions (1 ml) were collected and 200 μl of each fraction was individually processed for RNA extraction using the urea–SDS method (Gough, 1988). RNA samples were then treated with RNase-free DNase I (1 unit per 100 μl reaction) at 37 °C for 1 h and extracted further with phenol–chloroform and precipitated by ethanol. Purified RNA samples were reverse-transcribed using RAV2 RT (Amersham), then amplified by PCR for 25 cycles. The two HIV Gag primers used corresponded to nucleotides 1082 to 1099 (primer 1) and 1227 to 1244 (primer 2) of the HXB2R sequence (Myers *et al.*, 1990). Primer 1 was labelled with [γ-^{32}P]ATP and the amplified products were analysed on 8% polyacrylamide gels which were dried and subjected to autoradiography.

Results

Cell-associated and budding-associated RT activities in cells persistently infected with HIV

In an effort to identify the RT activity involved in the rapid synthesis of unintegrated HIV DNA following cell-to-cell transmission of virus infection, we first investigated the virus donor cells (H3B) used in our cell-to-cell transmission model. Virus donor cells were vortexed under controlled conditions of increasing severity to release viruses that were budded but trapped, and partially budded viruses. As budding, defined as a form of exocytosis (White & Fenner, 1986), is a continuous process, it is difficult to make a clear-cut distinction between RT activity associated with budding events and RT activity associated with cells but not yet involved in budding. We defined the RT activity found in the culture supernatant as 'naturally budded', that released by controlled but severe vortexing (short of damaging the cells) as 'budding-associated' and the remaining RT activity released by repeated freezing and thawing of the severely vortexed cells, as 'cell-associated'. The controlled vortexing experiments were repeated many times. Fig. 1 shows the average values of three independent measurements of such an experiment. When H3B cells were vortexed at 80% of the full force of the vortex machine (Maxi Mix II; Thermolyne Corporation, subsidiary of Sybron) for 3×10 s, followed by a more vigorous vortex at 100% force of the vortex machine for a further 3×1 min, the force-released budding-associated endogenous and exogenous RT activities (BA columns in Fig. 1) were comparable to those of the naturally budded RT in a 1 h period from the same number of H3B cells (NB columns in Fig. 1). After removal of budding-associated RT, the cells were immediately frozen and thawed in fresh cell culture medium three times and the cell lysate was found to contain less, but significant, cell-associated endogenous and exogenous RT activities (CA columns in Fig. 1).

When examined by transmission electron microscopy, undisturbed H3B cells (Fig. 2*a*, *b*) typically showed numerous budded HIV particles and some fully mature HIV virions (small arrows) trapped near the surface of the cell plasma membrane, whereas representative electron micrographs of vigorously vortexed H3B cells (Fig. 2*e*, *f*) showed that these cells were largely free of budding-associated virus particles. However, structures resembling early stages of budding (large arrows) could be found in all three preparations of H3B cells (Fig. 2*b*, *d* and *f*). Therefore the fraction we refer to as cell-associated RT must also include such structures as well as internal cellular contents.

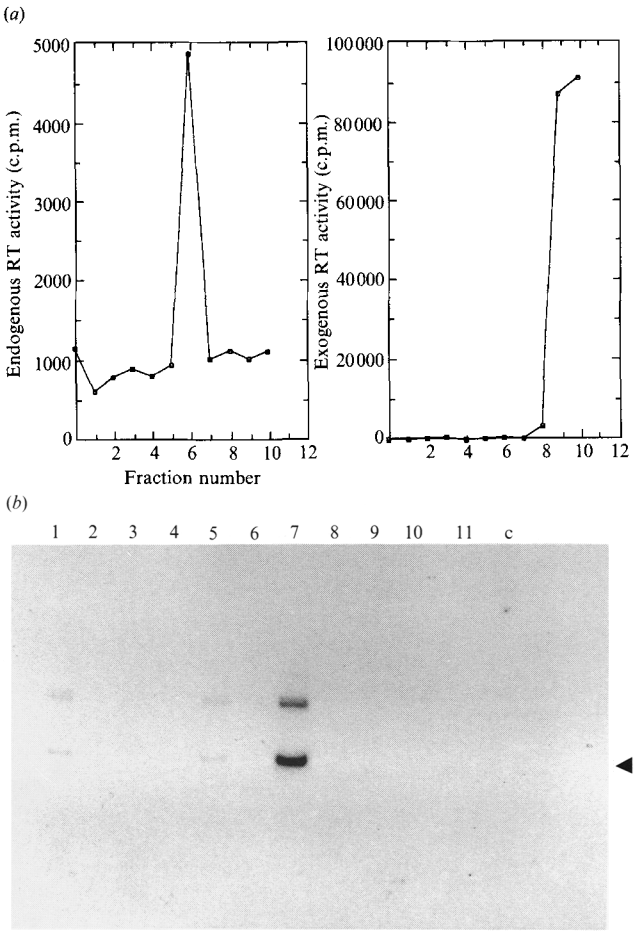

Fig. 3. Physical form of cell-associated HIV RT in H3B cells. A cell-associated RT preparation equivalent to 4×10^7 H3B cells was analysed by sucrose gradient centrifugation as described. Eleven 1 ml fractions (fraction 1, bottom; fraction 11, top) were collected. (*a*) Twenty μl of each gradient fraction was used for exogenous RT assay and 10 μl for endogenous RT assay. (*b*) Two-hundred μl of each fraction (1 to 11) was processed for genomic length HIV RNA detection by RT/PCR as described in Methods. Lane c, RT/PCR negative control. Arrow indicates the 160 bp amplification product.

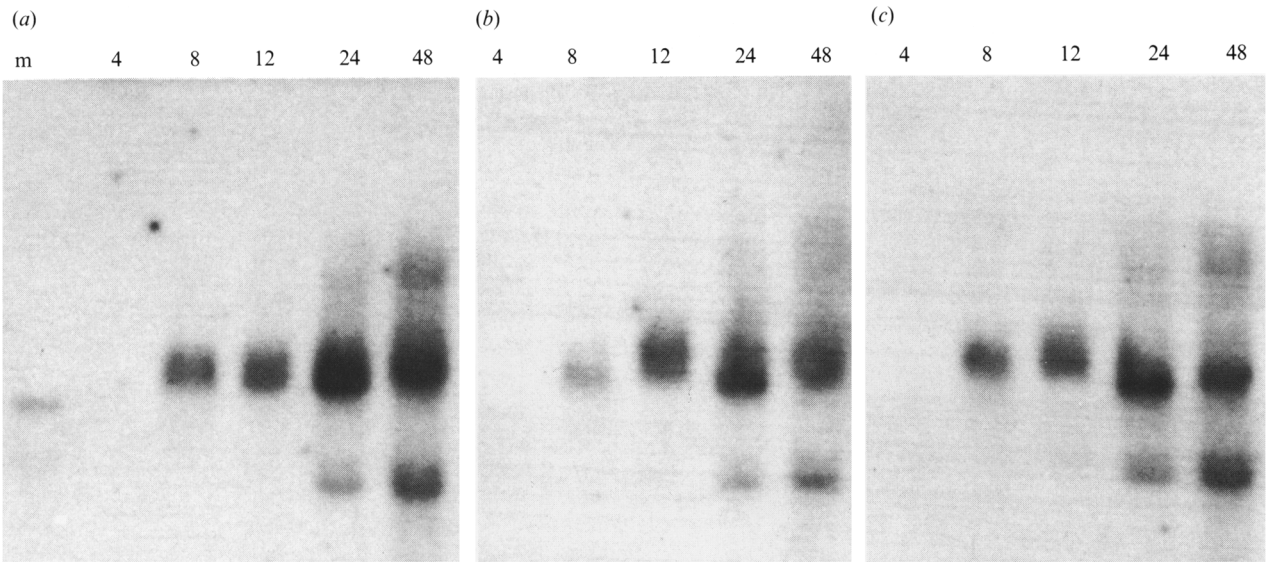

Fig. 4. Budding-associated virus was not required for HIV reverse transcription following cell-to-cell infection. Undisturbed H3B cells (*c*), H3B cells that had been vortexed 3 × 10 s (*b*), and H3B cells that had been vortexed 3 × 10 s followed by 3 × 1 min (*a*) were collected by low-speed centrifugation, resuspended in fresh medium and mixed with HUT 78 cells at a ratio of 1:4. At 4, 8, 12, 24 and 48 h post-mixing, extrachromosomal DNA equivalent to 6×10^5 cells was loaded onto each lane and viral DNA was analysed by Southern blotting as described in Methods. Lane m, *Sst*I fragment of HIV DNA from plasmid pBH10 (Hahn *et al.*, 1984) as marker, 100 pg.

Physical form of cell-associated RT in H3B cells

We next analysed the physical form of cell-associated RT, since only in specific association with viral RNA (not as free enzyme) can we expect RT to initiate the synthesis of viral DNA *in vivo*. The cell-associated RT preparation was first subjected to sucrose gradient sedimentation, then individual fractions were assayed for exogenous RT activity, endogenous RT activity and for the presence of viral RNA as described in Methods. Triton X-100 (0·5%) was included in the sucrose gradient to eliminate non-specific protein–RNA associations. As a result, the final endogenous RT reaction mix in this experiment contained 0·125% Triton X-100 and 15 μg/ml melittin. Fig. 3(*a*) shows that the majority of the cell-associated exogenous RT activity was found in the top two fractions (fractions 10 and 11) of the sucrose gradient, whereas endogenous RT activity was present mainly in one distinct peak, in fraction 7. These results indicated that whereas the majority of cell-associated exogenous RT was in a soluble (non-sedimentable) form, the endogenous activity was found in a particulate structure. Using HIV Gag-specific primers and RT/PCR, we demonstrated clearly that the gradient fraction containing this particulate structure (fraction 7) was also the major fraction containing genomic length, HIV viral RNA (Fig. 3*b*). This structure sedimented more slowly than the viral DNA-containing cytoplasmic replication complex found early after cell-to-cell transmission of HIV infection (Karageorgos *et al.*, 1993).

Budding-associated virus was not required for de novo *reverse transcription following cell-to-cell transmission of infection*

To examine whether budding-associated virus RT was required for full unintegrated HIV DNA synthesis following cell mixing, three types of H3B cells were prepared: undisturbed H3B cells, H3B cells that had been vortexed 3 × 10 s with the force-released RT removed (post-BA1), and H3B cells that had been further vortexed 3 × 1 min with the force-released RT removed (post-BA1 and -BA2). These three preparations were co-cultured with HUT 78 cells at a ratio of 1:4. Fig. 4 shows that, with all three types of cell preparation, the kinetics of unintegrated viral DNA synthesis were essentially identical. Further experiments, in which cell-free virus (in amounts equivalent to a 2 h natural release from the donor H3B cells) was added to the cell-to-cell transmission infection model, showed no change in the kinetics of unintegrated HIV DNA synthesis from that seen in a standard cell-to-cell transmission infection (data not shown). These results indicated that the extensively vortexed cell preparation contained all the factors required to initiate *de novo* reverse transcription following cell mixing.

Role of pre-existing donor cell RT in the initiation of reverse transcription following cell mixing

We next wished to examine whether pre-existing RT in

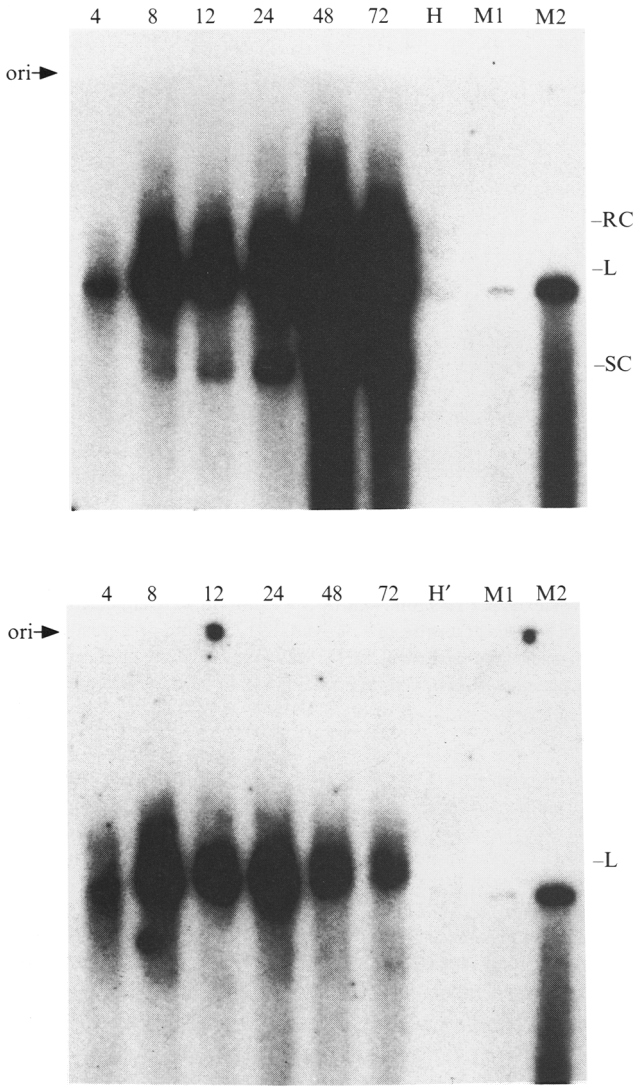

Fig. 5. *De novo* transcription from donor cell proviral DNA was not required for HIV reverse transcription following cell-to-cell infection. H3B cells (*a*), or H3B cells pre-treated with 5 μg/ml of actinomycin D for 2 h (*b*), were washed and co-cultured with HUT 78 cells. At 4, 8, 12, 24, 48 and 72 h p.i., extrachromosomal DNA equivalent to 1×10^6 cells was loaded onto each lane and viral DNA was analysed by Southern blotting. Lanes H and H′ represent extrachromosomal DNA from 1×10^6 untreated or actinomycin D-treated H3B cells alone, respectively. Lanes M1 and M2 represent 10 and 100 pg of the *Sst*I fragment of HIV DNA as a marker. RC, relaxed circular viral DNA; L, linear viral DNA; SC, supercoiled viral DNA (Li & Burrell, 1992). ori, Origin of migration.

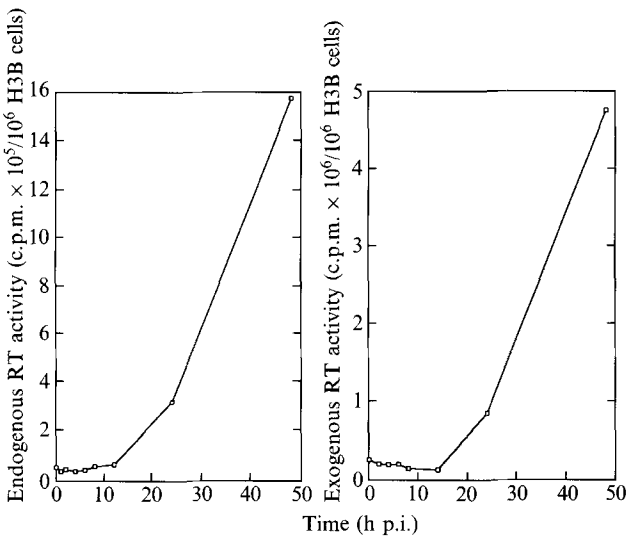

Fig. 6. RT activities following cell-to-cell HIV infection. H3B cells and HUT 78 cells were washed and co-cultured at a ratio of 1:4. At appropriate times after infection, cells were lysed in buffer A containing 0·5% Triton X-100 (see Methods). These lysates, which contain the combined budding-associated and cell-associated RT fractions, were assayed for endogenous (0, 1, 2, 4, 6, 8, 12, 24 and 48 h p.i.) and exogenous (0, 2, 4, 6, 8, 14, 24 and 48 h p.i.) RT activities. RT activities were expressed as c.p.m. per 10^6 virus donor (H3B) cells.

donor cells was sufficient for the dramatic level of reverse transcription seen following cell mixing. Three approaches were used. Firstly, H3B cells were pulse-treated with actinomycin D at 5 μg/ml for 2 h at 37 °C (Reich *et al.*, 1961; Goldberg & Rabinowitz, 1962). Under these conditions, actinomycin D treatment inhibited > 90% of total cellular RNA transcription as measured by [^{3}H]uridine incorporation, and RNA synthesis was not restored after washing to remove free actinomycin D (data not shown). When H3B cells pretreated with actinomycin D were washed and mixed with untreated HUT 78 cells in the absence of drug, viral circular DNA formation was largely inhibited but viral reverse transcription itself, as judged by the synthesis of linear viral DNA *in vivo*, was not affected (Fig. 5). As actinomycin D irreversibly inhibited > 90% of the RNA transcription from the virus donor cells, it was considered highly unlikely that induction of *de novo* viral RNA transcription from proviral DNA in donor cells was required for initiation of reverse transcription during cell-to-cell transmission of HIV infection.

Secondly, we examined whether co-cultivation of H3B cells and HUT 78 cells led to increased levels of budding- and cell-associated RT activity detectable in *in vitro* assays. H3B cells alone or H3B cells co-cultured with HUT 78 cells were harvested at different times after initiation of virus infection (or mock infection). Endogenous and exogenous RT activities were examined in cell lysates (prepared with 0·5% Triton X-100 and containing both budding- and cell-associated RT). Increased endogenous and exogenous RT activity was first seen 24 to 48 h after cell-to-cell transmission of infection (Fig. 6). Mock-handled H3B cells did not show a significant increase of RT activity within 48 h (not

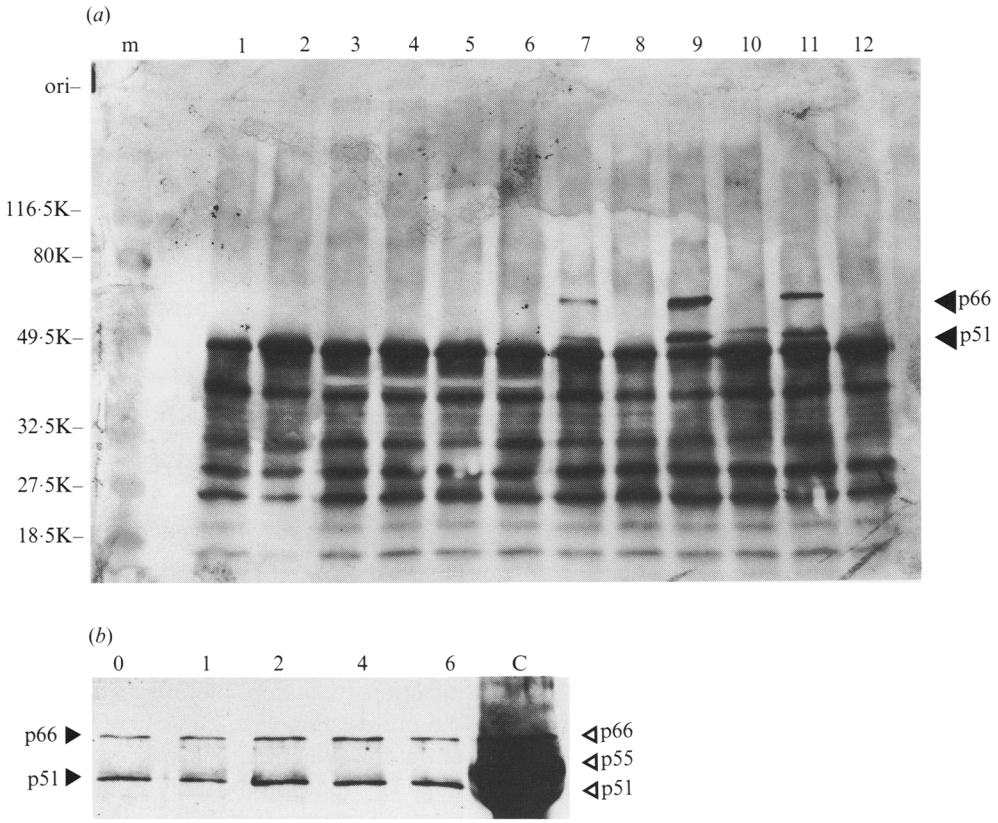

Fig. 7. Immunoprecipitation/Western blotting of HIV RT before and after cell-to-cell infection. (*a*) Naturally budded (NB, lanes 7 and 8), budding-associated (BA, lanes 9 and 10) and cell-associated (CA, lanes 11 and 12) fractions prepared from H3B cells (see Fig. 1) or mock preparations from uninfected HUT 78 cells (NB, lanes 1 and 2; BA, lanes 3 and 4; CA, lanes 5 and 6) were precipitated with anti-HIV RT MAb RTMAb6 (lanes 1, 3, 5, 7, 9 and 11) or mock-precipitated with normal mouse serum (lanes 2, 4, 6, 8, 10 and 12). Immunoprecipitated HIV RT samples were separated on a 10 to 20% gradient SDS–polyacrylamide gel and detected as described in Methods. Material from approximately 10^8 cells was loaded onto each lane. ori, Origin of polyacrylamide gel. Lane m, pre-stained protein M_r standards (Bio-Rad). (*b*) H3B cells and HUT 78 cells were washed and co-cultured at a ratio of 1:4. At 0, 1, 2, 4 and 6 h after co-cultivation cells were lysed and RT was precipitated with RTMAb6. Lane C is a 0 h sample precipitated with HIV-positive human serum. Material equivalent to approximately $1{\cdot}6 \times 10^7$ H3B cells was loaded onto each track and electrophoresed on a 10% SDS–polyacrylamide gel. Western blotting was carried out as described in Methods.

shown). However, and most importantly, there was no early [4 to 12 h post-infection (p.i.)] increase of either endogenous or exogenous *in vitro* RT activity at a time when *de novo* synthesis of unintegrated HIV DNA was maximal (Li & Burrell, 1992; Li *et al.*, 1992).

Thirdly, immunoprecipitation/Western blotting using anti-HIV RT MAb demonstrated that H3B cells contained, in cell-associated form, fully cleaved p66 and p51 RT polypeptide species but, surprisingly, very little if any RT precursor polypeptides (Fig. 7*a*). During the first 6 h after cell-to-cell transmission of HIV infection, there was no significant increase in either the p66 or p51 polypeptide of RT as judged by immunoprecipitation and Western blotting (Fig. 7*b*). Thus the dramatic *de novo* reverse transcription following cell-to-cell transmission of HIV infection seemed to take place without a detectable increase in *in vitro* RT activities or mature RT polypeptides p66 and p51.

Discussion

Earlier studies on type C oncovirus indicated that retrovirus RT maturation may occur at or after virion budding (Witte & Baltimore, 1978; Eisenman *et al.*, 1980) and recent studies with HIV suggested that the final maturation of virion morphology occurred after virion assembly and budding (Gelderblom, 1991; Arnold & Arnold, 1991; Haseltine & Wong-Staal, 1988). With this background, we wished to identify the source and molecular form(s) of RT activities involved in the *de novo* reverse transcription in the early phase of virus replication following cell-to-cell transmission of virus infection (Li & Burrell, 1992; Li *et al.*, 1992). In the present study, analysis by electron microscopy, tritiated thymidine triphosphate incorporation in exogenous and endogenous RT assays and immunoprecipitation/Western blotting indicated that enzymatically active

mature RT was still present in the persistently infected donor cells after removal of loosely trapped or budding-associated virus by vigorous vortexing. No conclusive evidence was found for a 160K Gag–Pol precursor in significant amounts in immunoprecipitates of the vortexed cell preparation. We also demonstrated that during the period of dramatic *de novo* reverse transcription following cell mixing *in vivo* (4 to 12 h p.i.), no detectable increase in *in vitro* endogenous or exogenous RT activity, or of RT polypeptides, occurred. Finally, we showed that donor cells from which the naturally budded and budding-associated RT fractions had been removed, and donor cells in which cellular transcription had been irreversibly inhibited by actinomycin D, could still initiate reverse transcription in the early phase of HIV replication as effectively as untreated cells. This suggests that persistently infected cells contained sufficient pre-existing RT for initiation of infection, but that this was in a form that was active *in vitro* but inactive *in vivo* until transmission of HIV infection to susceptible cells occurred.

The relationship between processing of Gag–Pol precursor polyproteins and both virion maturation and expression of RT activity is not clear. Hu & Kang (1991) reported that many forms of artificial HIV *pol* gene products, including partly cleaved forms, possessed RT activity in activity gels. However, using activity gel analysis of HIV RT, Starnes *et al.* (1988) demonstrated that p66 was the only polypeptide expressing HIV-specific DNA polymerase activity in infected H9 cells, and interpreted this polypeptide species as being derived from both intracellular RT and budded but trapped virions. Our recent unpublished data are consistent with this view. Recent structure–function studies (Jacobo-Molina & Arnold, 1991; Kohlstaedt *et al.*, 1992) indicate also that the active polymerization site is present only within a properly processed p66 subunit with appropriate conformation. In viral genetic studies, Peng *et al.* (1989) reported that transfection with protease mutants of HIV, in which cleavage of the Gag–Pol precursor would not occur, led to the production of non-infectious virions of abnormal appearance although RT activity was produced in some cases. Similarly, Park & Morrow (1991) described an HIV mutant that effectively processed the Gag–Pol precursor and produced significant RT activity without virion formation. Fully processed Gag products in the cytoplasm of wild-type HIV-infected cells has been reported by Kaplan & Swanstrom (1991) and intrinsic proteolytic activity of the precursor froms of HIV protease has been reported by Phylip *et al.* (1992). In the present study we have provided further evidence that in cells persistently infected with HIV, processing of the Gag–Pol polyprotein and maturation of viral RT may occur before or at budding (we consider our cell-associated RT forms some early budding structures; see Fig. 2). One of the antiviral strategies currently under evaluation is that of the viral protease inhibitors. In light of the discussion mentioned above, it would probably be important to test the efficacy and dosage of the potential viral protease inhibitors on both cell-free and cell-to-cell HIV infection models.

Initiation of reverse transcription by the enzyme present in cell-free virions may occur in two settings: (i) in *in vitro* assays, where partial or complete disruption of virion structure by detergents, together with provision of nucleotide precursors, the correct ionic environment and (for exogenous assays) a suitable template, may be sufficient to activate the reverse transcription process and (ii) during the early stage of replication in susceptible cells where the reverse transcription process is activated following partial virion uncoating in the cytoplasm and possible conformational changes in both the endogenous template and RT. The situation in cell-to-cell transmission of infection is not clear-cut. Initiation of *de novo* reverse transcription following transmission of HIV infection to susceptible cells is likely to involve mainly the pre-existing enzyme as demonstrated above. We do not know whether this process involves activation of relatively mature, partially budded virions by a similar mechanism to that for cell-free virus, or whether activation of RT in this setting uses a different mechanism following cell-to-cell contact and actually bypasses the mature virion stage. At least in our experiments, removal of as much partially budded material as possible by vigorous vortexing did not slow, to a detectable level, the ability of donor cells to initiate infection. In any case, the mechanism of suppression of reverse transcription in the cytoplasm of persistently infected cells is not known. Our findings of significant exogenous and endogenous *in vitro* RT activity in vigorously vortexed cell preparations, and of fully cleaved immunoprecipitable p66 and p51 RT polypeptides within the cells, suggest that neither a delay in cleavage of inactive RT precursor molecules to active, cleaved enzyme, nor a lack of native association between enzyme and template, is likely to be the reason for lack of ongoing reverse transcription in HIV persistently infected H3B cells. There are a number of other viral and cellular proteins that are specifically associated with the HIV reverse transcription–replication complexes following cell-to-cell transmission of viral infection (Karageorgos *et al.*, 1993) and the HIV nuclear capsid protein p7NC has also been reported to play an essential role in unintegrated viral DNA synthesis (Rice *et al.*, 1993). These and other factors may be involved in activation of reverse transcription. The activation process discussed above is different from the stimulation of virus replication in persistently infected cultures by

agents such as phorbol myristate acetate, which is likely to involve up-regulation of RNA transcription from integrated provirus (Pomerantz *et al.*, 1990; Michael *et al.*, 1991). We believe that further clarification of the mechanism of suppression of reverse transcription in persistently infected cells, and its activation after contact with susceptible cells, will highlight similarities and differences between cell-free virus and cell-to-cell infection models, and will add to our understanding of critical events during the initiation of HIV infection.

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