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Human Foamy Virus Reverse Transcription That Occurs Late in the Viral Replication Cycle

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Foamy viruses (FVs) are retroid viruses which use a replication strategy unlike those of other retroviruses and hepadnaviruses (S. F. Yu, D. N. Baldwin, S. R. Gwynn, S. Yendapilli, and M. L. Linial, Science 271:1579–1582, 1996). One of the striking differences between FVs and retroviruses is the presence of large amounts of linear genome-length DNA in FV-infected cells and in virions. We report here that large quantities of genome-length linear FV DNA accumulate in cells infected with FV, as determined by Southern blotting. To determine whether these unintegrated virus DNAs result solely from superinfection, we analyzed the occurrence of virus cDNA of the so-called human FV isolate (HFV) in cells transfected with a virus mutant deficient in the envelope gene and in cells which are resistant to superinfection due to stable expression of the envelope protein. We show that the synthesis of viral cDNA is independent of superinfection and that HFV synthesizes cDNA intracellularly as a late event in the replication cycle. To further confirm this finding, we performed inhibition studies with the reverse transcriptase inhibitor zidovudine (AZT). While AZT had no effect or only a minor effect on virus titers when added to cells prior to virus infection, viral titers were reduced by 3 or 4 orders of magnitude when the virus was produced from cells in the presence of AZT. Our results are most compatible with the hypothesis that the functional nucleic acid of the extracellular HFV consists of largely double-stranded linear DNA.

Foamy viruses (FVs) are a peculiar group of retroviruses (30, 42). Although their proviral genome structure is similar to those of other members of the family *Retroviridae*, some key features of the FV replication strategy are highly divergent. It has been shown recently that the so-called human FV isolate (HFV) prototype of *Spumavirinae* expresses its Pol protein independently from the Gag protein from a spliced mRNA (7, 15, 44). Moreover, a large amount of DNA was detected in gradient-purified extracellular virions of HFV (44), which raises the possibility of FVs being DNA viruses.

Basically, the retrovirus replication cycle can be divided into two phases (6). The early phase covers the steps of receptor-mediated entry, generation of cDNA by reverse transcription of the RNA genome, transport of the preintegration complex to the nucleus, and integration of the provirus. The late phase includes all steps involved in proviral gene expression, synthesis and processing of viral proteins, packaging of the diploid RNA genome, capsid formation, and release by budding from the cell membrane. Final processing of Gag and Pol precursor molecules (maturation) is believed to occur late in the budding process or after virus release (6).

The finding of DNA in extracellular virions (44) suggested to us that HFV may diverge from the retroviral replication cycle with respect to the point at which reverse transcription of the RNA genome occurs. We therefore wished to determine whether HFV is able to generate cDNA copies of the RNA pregenome late in the replication cycle by using conditions which prevent the early phase of viral replication.

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MATERIALS AND METHODS

Recombinant DNA. All recombinant-DNA work was carried out by established techniques (1, 32). Virus mutants were made in the pHSRV2 background (34), which is an infectious molecular clone bearing a deletion in the U3 region of the long terminal repeat (LTR) (35). All mutants were checked by DNA sequence analysis to exclude off-site mutations. DNA sequences were automatically determined by using AmpliTaqFS and the ABI 310 sequence analysis system (Perkin-Elmer).

Functional inactivation of the HFV *env* gene in M68 was made by Klenow filling-in of an *Mro*I site at the beginning of the *env* open reading frame. Protein analysis (21) of M68 following transient transfection into BHK-21 cells revealed that M68 is unable to express Env protein (data not shown). M69 harbors a mutation in the active site of the reverse transcriptase domain (17), which is highly conserved among reverse transcriptase enzymes from *Escherichia coli* to human retroviruses (23). The mutation was introduced by recombinant PCR (14) into a *Bsp*1407I/*Pac*I fragment which was then exchanged for the wild-type fragment of pHSRV2.

Plasmid pSP/HFV-1, which was used to generate the HFV riboprobe, was made by inserting a blunted 330-bp *Dra*III/*Kpn*I fragment spanning the 5′ part of the *pol* gene and excised from pGPZ-1 (15) into the *Sma*I site of pSP65. A 3-kb *Bam*HI fragment excised from pMOV-3 (13) was inserted into the pSP65 *Bam*HI site, leading to pSP/MLV-1. This plasmid was used to generate the murine leukemia virus (MLV)-specific riboprobe.

In plasmid pcHSRV2, the human cytomegalovirus (CMV) immediate early gene enhancer/promoter was substituted for the U3 region of the 5′ LTR of the proviral clone. In this plasmid, the transcriptional start site of the constitutive CMV promoter is identical to the HFV transcriptional start site in pHSRV2, analogous to a recent study with MLV (37). To create this plasmid, a double-stranded oligonucleotide which created the start of transcription and some convenient restriction sites was introduced into *Sac*I-cut pcDNA (Invitrogen). This was followed by stepwise addition of the pHSRV2 genome beginning from the *Sac*I site located at position +4 after the start of transcription as described previously (2).

The HFV *env* expression vector pENV3.1 was made by inserting a PCR fragment containing the *env* gene and incorporating *Eco*RI and *Bam*HI sites at the 5′ and 3′ ends, respectively, into expression vector pSG5 (Stratagene) under the control of the simian virus 40 early enhancer/promoter.

Cells and DNA transfections. BHK-21 and 293T cells were cultivated in minimal essential medium supplemented with 5 or 10% fetal calf serum, respectively. In addition, 1 μg of G418 (Sigma) per ml was used for the cultivation of BHK/LTRlacZ indicator cells. Titrations of cell-free (0.2-μm-pore-size filtrate) virus preparations were performed in these cells by a blue cell assay as described previously (34).

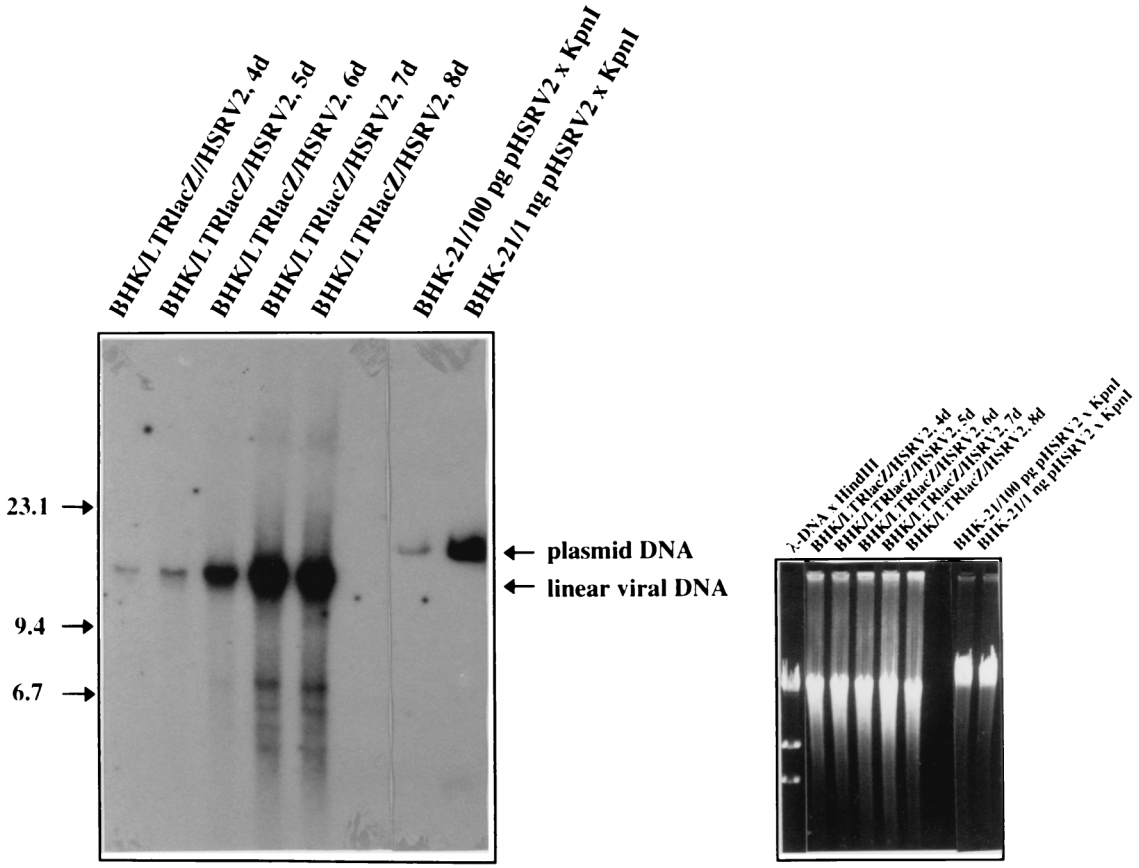


FIG. 1. Accumulation of linear HFV DNA in infected cells. BHK/LTRlacZ cells were infected with HSRV2 virus at an MOI of 0.05. From days 4 to 8 (4d to 8d) after infection, DNA from the infected cells (3 μg per lane) was analyzed by Southern blot hybridization with a ^{32}P-labeled riboprobe from the HFV *pol* gene (see Fig. 2). The indicated amounts of *Kpn*I-linearized pHSRV2 plasmid (15.66 kb) were diluted in BHK-21 cell DNA and served as a standard to calculate the viral copy numbers in the lytically infected cells. One hundred picograms of plasmid corresponds to approximately seven copies per cellular genome. Size markers (in kilobase pairs) are shown on the left.

BHK-21 cells were cotransfected with pENV3.1 and pBabepuro (26), and cells were selected and maintained in the presence of 0.5 μg of puromycin (Sigma) per ml. Following an initial phase of cell death due to the fusiogenic activity of the HFV Env protein, resistant colonies grew out and Env-expressing cells were detected by immunoperoxidase staining by using an FV-positive chimpanzee serum (4). A single cell clone (BHK/Env-1), which was used in all further experiments, was obtained by limiting dilution.

The amounts of plasmids indicated below (in the legends to Fig. 3 to 5 and in Table 1) were transfected into BHK-21, BHK/Env-1, and 293T cells as $CaPO_4$ coprecipitates (11). Upon transfection of the pcHSRV2 plasmid, transcription from the CMV promoter was enhanced by addition of 10 mM sodium butyrate (Sigma) to the culture medium and incubation for 12 h after transfection as described elsewhere (37). Zidovudine (AZT) was added to 293T, BHK-21, or BHK/LTRlacZ cells at a concentration of 5 μm, and the cells were incubated for the intervals indicated in the legend to Fig. 5.

Southern blot hybridization. DNA was extracted from infected or transfected cells by using a DNA preparation kit containing RNase A (Quiagen). DNAs were electrophoretically separated in 0.8% agarose gels and transferred to a nylon membrane (Schleicher & Schuell) in alkaline buffer. The generation of riboprobes by using SP6 polymerase (Boehringer Mannheim) and hybridizations were carried out according to standard protocols (1). Blots were washed under stringent conditions, with a final wash in 0.1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate)–0.1% sodium dodecyl sulfate at 65°C for 20 min, and were air dried and exposed to X-ray film at −70°C with an intensifying screen.

RESULTS AND DISCUSSION

Accumulation of linear genome-length HFV DNA in infected cells. It has long been noted that large amounts of unintegrated FV DNA are present in infected cells (24, 36). To determine exactly the virus copy numbers on a per-cell basis, we infected BHK/LTRlacZ indicator cells with HSRV2 at a multiplicity of infection (MOI) of 0.05. The viral DNA copy numbers in the cell culture were analyzed every day, from day 4 after infection until day 8, by Southern blot hybridization. In separate BHK/LTRlacZ cells infected with the same MOI, the number of virus-infected cells was determined via staining for β-galactosidase expression. As shown in Fig. 1, an average of seven copies of viral DNA per cellular genome was detected on day 4 after infection. At this time, 15% of the cells were infected. This amounts to approximately 45 virus DNA copies per infected cell. This value increased to 150 copies of viral DNA per infected cell on day 6 after infection (50% of the cells were infected). Thereafter, a value of more than 500 virus DNA copies per infected cell was calculated (80% infected cells on day 8). This value is in the same range of copy numbers determined previously for simian foamy virus type 3-infected cells (36). However, it is 10 to 100 times more than the amounts of unintegrated DNA reported for other retroviruses of avian, feline, and human origins (5, 16, 27, 43).

Superinfection has been implicated in the generation of unintegrated retrovirus DNA (reviewed in reference 38). To exclude this possibility and to investigate whether FVs may reverse transcribe the RNA pregenome late in the infection cycle, the following experiments were carried out.

Detection of cDNA transcripts in cells transfected with HFV mutants. Two virus mutants of the infectious molecular clone

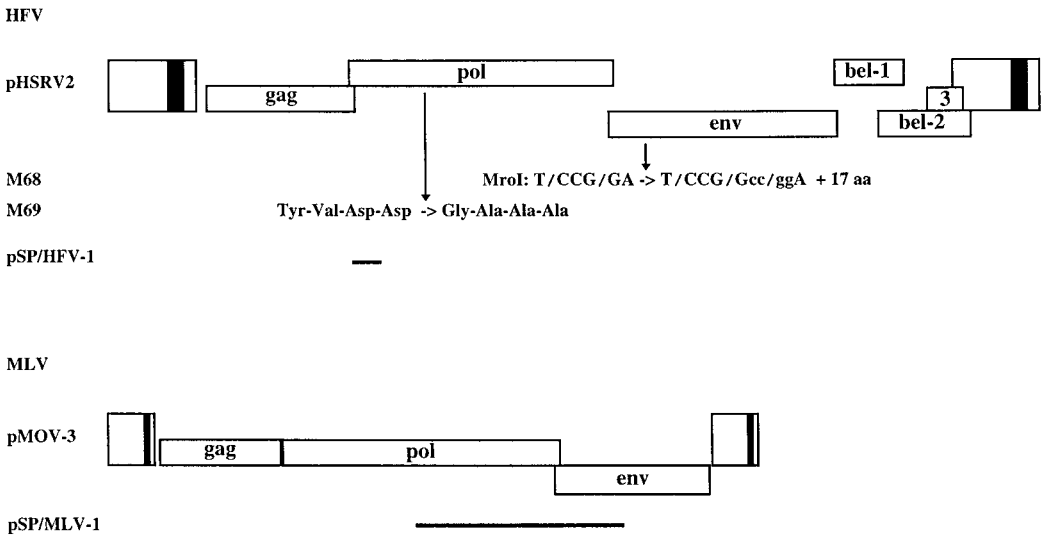


FIG. 2. DNA constructs used in this study. pHSRV2 is an infectious molecular clone which has a deletion in the U3 region of the LTR (35). M68 is a derivative of pHRSV2 with an inactivated *env* gene. In M69, the highly conserved active site (YVDD) of the HFV reverse transcriptase was mutated. Plasmid pSP/HFV-1 was used to generate the HFV riboprobe, and pSP/MLV-1 was used for the synthesis of the MLV-specific riboprobe.

pHSRV2 were constructed as shown in Fig. 2. M68 carries a frameshift mutation in the 5′ region of the *env* gene. Virus generated from this plasmid is not infectious, and the defect can be complemented in *trans* by Env-expressing cells (see below). Thus, M68 virus is unable to superinfect. M69 (Fig. 2), which carries a mutation of the active site of the reverse transcriptase, served as a control. Transient transfection of this mutant resulted in wild-type levels of expression of unprocessed and cleaved Gag and Pol proteins (data not shown). After introduction into BHK-21 cells, the plasmids were detected by Southern blot analysis in DNAs prepared up to day 4 after transfection (Fig. 3A). In addition to the plasmid, a band approximately 12 kb long was detected in DNA harvested from M68-transfected cells but not in DNA from M69-transfected cells. This DNA band comigrated with control DNA prepared from BHK-21 cells lytically infected with HSRV2 (Fig. 3A) and represents reverse-transcribed virus cDNA, since an active virus polymerase was required for its generation. When DNA from BHK-21 cells transfected with the pMOV-3 infectious molecular clone was analyzed by Southern blotting, the transfected plasmid DNA was detected but no reverse transcript approximately 8 kb in length was detected (Fig. 3B).

Detection of cDNA transcripts in cells resistant to superinfection. The detection of the reverse HFV transcript after transfection of an *env* gene-negative mutant may either indicate a physiological phenomenon or represent an artifact due to the assay conditions. Specifically, the generation of the reverse transcript could simply be a peculiar consequence of FV particles which are inhibited from leaving the cell. To exclude this possibility, we generated BHK-21 cells expressing the HFV Env protein (BHK/Env-1). Since no cell lines which lack the FV receptor are known, we reasoned that cells stably expressing the FV Env protein would down regulate the virus receptor and become resistant to superinfection. Such interference by receptor down regulation is well-known for murine and avian retroviruses (6, 12). Consequently, BHK/Env-1 cells were found to be completely resistant to superinfection at an MOI of 1 with an FV vector (pFOV-7) (34) encoding green fluorescent protein (pFOV-7/gfp) as a marker gene (data not shown). When these cells were transfected with pHSRV2 and the M68 plasmid, infectious virus was recovered from the supernatant, demonstrating that M68 and the integrated *env* gene of the BHK/Env-1 cells were functionally intact and that the defect of M68 could be complemented by Env-expressing cells (Table 1). As expected, the transfection was transient due to resistance of the BHK/Env-1 cells to superinfection (Table 1). DNA analysis of pHSRV2- and M68-transfected BHK/Env-1 cells revealed the generation of HFV reverse transcripts (Fig. 4). Together with the experiment whose results are shown in Fig. 3A, this result provides formal proof that reverse transcription of the HFV RNA pregenome can occur independently of and before the budding process as a late event in the viral replication cycle.

Influence of reverse transcription inhibitors on FV replication. AZT interferes with retroviral replication by inhibiting reverse transcription in the early phase of the replication cycle (25). AZT has already been shown to be effective against HFV replication (31). In that study, the drug was added during and after infection with HFV which was produced on human U373-MG glioma cells. While the viral 12-kb cDNA was clearly detected by Southern blotting in Hirt extracts of untreated cultures, only a very faint band appeared in AZT-treated cells (31). To determine the influence of AZT on virus replication, the following experiment was designed (Fig. 5). Plasmid pcHSRV2 is a derivative of the pHSRV2 infectious proviral molecular clone. The viral sequence of pcHSRV2 is identical to that of pHSRV2 with the exception of the 5′-LTR U3 region, which was replaced by the CMV immediate-early gene promoter. Transfection of pcHSRV2 leads to infectious virus which replicates continuously in susceptible cells (data not shown). However, pcHSRV2 has the advantage that transfection into 293T cells and virus production are more efficient than transfection of pHSRV2 into BHK-21 cells and virus production. pcHSRV2 was transfected into 293T and BHK-21 cells, and 48 h before the cell-free virus was harvested, the cells were either cultivated in the presence of 5 μM AZT or left untreated. Virus titers were determined on BHK/LTRlacZ indicator cells which had been pretreated with AZT for 4 or 24 h or left untreated. When virus was produced and assayed in the absence of AZT, pcHSRV2 led to appreciable amounts

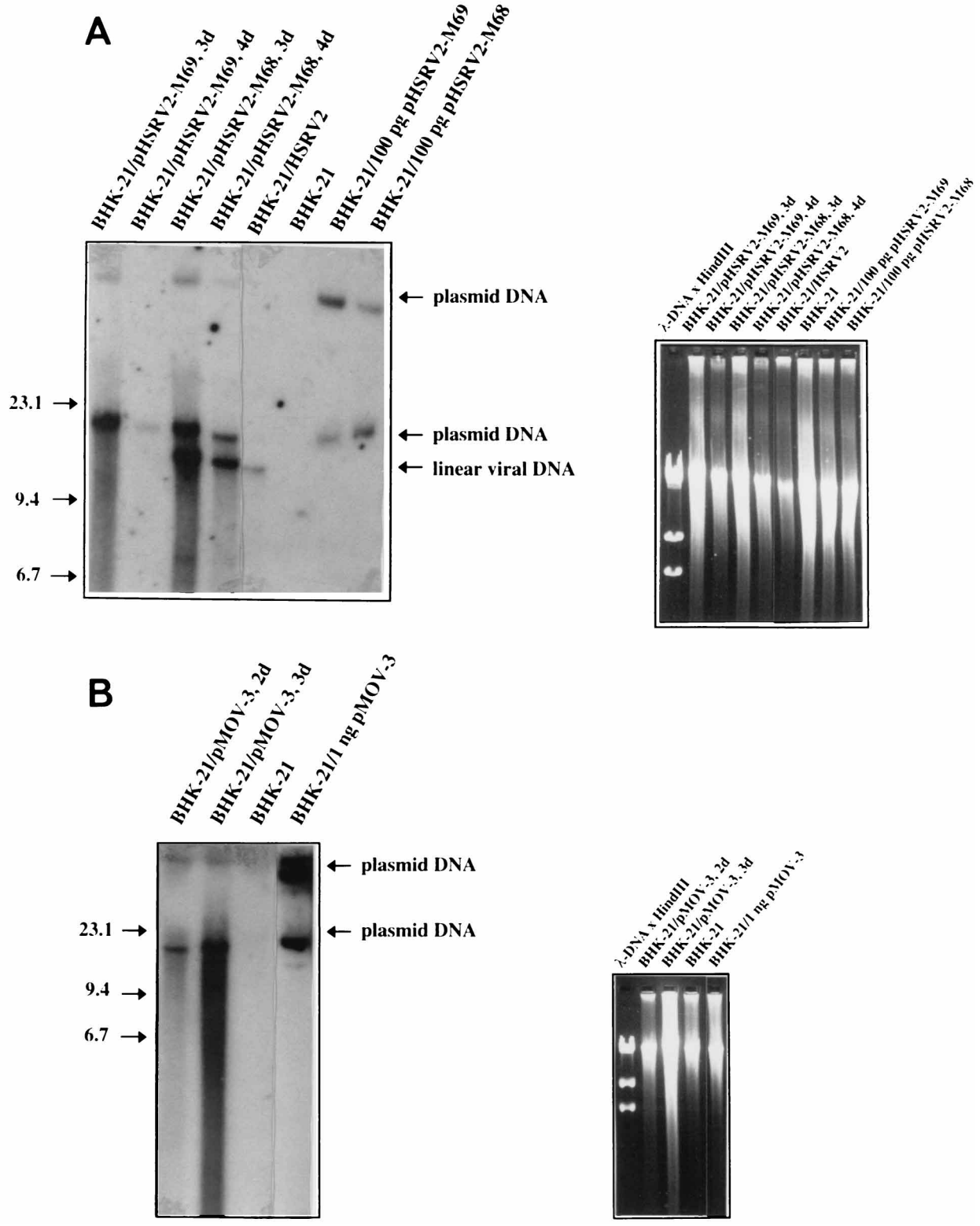


FIG. 3. Search for cDNA transcripts in cells transfected with HFV mutants (A) and the MLV plasmid (B). Plasmids (20 μg) were transfected into BHK-21 cells seeded in 75-cm^2 cell culture flasks. DNA was harvested on days 2 to 4 (2d to 4d) after transfection and analyzed by Southern blot hybridization. A 5-μg sample of DNA was loaded per lane, except in the control lane with DNA from HSRV2-infected BHK-21 cells (BHK-21/HSRV2), which was loaded with 1 μg of DNA. No significant difference in the result was noted when DNA was harvested on day 2 or 3 after transfection with HFV plasmids. Size markers (in kilobase pairs) are shown on the left.

TABLE 1. Complementation of the M68 *env* gene defect in cells stably expressing Env protein[a]

Day posttransfection	Virus titer[b]			
	BHK-21 cells		BHK/Env-1 cells	
	pHSRV2	M68	pHSRV2	M68
4	2.65×10^2	0	1.80×10^2	2.10×10^2
7	4.50×10^3	0	2.35×10^2	5
14	1.15×10^5	0	46.5	1

[a] BHK-21 cells and BHK/Env-1 cells were transfected with 10 μg of plasmid DNA. At the indicated days after transfection, the cell-free supernatant was assayed on BHK/LTRlacZ cells for infectious virus.

[b] Results are mean virus titers per milliliter of supernatant from two independent experiments with less than 20% variation.

of infectious virus 48 h after transfection of 293T cells or 112 h after transfection of BHK-21 cells (Fig. 5, positive control, experiment A). When AZT was present during virus production (in 293T cells or BHK-21 cells) and in the BHK/LTRlacZ indicator cells, no or almost no virus replication was detected (Fig. 5, negative control, experiment B). This demonstrates that AZT is effective against HFV replication at the concentration used. Interestingly, production of HFV in the absence of AZT and assay in the presence of AZT (experiment C in Fig. 5) led to only a modest decrease in virus titers, which depended on the cell type used to produce HFV and the preincubation time in the presence of AZT of the indicator cells. In sharp contrast, production of HFV in the presence of AZT and assay in the absence of AZT (experiment D in Fig. 5) resulted in virus titers similar to those of the negative control.

The reduction in the development of virus titers following transfection of BHK-21 cells and assaying in the absence or presence of AZT (Fig. 5, compare experiments A and C) is likely due to incomplete reverse transcription in the producer cells. An intermediate linear DNA with a single-stranded gap, which is located around the second polypurine tract in the middle of the HFV genome, has been detected in primate FVs (18, 36). It is tempting to speculate that this region is among the virus genome parts which have not been fully reverse transcribed in virion DNA. We also observed a slight decrease in titers of virus produced by transfection of 293T cells and assayed on indicator cells which had been preincubated with AZT for 24 h compared to preincubation for 4 h (Fig. 5, experiment C). This reduction is unlikely to be due to toxic effects of AZT on the cells and resulted probably from an increase in the amount of available triphosphorylated derivative (8).

This experiment clearly indicates that the majority of the reverse transcription of HFV has taken place before virus release from an infected cell. Partial reverse transcripts in virions have also been reported for human immunodeficiency virus and MLV, which may indicate some flexibility of retroviruses with respect to the timing of cDNA synthesis (22, 39). However, in contrast to the case for HFV, PCR methods had to be applied to detect mainly early transcripts. Furthermore, the addition of AZT to the virus-producing cells had little or no influence on viral infectivity, while AZT efficiently blocked replication when added to cells prior to infection with human immunodeficiency virus (3, 20, 39, 46).

Implications of reverse transcription timing for the FV replication strategy. The point at which reverse transcription oc-

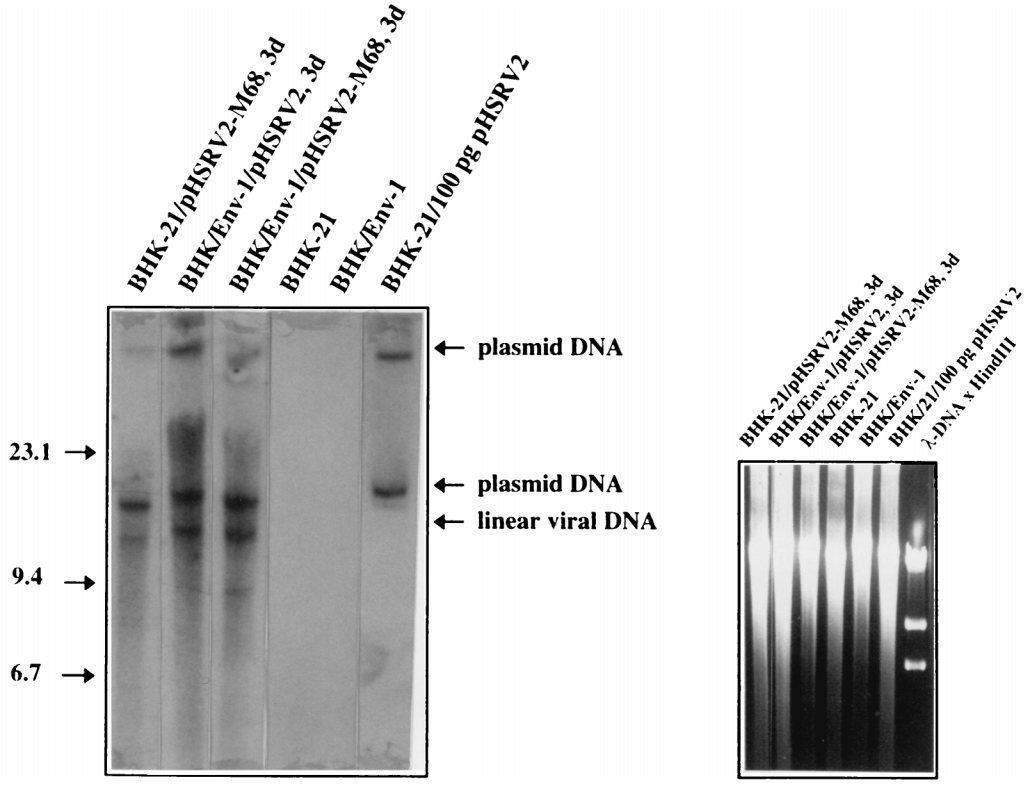
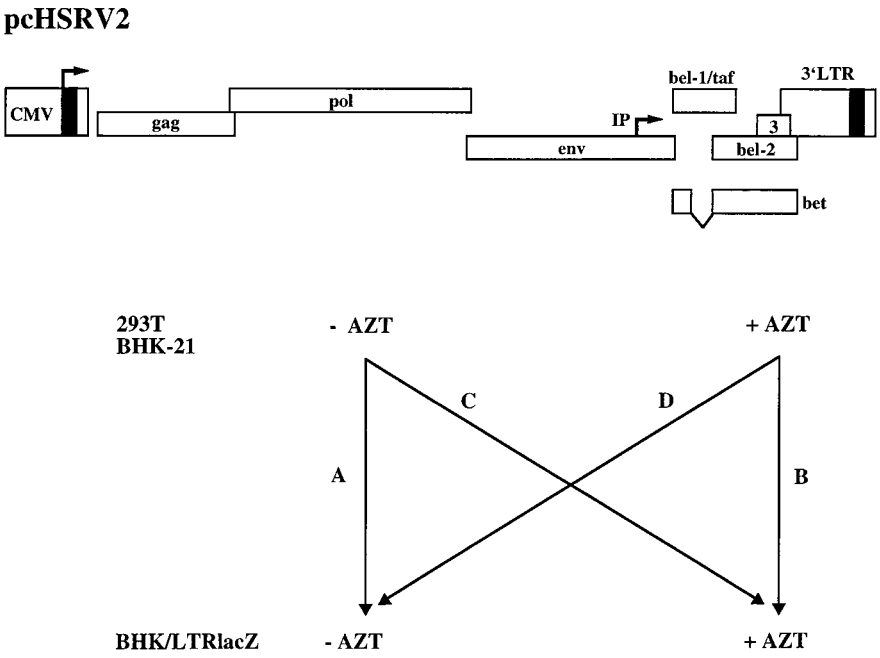


FIG. 4. HFV cDNA transcripts in cells stably expressing the Env protein. pHSRV2 and M68 were used to transfect BHK/Env cells, and DNAs were harvested on day 3 after transfection and analyzed by Southern blot. Size markers (in kilobase pairs) are shown on the left.



293T	BHK-21
A 3.93 x 10^3/ml	A 3.63 x 10^3/ml
B 1/ml (04 hrs AZT) 0/ml (24 hrs AZT)	B 2/ml (04 hrs AZT)
C 4.12 x 10^3/ml (04 hrs AZT) 1.43 x 10^3/ml (24 hrs AZT)	C 9.50 x 10^1/ml (04 hrs AZT)
D 2/ml	D 1/ml

FIG. 5. Influence of AZT treatment on the infectivity of HFV. The infectious molecular clone pcHSRV2 (20 μg) was used to transfect 293T or BHK-21 cells. Following transfection, the transcription from the CMV promoter was enhanced by the addition of sodium butyrate to the culture medium and incubation for 12 h. Cell-free virus was harvested 48 h after transfection of 293T cells and 112 h after transfection of BHK-21 cells and assayed for the virus titer on BHK/LTRlacZ indicator cells (experiment A). AZT was added to the virus-producing cells 48 h before virus harvest and to the indicator cells 4 h (virus produced from BHK-21 cells) or 4 and 24 h (virus produced from 239T cells) prior to the inoculation (experiment B). In experiment C, virus was produced in the absence and assayed in the presence of AZT, and in experiment D, virus was produced in the presence and assayed in the absence of AZT. The values shown are the means of two independent experiments with less than 20% variation.

curs is a major criterion by which retroviruses can be distinguished from hepadnaviruses (41). As described above, reverse transcription of the RNA genome occurs early in the retrovirus replication cycle. In contrast, reverse transcription of the RNA pregenome is a late event in hepadnavirus replication (29, 41). We have shown here that HFV is able to follow the latter strategy.

Close to full-length virion DNA has been detected in cell-free HFV produced in the human T-cell line H9 by Southern blot analysis, and no intermediate products which might have been expected if FVs were "mixed" viruses with respect to their genome were detected (44). However, only one in six to nine infectious particles was reported to contain DNA, leaving the curious possibility that the majority of viruses harbor an RNA genome (44). Such alternative RNA/DNA virus genomes would be unique (28) and difficult to analyze by biochemical methods (19).

In the light of the results reported here, we suggest that the functional FV genome consists of largely double-stranded linear DNA. The extent to which reverse transcription has proceeded may depend on the cell type used to produce the virus. The extracted virion DNA is not necessarily infectious, because virus enzymatic functions are needed for the completion of reverse transcription and for the integration of the virus genome into the new host cell genome. This model is compatible with previous reports on the FV replication strategy (31, 44). FVs would therefore combine the advantages of the retroviral and hepadnaviral replication pathways, i.e., the greater

stability of a DNA versus an RNA genome (42) and the possibility of integration into the host genome (40). Furthermore, it is tempting to speculate that FV reverse transcripts may be reimported into the nucleus (33, 45) and that the retention of the Env protein in the endoplasmic reticulum (9, 10, 30) may be advantageous for the virus to allow late reverse transcription to proceed.

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