Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3604–3608, May 1990
Biochemistry

Evidence that retroviral transduction is mediated by DNA, not by RNA

(Harvey murine sarcoma virus/Moloney murine retrovirus/provirus alteration/transfection/recombination)

DAVID W. GOODRICH* AND PETER H. DUESBERG

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

Contributed by Peter H. Duesberg, February 26, 1990

ABSTRACT **Retroviral transduction of cellular nucleic acid sequences requires illegitimate RNA or DNA recombination. To test a model that postulates transduction via efficient illegitimate recombination during reverse transcription of viral and cellular RNAs, we have measured the ability of Harvey sarcoma viruses (HaSVs) with artificial 3′ termini to recover a retroviral 3′ terminus from helper Moloney virus (MoV) by illegitimate and homologous recombination. For this purpose, mouse NIH 3T3 cells were transformed with Harvey proviruses and then superinfected with MoV. The proviruses lacked the 3′ long terminal repeat and an untranscribed region of the 5′ long terminal repeat to prevent virus regeneration from input provirus. Only 0–11 focus-forming units of HaSV were generated upon MoV superinfection of 3×10^6 cells transformed by Harvey proviruses with MoV-unrelated termini. This low frequency is consistent with illegitimate DNA recombination via random Moloney provirus integration 3′ of the transforming viral *ras* gene in the 10^6-kilobase mouse genome. When portions of murine viral envelope (*env*) genes were attached 3′ of *ras*, 10^2–10^5 focus-forming units of HaSV were generated, depending on the extent of homology with *env* of MoV. These recombinants all contained HaSV-specific sequences 5′ and MoV-specific sequences 3′ of the common *env* homology. They were probably generated by recombination during reverse transcription rather than by recombination among either input or secondary proviruses, since (*i*) the yield of recombinants was reduced by a factor of 10 when the *env* sequence was flanked by splice signals and (*ii*) HaSV RNAs without retroviral 3′ termini would be inadequate templates for provirus synthesis. We conclude that there is no efficient illegitimate recombination in retroviruses. In view of known precedents of illegitimate DNA recombination, the structure of known viral *onc* genes, and our evidence for illegitimate DNA recombination via provirus integration, we favor the DNA model of transduction over the RNA model.**

Retroviral transduction of oncogenic sequences requires either RNA- or DNA-mediated illegitimate recombination. Based on an experimental system that appeared to involve "surprisingly efficient" illegitimate recombination by RNA template switching during reverse transcription (1), the RNA model is currently favored (1–11). This model holds that a retrovirus would integrate upstream of a proto-*onc* gene, spontaneously lose its 3′ terminus, or fail to terminate during transcription (9), and produce a 5′ viral–3′ proto-*onc* mRNA. In a second step, the cellular 3′ region of this hybrid RNA would recombine with the nonhomologous 3′ region of an intact (helper) retrovirus during reverse transcription in order to obtain a retroviral 3′ terminus. A competing DNA model holds that all steps of retroviral transduction are mediated by illegitimate primary DNA recombination (12).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The experimental system on which the RNA model is based showed efficient regeneration of Harvey murine sarcoma virus (HaSV) from cells that were transformed by a 3′-truncated Harvey provirus and then superinfected with Moloney virus (MoV). The mechanism was assumed to reflect efficient illegitimate recombination between HaSV RNA and MoV RNA during reverse transcription (1). However, this system did not exclude HaSV regeneration involving recombination between elements of the input Harvey provirus alone. Indeed, the truncated input provirus contained an intact 5′ long terminal repeat (LTR), a packaging sequence, and a complete transforming *ras* gene (1) and thus all the elements essential for regeneration of infectious HaSV.

A reinvestigation of the above system demonstrated that the efficient regeneration of HaSV in this system depended entirely on an untranscribed U3 region from the 5′ LTR of the truncated provirus (12). Since the RNAs transcribed from Harvey provirus with an intact 5′ LTR, which did regenerate transforming virus, and from provirus with a truncated 5′ LTR, which did not regenerate transforming virus, were identical, we concluded that HaSV regeneration did not involve illegitimate recombination with MoV during or after reverse transcription. Instead we proposed that HaSV was regenerated from truncated proviral DNA elements with intact 5′ LTRs by illegitimate recombination during transfection (12). The high frequency of this process was suggested to reflect tandem integration during transfection of the Harvey provirus (12). Indeed, multiple Harvey proviruses were originally observed in cells transformed by 3′-truncated Harvey proviruses (1, 13).

In an effort to distinguish between the two models of transduction, we have determined conditions under which a Harvey provirus with an artificial 3′ terminus can recover a correct retroviral 3′ terminus from MoV by illegitimate and homologous recombination. HaSV regeneration from input provirus was precluded by studying proviruses with a single truncated, instead of a complete, 5′ LTR (12).

MATERIALS AND METHODS

Defective Harvey sarcoma proviruses with and without *env*-related sequences were constructed as follows (Fig. 1).

Harvey proviral plasmid pR5Ψ was derived from pH1/R5Nhe (12). The 1.1-kilobase (kb) *Xba* I [−151]–*Sac* II [940] restriction fragment, containing the presumed Harvey RNA packaging signal (14, 15), was removed from pH1/R5Nhe, and the remaining *Sac* II site [940] was modified by attachment of an *Xba* I linker to make pR5NΔSX. The numbers in brackets refer to sequence positions of HaSV (15). The

Abbreviations: HaSV, Harvey sarcoma virus; MoV, Moloney virus; LTR, long terminal repeat; ffu, focus-forming unit(s); pfu, plaque-forming unit(s).

*Present address: Department of Pathology, School of Medicine, University of California at San Diego, La Jolla, CA 92093.

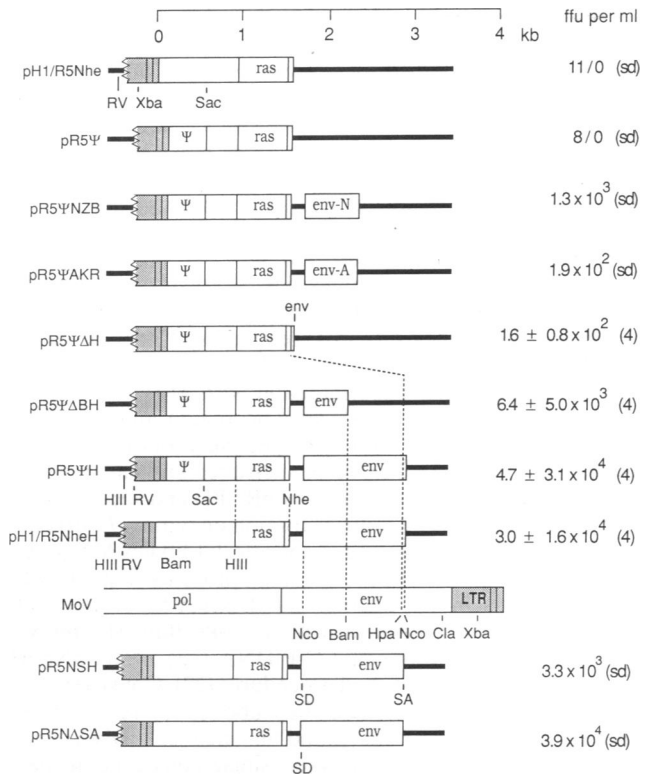

FIG. 1. Genetic structures of truncated Harvey sarcoma proviruses and their ability to recombine with MoV. The Harvey proviruses differ with regard to the presence or absence of artificially added sequences that are homologous to the *env* gene of MoV 3′ of the *ras* coding region and with regard to the HaSV- or MoV-derived (Ψ) origin of their RNA packaging sites. The single 5′ LTRs (gray) of all Harvey proviruses are truncated up to the *Eco*RV site (marked by a jagged line) of the U3 region (15). Transcription originates from the repeat (R) region within the LTR (15), which is marked by two vertical bars. Restriction enzyme sites (RV, *Eco*RV; HIII, *Hin*dIII; Sac, *Sac* II) essential for the construction and definition of the proviruses are indicated (*Materials and Methods*). SD is a splice donor and SA a splice acceptor. Focus-forming units (ffu) of recombinant HaSV obtained 3 days after superinfection of transformed cells with helper MoV are either from single determinations (sd) or means ± standard deviation of four determinations. The titers of MoV were 10^6–10^7 plaque-forming units (pfu)/ml, as determined by the XC plaque assay (21), in each of the experiments for which ffu are recorded.

716-base *Xba* I–*Pst* I restriction fragment from a proviral MoV clone, pZAP (12, 16), which includes the MoV RNA packaging site (14, 15), was modified by placing *Xba* I linkers on the blunt-ended *Pst* I site. The modified fragment, Ψ, was then inserted into the *Xba* I site of pR5NΔSX to make pR5Ψ.

Plasmid pR5ΨH was made from pR5Ψ by inserting an *Nco* I linker into the *Bam*HI site of pBR322, 196 bases 3′ of the *ras* gene, to create pR5NΔSXN, and then putting the 1.2-kb *Nco* I *env* restriction fragment from pZAP into this *Nco* I site. pH1/R5NheH was made from pR5Ψ by replacing the 1.7-kb *Eco*RV–*Nhe* I region with the 1.9-kb *Eco*RV [−232]–*Nhe* I [1695] fragment from pHI/R5Nhe. pR5ΨΔBH was made by partial *Bam*HI digestion and religation of pR5ΨH, which contains three *Bam*HI sites, two overlapping the *Nco* I linkers flanking the *env* region and one in the *env* region itself. A clone with a deletion from the *Bam*HI site in *env* to the 3′ *Bam*HI site was selected and termed pR5ΨΔBH. It had lost the 690 bases at the 3′ end of the *env* homology of pR5ΨH. pR5ΨΔH was constructed by *Nhe* I and *Hpa* I digestion of pR5ΨH and religation after modification of the *Nhe* I site with Klenow polymerase to create a blunt end. pR5ΨΔH retains only 32 nucleotides of *env*, namely, those 3′ of the *Hpa* I site to the *Nco* I site of *env*.

Plasmids containing *env* regions from AKR and NZB murine retroviruses were derived as follows. A 610-base *Bgl* II restriction fragment from the *env* gene of AKR provirus (15, 17) was ligated to the compatible pBR322 *Bam*HI site of pR5Ψ to generate pR5ΨAKR. pR5ΨNZB was constructed by insertion of a 634-base *Bgl* II–*Eco*RV *env* fragment from the xenotropic NZB provirus (15, 18), whose *Eco*RV blunt end had been modified by attachment of a *Bgl* II linker, into the *Bam*HI site of pBR322 3′ of the *ras* sequence of pR5Ψ.

Two plasmids containing *env* regions flanked by splicing signals were derived from pR5Ψ. A synthetic oligonucleotide with an *Nhe* I-compatible 5′ end followed successively by a MoV splice donor (15), an *Nco* I site, a MoV splice acceptor, and a *Bam*HI compatible end was inserted into the *Nhe* I and *Bam*HI sites of pBR322 to create pBR/S. The *Eco*RV–*Nhe* I *ras*-containing region of pHI/R5Nhe was then placed into the *Eco*RV and *Nhe* I sites of pBR/S to make pR5N/S. Finally, the 1.2-kb *Nco* I env fragment of MoV was inserted into the *Nco* I site of the oligonucleotide to create pR5NSH. The plasmid pR5NΔSA was made from pR5NSH by removing the 3′ end of the *env* region of MoV, including the splice acceptor, from a *Hpa* I site up to the *Nru* I site of pBR322 and religating the blunt ends.

RESULTS

Rare Illegitimate Recombination Between HaSV and MoV. To test the frequency of illegitimate recombination between HaSV and MoV, we measured regeneration of HaSVs from Harvey proviruses with artificial, heterologous 3′ termini by recombination with MoV. The Harvey proviruses pH1/R5Nhe and pR5Ψ each lacked the 5′-most 215 nucleotides of the 5′ LTR, up to the 3′-most *Eco*RV site of the U3 region (15), and all retroviral sequences 3′ of *ras* (Fig. 1). These defective Harvey proviruses were transfected into mouse NIH 3T3 cells (12, 19) together with pSV2neo, a plasmid carrying a neomycin-resistance gene, so that cells that had not taken up transfected DNA could be rapidly eliminated by selection with the neomycin analogue G418 (20). Two weeks after transfection, foci of morphologically transformed, G418-resistant cells were observed. These results confirmed and extended our previous observations that despite truncation of the 5′ LTR up to the *Eco*RV site, the Harvey proviruses tested expressed sufficient viral *ras* RNA for cell transformation and packaging by helper MoV (12) (see below).

Clonal cultures of morphologically transformed 3T3 cells were then prepared from isolated foci and HaSV regeneration was measured 3 days after superinfection of about 3×10^6 transformed cells with MoV. In most such experiments no HaSV was recovered and in some low yields were observed. For example, 11 ffu/ml were recovered in one experiment from cells transformed by pH1/R5Nhe and 8 ffu/ml from cells transformed by pR5Ψ (Fig. 1). These results extended our previous study in which no measurable titer of HaSV was observed, possibly because fewer cells were studied in fewer experiments (12). Indeed, the recovery of HaSV observed here was dependent on particular cell clones. The frequency of this event was about 1000-fold lower than previously reported for Harvey proviruses with intact 5′ LTRs (1, 12). The titer of helper MoV was 10^6–10^7 pfu/ml as measured by the XC plaque assay (21) (Fig. 1). The low yields of HaSV are consistent with illegitimate recombination between the integrated Harvey provirus and Moloney provirus via random provirus integration into the 10^6-kb mouse genome (see *Discussion*).

Efficient Homology-Dependent Recombination Between MoV and HaSV with Artificial MoV-Related Sequences. To

determine the efficiency with which Harvey proviruses with a nonviral 3′ terminus may acquire an authentic retroviral terminus from a helper virus by homologous recombination, we studied Harvey proviruses that carried defined sequences homologous to MoV 3′ of their selectable, transforming *ras* genes. The Harvey proviruses pH1/R5NheH and pR5ΨH each carried a 1.2-kb *Nco* I-resistant restriction fragment of the Moloney proviral *env* gene, which had been inserted 3′ of their *ras* genes. The Harvey constructs pR5ΨΔBH and pR5ΨΔH contained 512 and 32 bases, respectively, of the *env* gene of MoV. Each of these proviral plasmids also lacked the 5′-most 215 nucleotides of the 5′ LTR, up to the 3′-most *Eco*RV site of the U3 region (15), and all retroviral sequences 3′ of the added *env* region (Fig. 1). Plasmids pR5ΨH, pR5ΨΔH, and pR5ΨΔBH differed from pH1/R5NheH in that a 1.1-kb DNA region that included the presumed RNA packaging site (Ψ) of Harvey virus was replaced by the equivalent from Moloney proviral DNA (14–16). These packaging variants were constructed to avoid a possible bias of helper MoV against packaging HaSV RNA and hence against RNA-mediated recombination.

Three days after superinfection with MoV, clonal cultures of 3T3 cells transformed by Harvey proviruses pH1/R5NheH and pR5ΨH, which contained 1.2 kb of the MoV *env* gene, generated HaSV at $3 \pm 1.6 \times 10^4$ ffu/ml and MoV at 10^6–10^7 pfu/ml. The Harvey clone with 512 bases of the MoV *env* gene, pR5ΨΔBH, gave rise to HaSV at $6.4 \pm 5 \times 10^3$ ffu/ml, and the Harvey clone with 32 bases of the MoV *env* gene, pR5ΨΔH, generated $1.6 \pm 0.8 \times 10^2$ ffu of HaSV per 10^6–10^7 pfu of MoV (Fig. 1).

We also tested HaSV regeneration by recombination between MoV and Harvey proviruses lacking retroviral 3′ termini but carrying *env* regions derived from the AKR and NZB murine retroviruses (15, 17, 18) 3′ of *ras* (Fig. 1). The *env* sequences from these viruses are about 50% related to MoV (15, 17, 18). The Harvey provirus with a 610-base *Bgl* II-resistant fragment from the *env* gene of AKR virus, pR5ΨAKR, generated 1.9×10^2 ffu of HaSV per 10^6–10^7 pfu of MoV. Similarly, a Harvey provirus with a 636-base *Bgl* II–*Eco*RV fragment from the *env* gene of NZB virus, pR5ΨNZB, generated 1.3×10^3 ffu of HaSV per 10^6–10^7 pfu of MoV (Fig. 1).

It follows that efficient, homologous recombination had occurred between HaSV and MoV and that the efficiency of recombination was proportional to the extent of sequence homology between the respective HaSV construct and MoV.

The Harvey proviruses that differed only with regard to the MoV versus the HaSV origin of the RNA packaging sites, pH1/R5NheH and pR5ΨH, generated virtually identical yields of recombinants, namely, 3 ± 1.6 versus $4.7 \pm 3.1 \times 10^4$ ffu/ml (Fig. 1). This result indicated that the helper MoV did not discriminate between HaSV RNAs on the basis of these different packaging sites.

Mechanism of Homologous Recombination. The homologous recombination observed here could occur at three distinct stages after infection: (*a*) between the input Harvey provirus and unintegrated or integrated Moloney provirus, (*b*) between two viral RNAs generating a recombinant cDNA during reverse transcription in a newly infected cell, or (*c*) between secondary proviruses generated in newly infected cells. Three groups of experiments were carried out to distinguish between these alternatives.

(*i*) Since the removal of homology by splicing would lower the probability of recombination between viral RNAs or between DNAs transcribed from viral RNA, but not that between input DNAs, two Harvey proviruses were compared that differed only in the presence of splice signals flanking an otherwise identical MoV-derived *env* sequence, pR5NSH and pR5NΔSA (Fig. 1). Recombinant HaSV at about 3.3×10^3 ffu/ml was obtained from the provirus with a spliceable *env* region, compared to 3.9×10^4 ffu/ml from the provirus without two splice signals (Fig. 1). Thus, HaSV regeneration was decreased about 10-fold, probably by a 90% efficient removal of the *env* sequences from pR5NSH by splicing. HaSV regenerated from pR5NSH still contained the *env* region (data not shown), indicating that these viruses arose from unspliced RNA. Indeed, splicing from *env* splice signals was expected to be leaky, as in natural provirus transcription (15). It appears that homologous recombination in our system occurred after provirus transcription.

(*ii*) If homologous recombination occurred between the common *env* regions of the input Harvey provirus (e.g., pR5ΨH) and Moloney provirus, recombinant proviruses and viral RNAs of about 3.9 kb would be expected in provirus-transfected cells after superinfection with MoV. The 3.9-kb size is the sum of the HaSV-specific region and the MoV-specific region that lie 5′ and 3′, respectively, of their common *env* homology (Fig. 1). To test for such a recombinant provirus the DNA from cells transformed by pR5ΨH and superinfected with MoV was digested with restriction enzymes *Hin*dIII and *Cla* I to generate a recombinant-specific 2.4-kb DNA fragment from the predicted 3.9-kb recombinant provirus (Fig. 1). After electrophoresis in 1% agarose the DNA was blotted onto nitrocellulose and hybridized with a 1.3-kb *Bam*HI- and *Nhe* I-resistant HaSV *ras*-specific [^{32}P]DNA (see pHI/R5NheH, Fig. 1) according to published procedures (22). Consistent with the splicing experiment described above, no recombinant-specific 2.4-kb DNA was found in such cell DNA under hybridization conditions that detect single-copy genes in mammalian cells—namely, hybridization of 5 μg of cell DNA with 10^8 cpm of *ras*-specific [^{32}P]DNA of $>10^8$ cpm/μg (Fig. 2*A*, lane 1).

Instead, *ras*-containing pR5ΨH DNA fragments of unpredictable sizes were present in well over two copies per cell, based on comparison to a probable proto-*ras* restriction fragment of about 5 kb (see below). Their concentration also exceeded the concentration of the Harvey proviral DNAs found in virus-infected cells (Fig. 2*A*, lane 2). As expected from the transformed phenotype of these cells, the *ras* gene of pR5ΨH was intact, generating a 1.1-kb *Hin*dIII-resistant fragment (Fig. 2*A*, lane 1) that initiated in pBR322 just 5′ of the *Eco*RV site of the truncated LTR and terminated at the *Hin*dIII site of *ras* (Fig. 1).

Further, we tested whether 3.9-kb recombinant HaSV RNA was detectable in virus released by provirus-transformed cells superinfected with MoV. The viral RNAs were prepared from virus harvested at 12-hr intervals to avoid thermal degradation (23). Despite these precautions against degradation, heterogeneous HaSV RNAs of >9 to <2 kb were observed in virus from pR5ΨH-transformed 3T3 cells superinfected with MoV (Fig. 2*B*, lane 1). The heterogeneity of this RNA is probably genuine, because in the absence of an authentic 3′ terminus the RNAs from the truncated Harvey proviruses terminate at nonspecific sites in plasmid or cellular DNA sequences. Clearly this experiment does not exclude a small amount of 3.9-kb recombinant RNA obscured by the high background of heterogeneous HaSV RNAs.

Since the probability of HaSV recombination was reduced by elimination of homology with MoV through splicing and since no recombinant HaSV DNA or RNA was found in provirus-transformed cells, we conclude that there is no detectable homologous recombination between truncated input Harvey proviruses and MoV.

(*iii*) If recombination in our system occurred during or after reverse transcription, cells infected and transformed with virus released from pR5ΨH-transformed and MoV-infected cells, termed R5ΨH 1^0 virus, would be expected to contain 3.9-kb recombinant proviral DNA and to release virus with 3.9-kb recombinant RNA. As shown in Fig. 2*A*, lane 2, a *Hin*dIII/*Cla* I digest of the DNA from R5ΨH 1° virus-

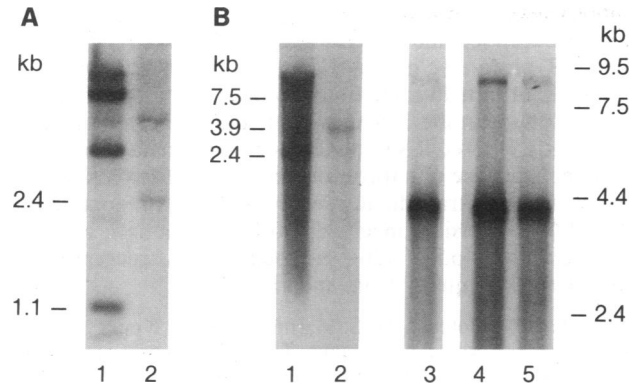

FIG. 2. Electrophoretic analysis of proviral DNAs and viral RNAs. (*A*) Lane 1, DNA of mouse NIH 3T3 cells transformed by the truncated Harvey provirus pR5Ψ; lane 2, DNA of 3T3 cells transformed by virus released from pR5Ψ provirus-transformed cells. DNA was isolated from nuclei of cells disrupted by the nonionic detergent Nonidet P-40 (22) and was digested with restriction enzymes *Cla* I and *Hin*dIII. About 5 μg of each digest was electrophoresed in a 1% agarose gel, transferred to a nitrocellulose filter, hybridized with a 1.3-kb *Bam*HI- and *Nhe* I-resistant HaSV *ras*-specific [^{32}P]DNA (Fig. 1), and then autoradiographed (22). The DNA probe was ^{32}P-labeled with a commercial nick-translation kit (Amersham N 5000) to a specific activity of $>10^8$ cpm/μg. The sizes of DNA fragments were estimated from the positions of commercial *Hin*dIII-digested λ phage DNA markers (BRL). (*B*) Lane 1, RNA from virus released by mouse NIH 3T3 cells transformed with truncated Harvey provirus pR5Ψ and superinfected with MoV; lanes 2–5, RNA from virus released by NIH 3T3 cells infected with virus from four stocks of viruses, each derived from an independent clonal culture of cells transformed by Harvey provirus pR5Ψ and superinfected with MoV. RNA was prepared from purified virus (23) and electrophoresed in 1% agarose containing 1% formaldehyde (22) in parallel with commercial RNA size markers (BRL). After electrophoresis the RNA was transferred to nitrocellulose, hybridized with [^{32}P]DNA probe, and subsequently autoradiographed. Lanes 1 and 2 were probed with the *ras* [^{32}P]DNA described in *A*. Lanes 3–5 were probed with a 1.2-kb *Nco* I-resistant *env* [^{32}P]DNA from Moloney provirus (Fig. 1).

transformed cells contained the HaSV–MoV recombinant-specific 2.4-kb DNA fragment, in addition to a fragment of about 5 kb. Since this larger fragment was also seen in pR5ΨH-transformed cells at the same relative concentration (Fig. 2*A*, lane 1), it appears to be from a cellular proto-*ras* sequence.

Consistently, cells infected by R5ΨH 1° virus stocks, derived from four independent clonal cultures of pR5ΨH-transformed NIH 3T3 cells, each released viruses with 3.9-kb recombinant HaSV RNAs (Fig. 2*B*, lanes 2–5). After electrophoresis in 1% agarose the RNA of one recombinant virus was hybridized with the above described *ras*-specific [^{32}P]DNA (Fig. 2*B*, lane 2). The RNA of three other recombinant HaSVs was hybridized with the 1.2-kb *Nco* I-resistant *env* fragment (Fig. 1) shared by pR5ΨH and MoV (Fig. 2*B*, lanes 3–5). We conclude that homology-directed recombination occurred between HaSV and MoV RNAs either during or after reverse transcription (see below).

In view of the infectious titers reported in Fig. 1, it was unexpected that the 8.5-kb MoV RNA was barely detectable with the *env* [^{32}P]DNA probe and that the 3-kb *env* mRNA was not detectable against the trailing background of the 3.9-kb recombinant HaSV RNA signals (Fig. 2*B*, lanes 3–5). According to these titers an excess of MoV over HaSV was expected. The result may reflect different sensitivities of the different assays used to titer HaSV and MoV (see Fig. 1 legend) or may reflect an early, nonfinal titer of the recombinant HaSV at the time it was titered, 3 days after superinfection with MoV (Fig. 1).

DISCUSSION

Homology Dependence of Retrovirus Recombination. The experiments reported here confirm and extend our proposal that retroviruses do not efficiently and reproducibly recombine with heterologous sequences upon simultaneous infections (12). However, in the same system, efficient recombination was observed between homologous retroviral sequences. We conclude that efficient retroviral recombination depends on, and is directed by, sequence homology between parental viral genomes and that there is no efficient illegitimate retroviral recombination.

Our results are at variance with a previous hypothesis that recombination among retroviral genomes during reverse transcription is "surprisingly efficient" even in the absence of homology (1). A plausible resolution of this discrepancy suggests that virus regeneration in that system was initiated during transfection by tandemly integrated, truncated Harvey proviruses (see Introduction). Recombination and concatenation of input DNAs are known artifacts of the transfection method used to introduce DNA into cells (3, 24, 25). Under our conditions, which prevented HaSV regeneration by recombination of input Harvey proviruses during transfection but allowed sufficient transcription for transformation and efficient packaging of transcripts (Fig. 2*B*), HaSV regeneration by recombination with MoV was very inefficient (see below).

Homologous Recombination During Reverse Transcription. The efficient homologous recombination observed here could have occurred during reverse transcription or between proviral DNAs. Since the HaSV RNAs with MoV-related sequences 3′ of *ras* lack a retroviral 3′ terminus, cDNA can be initiated only by the retroviral RNA primer (15) but cannot be extended from the 5′ repeat (R) region to the missing 3′ repeat (Fig. 1). Thus the truncated HaSV RNA will not be converted to a cDNA that could recombine or integrate and hence be further replicated by helper virus, except by template switching of cDNA initiated from the 3′ terminus of MoV RNA. It is consistent with this view that HaSV RNA without a retroviral terminus is not infectious and not, or practically not, subject to recombination with helper MoV [see pH/R5Nhe and pR5Ψ (Fig. 1) and ref. 12]. It follows that most if not all homologous recombination observed here occurred during reverse transcription. Indeed, homologous recombination during reverse transcription between the same HaSV and MoV RNAs that were studied here *in vivo* has recently been directly demonstrated *in vitro* (26).

Mechanism of Retroviral Transduction. Since there is now no experimental evidence for the RNA model of transduction via efficient, illegitimate retroviral recombination during reverse transcription, the DNA model appears preferable on three grounds.

(*i*) It is based on known precedents of illegitimate DNA recombination such as chromosomal DNA rearrangements and integrations of deleted or permuted proviruses (12).

(*ii*) The low level of HaSV regeneration observed here in the absence of homology with MoV is numerically consistent with illegitimate DNA recombination by random provirus integration. The chances for a superinfecting Moloney provirus to integrate 3′ of the transforming *ras* gene of a truncated Harvey provirus are about 1 in 10^6, considering that the mouse genome measures about 2×10^6 kb (11) and that multiple proviruses are present per cell (Fig. 2*A*). HaSV would then be regenerated spontaneously by transcription beginning in the 5′ LTR of the truncated Harvey provirus and terminating in a Moloney proviral LTR. Thus, the yields of HaSV regeneration in the absence of homology 3′ of *ras* observed here, 0–10 ffu/ml per 3×10^6 cells, are entirely consistent with this mechanism. Studying a similar system, we found previously that truncated Rous sarcoma virus can also be recovered from about 1 in 10^6

transformed cells by insertional recombination with helper virus (27). This phenomenon generates a predictable background that sets the upper limit for the sensitivity of this assay to detect illegitimate recombination.

Nevertheless, it may be argued that illegitimate RNA recombination was responsible for the low yields of HaSV regeneration in the absence of homology with MoV. This seems unlikely, however, because the frequency of illegitimate recombination between related retroviruses is much lower than the limit of sensitivity of the assay described here. This assertion is based on numerous observations that nondefective Rous sarcoma virus (RSV) or Moloney sarcoma virus (MoSV) was never generated in coinfections of either defective RSV (28, 29) or MoSV (30) with their helper viruses. Thus, illegitimate RNA recombination is numerically unlikely to be the source of the HaSVs generated here in the absence of homology with MoV. The RNA recombination model predicts that recombinant sequences immediately upstream of the 3′ LTR of regenerated HaSV would frequently be derived from MoV, whereas the DNA recombination model via random Moloney provirus integration predicts HaSV- or cell-derived sequences upstream of the MoV-derived 3′ LTR. Therefore, analysis of the recombinant RNAs could identify their origin.

(*iii*) The DNA model provides more plausible explanations for key observations in retroviral transduction than the RNA model. For example, a stretch of 10 adenine residues in the RNA of avian sarcoma virus PRCII, following the 3′ border of a transduced proto-*fps* sequence, has been interpreted in support of the RNA recombination model. It was postulated that the 10 adenines were derived from recombination with the poly(A) terminus of proto-*fps* mRNA (7). However, the 10 adenines could have been derived from the transducing retrovirus instead. This is especially likely since the 3′ junctions of the *fps* sequences of this virus and Fujinami sarcoma virus are exactly the same (31), and since the 3′ *fps* region of neither virus functions as a polyadenylylation signal. Further, a stretch of 10 adenines is not compelling evidence for an origin from the 3′ terminus of a mRNA; for example, HaSV and avian musculo-aponeurotic fibrosarcoma virus each contain one (15) or even two (32) internal poly(A) regions of 24–38 adenines far from borders of cell- and retrovirus-derived sequences. Moreover, no oligo(A) or poly(A) regions are found at the interfaces between transduced cellular and retroviral sequences of over 50 other oncogenic retroviruses (15), although most of the respective recombinations must have occurred 3′ of the proto-*onc* coding regions and thus near polyadenylylation sites, because most viral *onc* proteins are coterminal with proto-*onc* proteins (15, 33, 34). Thus, a single case of 10 adenines at the proto-*onc* retroviral border does not lend compelling support to the RNA model and certainly does not exclude the DNA model.

Clearly the DNA model provides the only plausible explanation for the existence of at least six oncogenic retroviruses with two distinct heterologous sequences: E26, AEV, MH2, GR-FeSV, HaSV, and KiSV (in fact, three heterologous sequences are present in HaSV and KiSV) (15). To explain the origin of these viruses in terms of the RNA model, one would have to postulate that after acquiring one heterologous sequence by illegitimate RNA recombination, the same retrovirus subsequently acquired a second (and third) one, again by illegitimate recombination. This is highly improbable because retroviruses transduce heterologous sequences very rarely—so far, fewer than 100 oncogenic viruses with transduced sequences, including 6 with *ras* sequences, have been described (15, 33, 35)—and because all transduced and hence nonessential sequences have half-lives of only several replicative cycles in the retrovirus vector (33, 34). By contrast, illegitimate DNA recombinations can be stored in cells as chromosomal abnormalities that are consistently found in tumor cells and also occur in normal tissue (33, 36). Indeed, untransduced recombinant retroviral–proto-*onc* genes such as the LTR–proto-*myc* genes of certain chicken leukemias with and without transforming function have been identified (37).

It follows that the DNA model is more plausible than the previously favored RNA model (1–11) of transduction. In the hypothetical case of a fortuitous homology between a proto-*onc* gene and a transducing retrovirus, RNA recombination would be a plausible mechanism of transduction, as shown here and previously (26), although DNA recombination would benefit equally from such homology.

We thank M. Botchan, G. S. Martin, G. Vande Woude, and R.-P. Zhou for many critical discussions. This research was supported by Outstanding Investigator Grant 5-R35-CA39915-05 from the National Cancer Institute.

1. Goldfarb, M. P. & Weinberg, R. A. (1981) *J. Virol.* **38,** 136–150.
2. Coffin, J. M. (1978) *J. Gen. Virol.* **42,** 1–26.
3. Goff, S. P., Tabin, C. J., Wang, J. Y.-J., Weinberg, R. & Baltimore, D. (1982) *J. Virol.* **41,** 271–285.
4. Linial, M. & Blair, D. (1982) in *RNA Tumor Viruses,* eds. Weiss, R., Teich, N., Varmus, H. E. & Coffin, J. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 649–783.
5. Swanstrom, R., Parker, R. C., Varmus, H. E. & Bishop, J. M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2519–2523.
6. Varmus, H. E. (1984) *Annu. Rev. Genet.* **18,** 553–612.
7. Huang, C., Hay, N. & Bishop, J. M. (1986) *Cell* **44,** 935–940.
8. Darnell, J., Lodish, H. & Baltimore, D. (1986) *Molecular Cell Biology* (Scientific American Books, New York).
9. Herman, S. A. & Coffin, J. M. (1987) *Science* **236,** 845–848.
10. Temin, H. M. (1988) *Cancer Res.* **48,** 1697–1701.
11. Lewin, B. (1990) in *Genes IV* (Wiley, New York), pp. 368–370.
12. Goodrich, D. W. & Duesberg, P. H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3733–3737.
13. Goldfarb, M. P. & Weinberg, R. A. (1981) *J. Virol.* **38,** 125–135.
14. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33,** 153–159.
15. Weiss, R., Teich, N., Varmus, H. E. & Coffin, J. (1985) *RNA Tumor Viruses: Molecular Biology of Tumor Viruses* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
16. Shoemaker, C., Hoffmann, J., Goff, S. P. & Baltimore, D. (1981) *J. Virol.* **40,** 164–172.
17. Herr, W. (1984) *J. Virol.* **49,** 471–478.
18. O'Neill, R. R., Buckler, C. E., Theodore, T. S., Martin, M. A. & Repaske, R. (1985) *J. Virol.* **53,** 100–106.
19. Zhou, R.-P., Kan, N., Papas, T. & Duesberg, P. H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6389–6393.
20. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1,** 327–341.
21. Gautsch, J. W. & Meier, H. (1976) *Virology* **72,** 509–513.
22. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
23. Canaani, E., Helm, K. V. D. & Duesberg, P. H. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 401–405.
24. Perucho, M., Hanahan, D. & Wigler, M. (1980) *Cell* **22,** 309–317.
25. Robbins, D., Ripley, S., Henderson, A. & Axel, R. (1981) *Cell* **23,** 29–39.
26. Goodrich, D. & Duesberg, P. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2052–2056.
27. Martin, G. S., Lee, W.-H. & Duesberg, P. H. (1980) *J. Virol.* **36,** 591–594.
28. Hanafusa, H., Hanafusa, T. & Rubin, H. (1963) *Proc. Natl. Acad. Sci. USA* **49,** 572–580.
29. Weiss, R. A., Mason, M. S. & Vogt, P. K. (1973) *Virology* **52,** 535–552.
30. Maisel, J., Dina, D. & Duesberg, P. H. (1977) *Virology* **76,** 295–312.
31. Pfaff, S. L., Zhou, R.-P., Young, J. C., Hayflick, J. & Duesberg, P. H. (1985) *Virology* **146,** 307–314.
32. Nishizawa, M., Kataoka, K., Goto, N., Fujiwara, K. T. & Kawai, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7711–7715.
33. Duesberg, P. H. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2117–2124.
34. Duesberg, P. H., Zhou, R.-P. & Goodrich, D. (1989) *Ann. N.Y. Acad. Sci.* **567,** 259–273.
35. Frederickson, T. N., O'Neill, R. R., Rutledge, R. A., Theodore, T. S., Martin, M. A., Ruscetti, S. K., Austin, J. B. & Hartley, J. W. (1987) *J. Virol.* **61,** 2109–2119.
36. Heim, S., Mandahl, N. & Mittelman, F. (1988) *Cancer Res.* **48,** 5911–5916.
37. Zhou, R.-P. & Duesberg, P. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7721–7725.