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Retroviral recombination during reverse transcription

(Harvey murine sarcoma virus/Moloney murine retrovirus/polymerase chain reaction/viral protein requirement/identical recombinants *in vitro* and *in vivo*)

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ABSTRACT **After mixed infection, up to half of related retroviruses are recombinants. During infection, retroviral RNA genomes are first converted to complementary DNA (cDNA) and then to double-stranded DNA. Thus recombination could occur during reverse transcription, by RNA template switching, or after reverse transcription, by breakage and reunion of DNA. It has not been possible to distinguish between these two potential mechanisms of recombination because both single-stranded cDNA and double-stranded proviral DNA exist in infected cells during the eclipse period. Therefore we have analyzed for recombinant molecules among cDNA products transcribed *in vitro* from RNA of disrupted virions. Since recombinants from allelic parents can only be distinguished from parental genomes by point mutations, we have examined the cDNAs from virions with distinct genetic structures for recombinant-specific size and sequence markers. The parents share a common internal allele that allows homology-directed recombination, but each contains specific flanking sequences. One parent is a synthetically altered Harvey murine sarcoma virus RNA that lacks a retroviral 3′ terminus but carries a Moloney murine retrovirus-derived envelope gene (*env*) fragment 3′ of its transforming *ras* gene. The other parent is intact Moloney virus. Using a Harvey-specific 5′ primer and a Moloney-specific 3′ primer, we have found recombinant cDNAs with the polymerase chain reaction, proving directly that retroviruses can recombine during reverse transcription unassisted by cellular enzymes, probably by template switching during cDNA synthesis. The recombinants that were obtained *in vitro* were identical with those obtained in parallel experiments *in vivo*.**

Upon simultaneous infection, up to 50% of the allelic sequences of distinct retroviruses recombine, indicating efficient homologous recombination (1–3). Even illegitimate recombination among retroviruses has been reported to be "surprisingly efficient" (4). Illegitimate recombination during reverse transcription has been proposed to explain the characteristic ability of retroviruses to transduce cellular sequences (4–11, 28). This hypothesis was based on the efficient regeneration of Harvey murine sarcoma virus (HaSV) from a truncated provirus that lacked a 3′ long terminal repeat (LTR) upon transfection in the presence of helper Moloney murine retrovirus (MoV) (4). However, reinvestigation of this experimental system indicated that recombination during reverse transcription is efficient only when sequence homology exists between the parental genomes (12, 13). Efficient HaSV regeneration from the truncated provirus was instead due to tandem recombinations of proviral DNAs during transfection (12, 13).

Since retroviral RNA genomes in infected cells are converted first to complementary DNA (cDNA) by reverse transcriptase and then to double-stranded proviral DNA, recombination could occur by template switching during cDNA synthesis or by breakage and reunion of proviral DNA (14–16). As both cDNA and double-stranded proviral DNA exist simultaneously in the cell, it is not possible to distinguish between these two mechanisms *in vivo* (14, 15). One previous study (16) investigating recombination during reverse transcription *in vitro* by electron microscopy showed largely double-stranded DNA structures with single-stranded crossovers between molecules. These structures have been interpreted as the results of fragmented plus-strand DNAs invading gapped regions of double-stranded DNAs. Therefore it was proposed that such structures serve as precursors for conventional DNA recombination in the cell (16).

Here we have investigated cDNAs transcribed *in vitro* from distinct parental RNAs for recombinant structures. For this purpose it was critical to develop a system that allowed biochemical detection of recombinants without the help of allelic markers that are biologically distinguishable *in vivo* (1–3). Since homologous recombinations between allelic parents would produce a series of recombinants that would differ only in the parental origins of point mutations and, hence, be extremely tedious if not impossible to sort out biochemically, we studied homologous recombination between parents that shared a homologous sequence flanked by specific heterologous sequences. Crossovers within the shared homology would thus generate recombinants with termini from each parent. Specifically, we searched for recombinants in which a truncated 3′ terminus of Harvey sarcoma provirus was replaced by a 3′ terminus from Moloney provirus by a crossover within an internal envelope gene (*env*)-specific homology. Detection of recombinant molecules was based on selective amplification with the polymerase chain reaction (PCR), using one primer that was specific for the HaSV parent and another that was specific for the MoV parent. By this method recombinant cDNAs were shown to be generated directly during reverse transcription. These results confirm the template-switching model (14) but not the DNA strand-invasion model (16) for homologous recombination upon reverse transcription.

MATERIALS AND METHODS

Transcription of Harvey and Moloney Virus RNAs to cDNAs *in Vitro*. Purified virus (17, 18) at 2–4 mg-protein equivalents per ml was incubated for 12–24 hr in a solution containing the four deoxynucleoside triphosphates (6 μM each), 10 mM Mg^{2+}, 50 mM KCl, 20 mM dithiothreitol, 50 mM Tris/HCl at pH 8, and 0.01% nonionic detergent (Nonidet P-40) (19–21). The cDNA was isolated from the reaction mixture by phenol extraction. In some experiments the viral RNA was eliminated from the cDNA by treatment for 4 hr at 40°C in 0.3 M NaOH (22).

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Abbreviations: HaSV, Harvey murine sarcoma virus; MoV, Moloney murine retrovirus; LTR, long terminal repeat; PCR, polymerase chain reaction.

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Hybrid Selection of *ras*-Specific cDNA. The total cDNA obtained by reverse transcription of HaSV–MoV complexes was subjected to hybrid selection for *ras*-containing species. The *ras* DNA used for hybrid selection was a plasmid, termed p1.7, that contained a 1.7-kilobase (kb) DNA fragment from Harvey provirus bordered by *Bam*HI and *Xba* I restriction enzyme sites (23). Hybridization between cDNA and nitrocellulose filter-bound plasmid DNA was carried out in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) with 50% (vol/vol) formamide at 42°C for 12–24 hr, as described (12). The filters were washed three times at room temperature in 2× SSC and once at 65°C in 0.1× SSC (12) before the bound cDNA was eluted by incubation in water at 95°C for 10 min. After washing, the filters typically retained about 0.5% of the total cDNA made in the reaction, as measured with radioactive tracer.

Specific Amplification of Recombinant cDNA by the PCR. The cDNA from about 0.1 A_{260} unit (≈40 μg) of purified virus was incubated in 50 μl of 50 mM KCl/10 mM Tris, pH 8.3/2.5 mM $MgCl_2$ containing 10 μmol of each deoxynucleoside triphosphate, 0.05 μmol of each of a set of two recombinant-specific primers (shown in Fig. 1), 10 μg of gelatin, and 1 unit of *Thermus aquaticus* (*Taq*) DNA polymerase. The mixture was heated to 94°C and then carried through 30–60 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 54°C–62°C, and polymerization for 3 min at 72°C, as described (24, 25). An aliquot of the reaction mixture was then analyzed for size and sequence content by agarose gel electrophoresis.

RESULTS

The System Used to Study Recombination During Reverse Transcription *in Vitro*. To obtain recombinant cDNA molecules that could be physically and chemically distinguished from parental cDNAs, two viral RNA sequences were chosen that shared an internal region of homology but differed from each other in specific flanking regions. One parent was the RNA of an artificially altered HaSV and the other was the RNA of MoV. The two RNAs were obtained as a defective HaSV–helper MoV complex, termed R5ΨH 1° virus, that was harvested from cells transformed by a synthetically altered Harvey provirus and superinfected with MoV (12). The HaSV RNA of R5ΨH 1° virus carried an artificial 1.2-kb *env* sequence, derived from sequence positions 6025–7227 of MoV RNA (23), 3′ of the *ras* coding region to allow homology-directed recombination with MoV (Fig. 1 and ref. 13). Crossovers between HaSV and MoV RNAs via this homology would define the sizes of the recombinant cDNA molecules (see Fig. 1 and below). Moreover, the HaSV RNA-packaging signal between the *Xba* I and *Sac* II restriction sites of the Harvey provirus (Fig. 1) was replaced via an *Xba* I linker by that of MoV (23), which is termed Ψ in Fig. 1. The purpose of this was to avoid a possible packaging bias of the MoV against HaSV RNA and thus a possible reduced yield in recombination during reverse transcription (13).

Other modifications of the Harvey provirus studied here had been introduced for parallel experiments studying recombination *in vivo*. (*i*) The untranscribed region of the 5′ LTR of the Harvey provirus was truncated to prevent recovery of replicating HaSV by recombination among input HaSV DNAs during transfection (12, 13). (*ii*) The 3′ terminus of the Harvey provirus was truncated. This prevented efficient synthesis of Harvey cDNA by reverse transcription, since it eliminated the terminally repeated RNA sequences necessary to transfer the cDNA, initiated from a primer near the 5′ end of the retroviral RNA, to the 3′ terminus (23). The 3′ truncation thus forced selection for recovery of the 3′ LTR

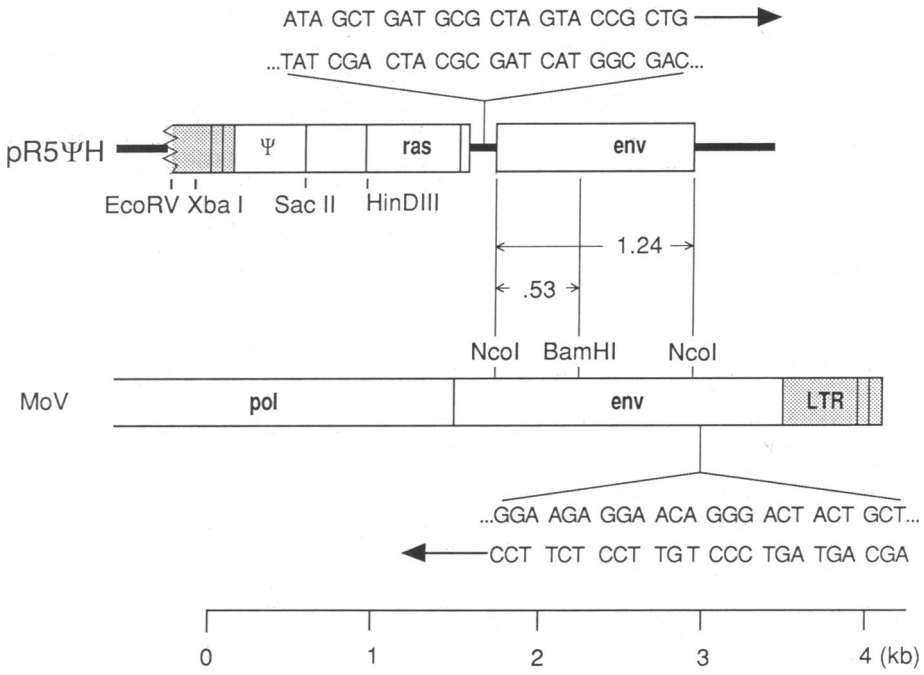

FIG. 1. The proviral structures of a synthetically altered HaSV (pR5ΨH) and of MoV examined for recombination during reverse transcription. The boxes represent proviral DNA sequences, of which the LTRs are shaded. The two vertical lines within the LTR delineate the R region, from which transcription of viral RNA originates (23). The thick horizontal lines depict sequences from the plasmid pBR322. *Eco*RV, *Xba* I, *Hin*dIII, *Nco* I, and *Bam*HI restriction endonuclease sites in the proviruses are indicated. The 5′ LTR of the Harvey provirus is truncated up to an *Eco*RV site, designated by a jagged line. Ψ represents a 714-base MoV-derived RNA-packaging region bordered on the 5′ side by the *Xba* I site of the LTR and on the 3′ side by a *Pst* I site joined with the *Sac* II site of HaSV with an *Xba* I linker. *ras* designates the coding region of the Harvey sarcoma viral transforming gene, and *env* is a 1.2-kb *Nco* I-bordered region derived from the envelope gene of MoV (23). The diagram shows the 3′ half of MoV, including part of the reverse transcriptase gene (*pol*) and all of the *env* gene, aligned with HaSV based on their common 1.2-kb *env* region. The pBR322-derived, pR5ΨH-specific nucleotide sequence 5′ of the common *env* region and the MoV-specific sequence 3′ of it were used as primers in PCRs carried out to detect HaSV–MoV recombinant cDNAs.

by recombination with MoV in the biological assay system (4).

Identification of Recombinant cDNAs Made *in Vitro*. Recombination between the altered HaSV and MoV RNAs of the R5ΨH 1° virus complex during reverse transcription was examined by analysis of recombinant Harvey *ras*-containing cDNAs made *in vitro* after selective amplification by the PCR. Before amplification by the PCR, Harvey *ras*-containing cDNA was separated from MoV cDNA by hybrid selection using *ras* DNA bound to a nitrocellulose filter. This selection precluded recombination between HaSV and MoV cDNAs during the subsequent PCR, since only MoV-specific sequences that were already covalently linked to *ras* sequences would survive the selection. Further, the hybrid selection eliminated PCR "noise" that could arise from nonspecific priming by MoV cDNAs and possibly nonviral cDNAs. The yield of HaSV cDNA from our HaSV–MoV complex (R5ΨH 1° virus) was only 0.5% of the total cDNA. This low yield was consistent with the truncated structure of the Harvey RNA in this virus, which prevents efficient cDNA synthesis prior to recombination (see below).

The *ras*-specific cDNA of the R5ΨH 1° virus was then specifically amplified for HaSV–MoV recombinant molecules by PCR using the Harvey-specific 5′ primer and the Moloney-specific 3′ primer, which flank the 1.2-kb *env* homology region. Based on the positions of the primers used, a recombinant DNA of 1.2 kb was expected (Fig. 1). A DNA of the expected size was indeed detected after agarose gel electrophoresis of the PCR products whether or not viral RNA was removed from cDNA by NaOH (*Materials and Methods*) prior to amplification (Fig. 2*A*, lane 2). In addition, this reaction and other reactions, described below, yielded DNAs of about 1 kb and 0.5 kb (Fig. 2*A*), which appear to be artifacts of the PCR.

The following controls indicated that the 1.2-kb DNA was generated by recombination between the RNA genomes of HaSV and MoV during reverse transcription. The 1.2-kb DNA band was not amplified when either of the primers was left out of the reaction (data not shown). Further, no 1.2-kb DNA was generated when the two primers were used to amplify cDNA from *in vitro* transcription of MoV RNA in detergent-disrupted virus, although the nonspecific 0.5-kb product was generated (Fig. 2*A*, lane 1). In addition, recombinant RNA was shown not to have preexisted in R5ΨH 1° virus by two methods. (*i*) Genomic DNA of R5ΨH 1° virus-producing cells was isolated and examined by PCR for the 1.2-kb recombinant DNA; no such DNA was found (Fig. 3, lanes 1–3). (*ii*) When purified RNA of R5ΨH 1° virus was transcribed *in vitro* with commercial reverse transcriptase using random primers (Amersham), and then amplified by the PCR with the 5′ HaSV-specific 5′ primer and the MoV-specific 3′ primer, no 1.2-kb recombinant DNA was found (Fig. 2*A*, lane 4). Further, no 1.2-kb DNA was detected if this cDNA was mixed with MoV cDNA prepared in detergent-disrupted virus prior to amplification by the PCR (Fig. 2*A*, lane 5). Likewise, no recombinant RNA or proviral DNA was found by direct analysis of cellular RNAs and DNAs from R5ΨH 1° virus-producing cells in a parallel study (13). It followed that the 1.2-kb HaSV–MoV recombinant DNA generated during reverse transcription of detergent-disrupted R5ΨH 1° virus originated from homology-directed recombination involving the 1.2-kb *env* homology shared by the proviral RNAs (Fig. 1).

The recombinant nature of the 1.2-kb DNA fragment from R5ΨH 1° virus cDNA was confirmed by two methods. (*i*) Digestion of the *env* sequence within the 1.2-kb DNA fragment with restriction endonuclease *Bam*HI should produce two fragments 710 and 530 bases long, based on the locations of *Bam*HI and *Nco* I sites in the *env* gene of MoV (ref. 23 and Fig. 1). Fragments of these sizes were observed, along with

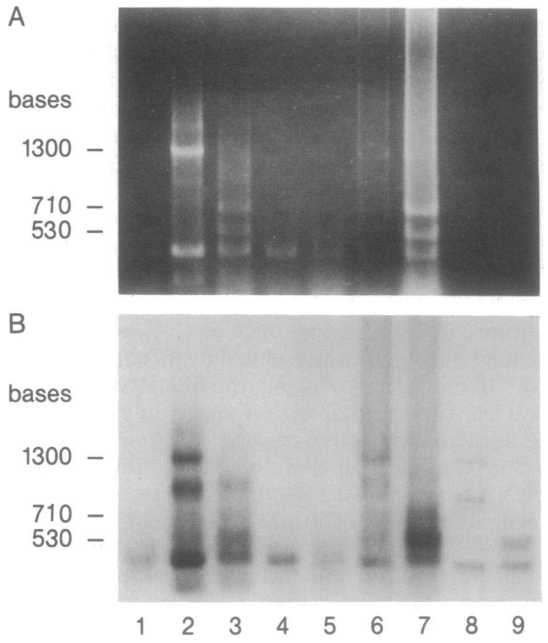

FIG. 2. Electrophoresis of HaSV–MoV recombinant DNAs generated by reverse transcription *in vitro* and amplified by the PCR. For all amplifications, templates were cDNAs made either by detergent-disrupted virions or by transcription of viral RNA with purified reverse transcriptase. The HaSV-specific 5′ primer and the MoV-specific 3′ primer shown in Fig. 1 were used. (*A*) Aliquots of the PCR products were electrophoresed in a 1.2% agarose gel and stained with ethidium bromide. Lane 1: DNA amplified from MoV cDNA generated by detergent-disrupted virus. Lane 2: DNA amplified from cDNA generated by detergent-disrupted R5ΨH 1° virus including HaSV and MoV RNAs. Lane 3: *Bam*HI digest of the DNA analyzed in lane 2. Lane 4: DNA amplified from cDNA generated by transcription of purified R5ΨH 1° virus (HaSV) RNA and MoV RNA with purified reverse transcriptase. Lane 5: DNA amplified from the template described for lane 4, mixed with cDNA made from MoV generated by detergent-disrupted virus. Lane 6: DNA amplified from cDNA generated by detergent-disrupted recombinant HaSV–MoV, termed R5ΨH 2° virus (see text). Lane 7: DNA described for lane 6 after digestion with *Bam*HI. Lane 8: DNA amplified from cDNA transcribed from purified HaSV–MoV (R5ΨH 2° virus) RNA and purified reverse transcriptase. Lane 9: Same DNA as in lane 8, after digestion with *Bam*HI. (*B*) The DNA from the agarose gel described for *A* was transferred to nitrocellulose. After hybridization with two MoV *env*-specific ^{32}P-labeled oligonucleotides, Nos. 6480 and 7247 (see text), the nitrocellulose filter was autoradiographed.

the disappearance of the 1.2-kb fragment, upon *Bam*HI digestion of the amplified cDNA products of R5ΨH 1° virus (Fig. 2*A*, lane 3). (*ii*) The 1.2-kb DNA amplified from R5ΨH 1° virus cDNA hybridized with two ^{32}P-labeled *env* oligonucleotides from sequence positions 6480–6500 and 7247–7270 in MoV (23) (Fig. 2*B*, lane 2). The 710- and 530-base *Bam*HI restriction fragments also hybridized with oligonucleotides 6480 and 7247, respectively (Fig. 2*B*, lane 3). In addition, the above-mentioned 1- and 0.5-kb fragments, which were not digested by *Bam*HI, hybridized with these oligonucleotides, suggesting that they are *env*-related sequences (Fig. 2*B*, lanes 2 and 3). As expected, no specific signals were obtained upon hybridization of DNA amplified from MoV cDNAs with the recombinant DNA-specific primers (Fig. 2*B*, lane 1).

Conditions for Recombination During Reverse Transcription *in Vitro*. In an effort to define the requirements of recombination during reverse transcription in detergent-disrupted virus, we set out to reconstruct a recombination system from purified components *in vitro*. For this purpose, phenol-extracted RNA from R5ΨH 1° virus (which contains both parental RNAs) was incubated with purified reverse transcriptase but otherwise under the conditions described

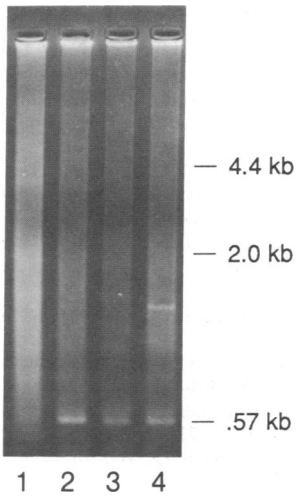

FIG. 3. Cellular and proviral DNA from cells transformed by truncated Harvey provirus (pR5ΨH) and then infected with MoV, and from cells transformed by virus harvested from Harvey provirus-transformed cells. Lanes 1–3: genomic DNA (4, 2, and 1 μg, respectively) from NIH mouse 3T3 cells transformed by pR5ΨH Harvey provirus (Fig. 1) and superinfected with MoV was subjected to PCR amplification using the HaSV-specific 5′ and MoV-specific 3′ primers described for Fig. 1; the total reaction product was electrophoresed as described for Fig. 2 and stained with ethidium bromide. Lane 4: genomic DNA (1 μg) of cells transformed by virus harvested from the Harvey provirus-transformed cells analyzed in lanes 1–3 was subjected to amplification and analyzed as for lanes 1–3. DNA sizes were estimated based on *Hin*dIII-resistant λ phage DNA size markers.

above for detergent-disrupted virus. The cDNA was subsequently amplified with the HaSV-specific 5′ and MoV-specific 3′ primers and examined for the characteristic 1.2-kb recombinant DNA. No 1.2-kb DNA was found (Fig. 2*A*, lane 4). Likewise, no 1.2-kb recombinant DNA was produced when the same cDNA template was amplified in the presence of MoV cDNA generated by reverse transcription in detergent-disrupted virus (Fig. 2*A*, lane 5). It appears that undefined viral structural proteins, together with the proximity of two parental genomes within a viral particle (26, 27), are essential for crossing-over during reverse transcription *in vitro*. Such proteins have also been postulated to be necessary for *in vitro* transcription of full-length cDNA from viral RNA (19). The absence of a 1.2-kb fragment from cDNA transcribed from purified R5ΨH 1° viral RNA with purified reverse transcriptase, even in the presence of MoV cDNA generated by disrupted virus, also indicated that recombinant cDNA obtained by reverse transcription of detergent-disrupted R5ΨH 1° virus was not an artifact of the PCR technique.

***In Vivo* Recombination Generates the Same Products as Recombination During Reverse Transcription *in Vitro*.** If the same recombination that we observed *in vitro* were to occur during reverse transcription *in vivo*, infection with R5ΨH 1° virus (which contains the two parental RNAs) would be expected to generate recombinant cDNA. Further, R5ΨH 1° virus-infected cells would be expected to produce recombinant virus.

Indeed, *in vitro* transcription with purified reverse transcriptase of phenol-extracted RNA from virus produced by R5ΨH 1° virus-infected cells generated the expected 1.2-kb recombinant DNA fragment after amplification (Fig. 2*B*, lane 8). As expected, this 1.2-kb DNA contained the 710- and 530-base *Bam*HI-resistant fragments (Fig. 2*B*, lane 9). (Not enough DNA was applied to visualize the DNAs by ethidium bromide staining in Fig. 2*A*, lanes 8 and 9.) By contrast, no recombinant DNA was obtained by reverse transcription of the purified RNAs from parental R5ΨH 1° virus (Fig. 2, lanes 4). Moreover, the genomic DNA of R5ΨH 1° virus-infected cells contained 1.2-kb recombinant proviral DNA, as evidenced by PCR amplification (Fig. 3, lane 4).

When cDNA made by detergent-disrupted virus from cells infected by R5ΨH 1° virus was amplified with the HaSV-specific 5′ and MoV-specific 3′ primers and analyzed by electrophoresis, the 1.2-kb recombinant DNA was detected by staining with ethidium bromide (Fig. 2*A*, lane 6) and by hybridization with the *env*-specific oligonucleotides (Fig. 2*B*, lane 6). This DNA also contained the expected 710- and 530-base *Bam*HI-resistant fragments (Fig. 2 *A* and *B*, lane 7). However, the background of nonspecific DNAs was higher in this cDNA than in the cDNA prepared from R5ΨH 1° virus, presumably because this cDNA was not hybrid-selected for *ras*-specific species prior to amplification.

It appears that the same recombination that was observed *in vitro* during reverse transcription also occurred *in vivo*—possibly by the same mechanism.

DISCUSSION

Recombinant molecules that did not exist prior to reverse transcription were generated by homologous recombination during reverse transcription within disrupted virions *in vitro*. Due to the distinct genetic structures of the parental and recombinant genomes in our system, such recombinant molecules could be amplified selectively by the PCR among primary cDNA transcripts. Similar recombinant molecules were also observed *in vivo* in cells infected by the same virus used for reverse transcription *in vitro*. Hence, retroviral RNA genomes can presumably recombine during reverse transcription *in vivo*.

Several observations indicate that most, if not all, recombinations observed here *in vitro* occurred by RNA template switching during cDNA synthesis rather than during or after synthesis of double-stranded DNA. Since the HaSV RNA studied here lacks a retroviral 3′ terminus, cDNA can be initiated by the retroviral primer near the 5′ end but cannot be extended from the 5′ repeat (R)-region (Fig. 1) to the missing 3′ repeat. Thus the 3′ truncated HaSV RNA will not be converted to cDNA, except by chance via random primers or by template switching of cDNA initiated from MoV RNA. It is consistent with this view that only about 0.5% of the total cDNA generated *in vitro* by our R5ΨH 1° virus, which contains both MoV RNA and 3′ truncated HaSV RNA, was HaSV cDNA—most of which presumably represents HaSV–MoV cDNA recombinants. This is not due to a shortage of HaSV RNA templates, because we have shown previously that HaSV (MoV) complexes with 3′ truncated HaSV RNAs like those described here carry just as much HaSV RNA as wild-type HaSV (12). The observation that 3′-truncated HaSV RNA in the absence of homology 3′ of *ras* is not infectious and not subject to recombination with helper MoV (12) is also entirely consistent with the proposal that it is not an adequate template for reverse transcription.

Our results are at variance with a previous study investigating recombination during reverse transcription (16). That study concluded that recombination is initiated only during synthesis of the second DNA strand. The second strand was reported to invade double-stranded DNA molecules at homologous gapped regions and the resulting structures were postulated to initiate conventional DNA recombination in the cell (16). Recombination during reverse transcription may have been overlooked because RNA·DNA hybrids were not distinguishable from DNA·DNA hybrids by the electron microscopy and because physically indistinguishable parental genomes were analyzed (16). Other studies of retrovirus recombination did not distinguish between recombination mechanisms that involved RNA·cDNA complexes and dou-

ble-stranded proviral DNAs, because the assay depended on biological selections of recombinant viruses that appeared only after one or several rounds of virus replication *in vivo*, rather than on direct selection of recombinant molecules (1–3, 14).

We have not attempted to analyze illegitimate recombination in our system for two reasons. (*i*) The sizes of recombinants that might arise by illegitimate recombination *in vitro* would be unpredictable and heterogeneous; hence, it would be difficult, if not impossible, to distinguish such recombinants from the background noise generated by the PCR. Potential recombinants would thus have to be cloned and analyzed individually. (*ii*) Since there is no evidence for efficient or reproducible illegitimate recombination *in vivo* between nonhomologous sequences of retroviruses (12, 13), any illegitimate recombination detected *in vitro* might have no biological relevance.

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