JOURNAL OF VIROLOGY, Feb. 1990, p. 886–889
0022-538X/90/020886-04$02.00/0

Vol. 64, No. 2

NOTES

Presence of a Retroviral Encapsidation Sequence in Nonretroviral RNA Increases the Efficiency of Formation of cDNA Genes

RALPH DORNBURG† AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, 1400 University Avenue, University of Wisconsin, Madison, Wisconsin 53706

Received 30 June 1989/Accepted 6 October 1989

We showed previously that retrovirus vector particles can encapsidate RNAs without retroviral *cis*-acting sequences, that such RNAs are reverse transcribed in infected target cells, and that the cDNA copies are inserted into the host genome resulting in cDNA genes (R. Dornburg and H. M. Temin, Mol. Cell. Biol. 8:2328–2334, 1988). To provide further evidence that this retrovirus-mediated gene transfer occurred through an RNA intermediate, we constructed retroviral vectors containing an intron from a cellular gene. This intron was lost in a cDNA gene formed after infection with retroviral particles, establishing that an RNA intermediate had existed. Retroviral vectors with additional encapsidation sequences were constructed. The presence of a murine leukemia virus encapsidation sequence in an mRNA transcribed from the hygromycin B phosphotransferase gene increased the efficiency of encapsidation into spleen necrosis virus vector particles and the formation of cDNA genes by approximately 2 orders of magnitude.

The flow of genetic information through an RNA intermediate into the DNA genome plays an important role in shaping vertebrate genomes during evolution. Data obtained from molecular cloning and DNA sequencing of numerous eucaryotic genes suggest that more than 10% of the mammalian genome consists of sequences that arose by this retroposition of RNA. These sequences include retroviruses, retrotransposons, and retroposons as well as copies of other RNA polymerase II and III transcripts, processed pseudogenes of protein coding genes, and short interspersed sequences (2, 3, 13, 16–21, 23).

Recently, it was shown that retrovirus proteins can contribute to the genesis of cDNA genes (4, 11). We established a retroviral vector system (using spleen necrosis virus-derived vectors) to study cDNA gene formation of a protein-coding gene (hygromycin B phosphotransferase gene [7]). We found that RNAs without retroviral *cis*-acting sequences were encapsidated into retrovirus particles. Such RNAs were reverse transcribed and integrated into the genome of infected target cells. To provide further evidence that the transfer of the hygromycin resistance gene by retrovirus vector particles occurred through an RNA intermediate, we constructed a retroviral vector containing an intron (pRD35; Fig. 1) and followed the same experimental protocol described recently (4). pRD35 contains the second intron (including the splice donor and acceptor sites) of the human beta-globin gene (*Bam*HI-*Eco*RI fragment isolated from pHb-1S [10]). This intron was inserted into a *Bam*HI site located between the murine leukemia virus (MLV) U3 promoter and the thymidine kinase (TK) promoter (derived from the herpes simplex virus type 1 TK gene). The orientation of the intron was the same as that of the hygromycin B phosphotransferase (*hygro*) gene.

C3A2 helper cells (22) were transfected (8) with this

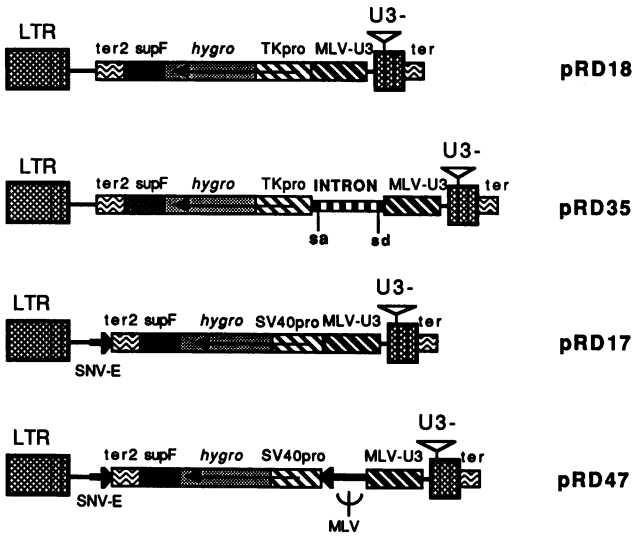

FIG. 1. Retroviral vectors. All vectors were derived from spleen necrosis virus (SNV) and contain spleen necrosis virus LTRs. In all vectors, the U3 region in the right LTR was deleted (U3−) (4, 5). Abbreviations: ter2 and ter, polyadenylation processing sites of the TK gene of herpes simplex virus and polyadenylation processing site of simian virus 40, respectively; supF, suppressor tRNA gene of *Escherichia coli*; TKpro, promoter of the TK gene of herpes simplex virus; MLV-U3, U3 promoter of MLV; sd, splice donor; sa, splice acceptor. pRD35 contains the second intron of the human beta-globin gene. pRD47 was derived from pRD17 and contains the MLV encapsidation sequence (Psi) between the two internal promoters. pRD17 differs from pRD18 in that the early promoter of simian virus 40 is substituted for the TK promoter. For more explanations, see the text.

* Corresponding author.
† Present address: University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Department of Molecular Genetics and Microbiology, Piscataway, NJ 08854-5635.

TABLE 1. Virus titers at steps 2 and 3[a]

Virus	Step 2			Virus titer (CFU/ml) at step 3 (D17 cells)
	Virus titer (CFU/ml)		No. of clones with intact provirus/total[b]	
	D17 cells	C3A2 cells		
RD18	8×10^3	2×10^2	8/9	1
RD35	5×10^2	3×10^1	4/5	0.5
RD17	2×10^4	7×10^2	16/23	1
RD47	2×10^4	5×10^2	5/10	1×10^{2c}

[a] Helper cells (C3A2) and D17 cells were infected with viruses harvested from transfected C3A2 helper cells. Titers (expressed as hygromycin resistant colony-forming units per milliliter of tissue culture supernatant medium) at step 2 (see the text) were determined as described previously (4).

[b] *Xho*I digestion of chromosomal DNAs isolated from step 2 helper cells gave a band of the predicted size. The lower titers on helper cells in comparison to D17 cells resulted from superinfection interference (9).

[c] Titer at step 3 of virus harvested from helper cells containing an intact provirus.

vector, selected for resistance to hygromycin B (hygro^r) (4), and passaged. (Transfected helper cells are designated step 1 cells.) These cells contained viral DNA like that in the plasmid. RNAs transcribed from the left long terminal repeat (LTR) promoter contain all *cis*-acting sequences for retroviral replication. Thus, these RNAs are efficiently encapsidated by virion proteins supplied by the helper cells (4). Virus was collected from confluent step 1 helper cell cultures, and fresh helper cells or D17 cells were infected (designated step 2 cells). The cells were selected for hygro^r, and the virus titers were determined as described recently (4). The titer of RD35 at step 2 was reduced by a factor of approximately 20 to 80 in comparison with that of pRD18, the vector from which pRD35 was derived (Table 1).

Analysis of step 2 cell lines. In the pRD35 vector construct, the promoter containing the U3 region of the right LTR was deleted (Fig. 1) and substituted by an *Xho*I linker (4, 5). As a result of retroviral replication, infection of step 2 cells resulted in the formation of a provirus with two U3-deleted (promoterless) LTRs, each containing an *Xho*I site (Fig. 2A). In our experiments, five single step 2 hygro^r helper cell colonies were isolated to give step 2 cell lines. Chromosomal DNAs were isolated (12), digested with *Xho*I, and subjected to Southern blot analysis (15). Four helper cell clones had a band of the expected size (Table 1, Fig. 2B, and data not shown). This result indicates that a provirus was formed, as expected, coinciding with our earlier observations (4). Further digestion of chromosomal DNAs with several more enzymes and analysis by Southern blots established that a correct provirus was formed in four such helper cell clones (data not shown). In these proviruses, RNAs were transcribed from the internal promoters only and were terminated in the TK polyadenylation processing site (Fig. 2A) (4, 5). These RNAs contained the intron in the correct orientation for RNA splicing. Thus, this intron should be spliced out (Fig. 2A).

Virus was harvested from confluent step 2 cultures con-

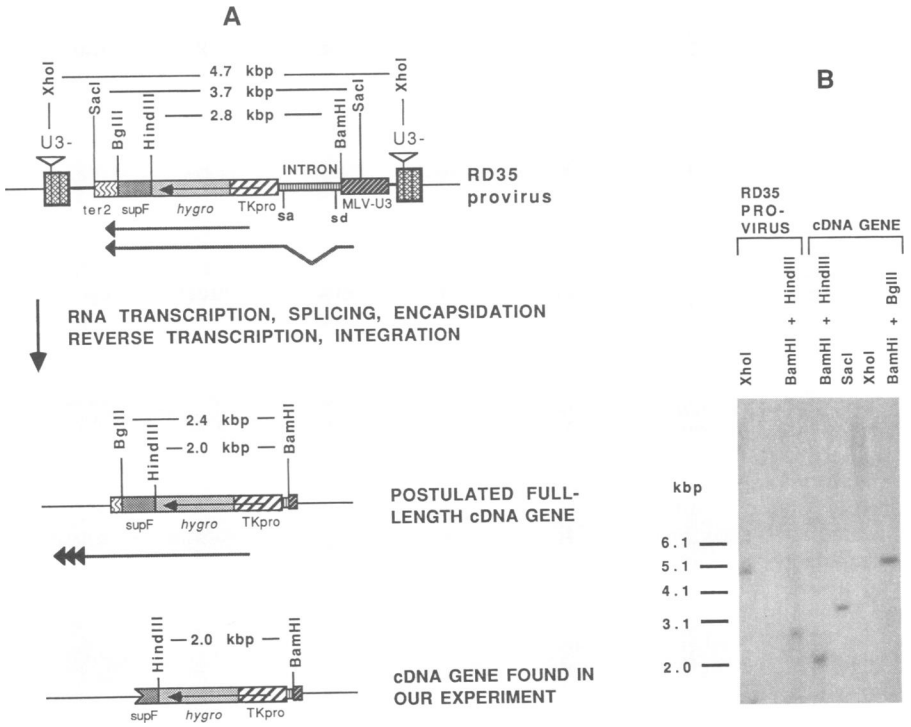

FIG. 2. Formation of cDNA genes by infection with retrovirus vector particles. (A) An RD35 provirus with U3-negative LTRs formed after infection with virus harvested from pRD35-transfected helper cells is shown at the top. RNAs expressed from the internal promoters are shown below. Transcripts starting from the MLV U3 promoter will be spliced. If these transcripts were encapsidated into retrovirus particles, reverse transcribed, and integrated in a infected target cell, the resulting postulated cDNA genes (middle) would lack the intron sequences. Expression of the *hygro* gene is driven by the internal TK promoter. Polyadenylation will depend on the presence of a polyadenylation signal in the chromosomal DNA. The structure of a cDNA gene formed in our experiments (as determined by Southern blot analysis) is shown at the bottom. (B) Southern blot analysis of chromosomal DNAs isolated from RD35-infected step 2 (RD35 provirus) and step 3 (cDNA gene) cell clones. The blot was hybridized with a purified *hygro* probe. The abbreviations are as in the legend to Fig. 1. For more explanations, see the text.

taining a correctly sized provirus, and fresh D17 cells were infected (step 3). The number of hygro^r cell colonies was determined (Table 1). The titer of RD35 at step 3 was similar to that of RD18, from which RD35 was derived (Table 1, step 3).

Analysis of cDNA genes. To investigate whether the hygromycin gene transfer from step 2 to step 3 was through an RNA intermediate, chromosomal DNA of one step 3 cell clone was analyzed by Southern analysis. Functional cDNA genes derived from the transcript starting from the MLV U3 promoter in the RD35 provirus should lack the intron sequences (Fig. 2A).

If the intron were spliced out, digestion of chromosomal DNAs of step 2 and step 3 cells with *Bam*HI plus *Hin*dIII and analysis of a Southern blot with a labeled *hygro* probe should result in bands with different mobility (Fig. 2A). The difference in size would reflect the loss of the intron. Our experiments confirmed this prediction (Fig. 2B). Moreover, digestion of the step 3 DNA with *Xho*I or *Sac*I resulted in bands different from the parental step 2 cell DNA. The cleavage sites of these restriction enzymes were located outside of the postulated RNA transcript. Thus, these data confirm that the transfer of the *hygro* gene by retroviral particles occurred through an RNA intermediate. In addition, the Southern analysis indicates that the cDNA gene was truncated at its 3′ end, as previously reported (4). This conclusion is based on the finding that digestion of the chromosomal DNA with *Bam*HI plus *Bgl*II resulted in bands larger than expected from the restriction enzyme cleavage sites present within a postulated full-length cDNA gene (Fig. 2).

Vectors with additional encapsidation sequences. The frequency of cDNA gene formation by infection with retroviral particles was about 8 orders of magnitude less than that of the formation of a retroviral provirus. RNA packaging was the least efficient step. Encapsidation of RNAs transcribed from RD17 and RD18 proviruses was approximately 4 orders of magnitude less efficient than that of RNAs with E sequences (in the absence of competition for encapsidation by retroviral RNAs) (4). To investigate whether more efficient encapsidation of nonretroviral RNAs would increase the frequency of cDNA gene formation, we constructed vectors with additional E sequences (pRD47; Fig. 1). Previously, it was shown that spleen necrosis virus virions efficiently encapsidate MLV-derived vector RNAs in the absence of competition for encapsidation by spleen necrosis virus RNAs (6). Thus, the encapsidation sequence of MLV (*Pst*I-*Bal*I fragment, map units 0.563 to 0.747) (1, 14) was inserted between the two internal promoters (*Bam*HI site) of pRD17. (pRD17 differs from pRD18 only by a substitution of the TK promoter for the simian virus 40 early promoter [4].)

Helper cells were transfected, selected for hygro^r, and passaged as described above. Virus was harvested from confluent plates. Fresh D17 cells and C3A2 helper cells were infected and selected for hygro^r, and the virus titers were determined. At step 2, RD47 gave titers similar to those of RD17, the vector from which RD47 was derived (Table 1, step 2).

Ten helper cell colonies were picked, and step 2 helper cell lines were established. Formation of the step 2 provirus was monitored by Southern blot analysis of chromosomal DNAs digested with *Xho*I as described above. Five step 2 helper cell lines revealed bands of the expected sizes (Table 1; Fig. 3, lanes a through c; data not shown). Digestion with several other enzymes confirmed that a correctly sized provirus was formed in these cells (data not shown). In the five other

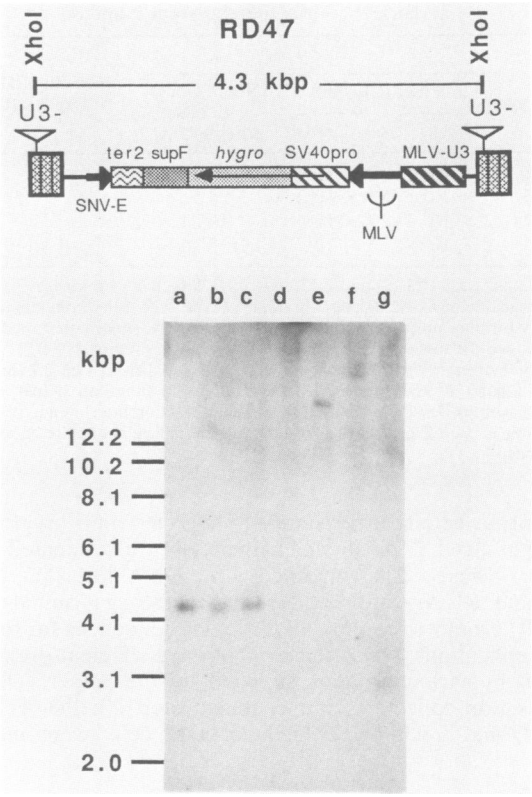

FIG. 3. Southern analysis of step 2 helper cell lines infected with RD47. The map of an RD47 provirus is shown at the top. Chromosomal DNAs were digested with *Xho*I and subjected to Southern analysis. The filter was hybridized with a *hygro* specific probe. For more explanations, see the text.

RD47-derived clones, proviruses with no reconstituted *Xho*I sites were found (Fig. 3; data not shown).

Virus was harvested from supernatant medium of step 2 helper cell lines containing a correctly sized provirus. Fresh D17 cells were infected (step 3), and virus titers were determined. Titers of approximately 100 CFU were observed with RD47, in comparison to 1 CFU observed with RD17 (Table 1, step 3). These data suggest that insertion of MLV encapsidation sequences increased the efficiency of formation of cDNA genes by 2 orders of magnitude.

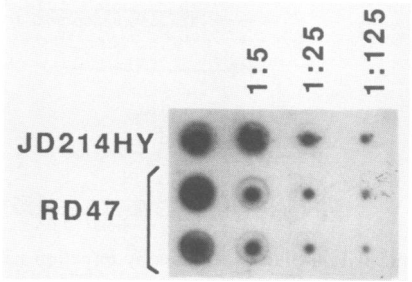

FIG. 4. RNA spot blot of viral RNAs encapsidated into retroviral vector particles: JD214HY RNA isolated from virus particles from 0.5 ml of tissue culture supernatant medium and RD47 RNA isolated from virus particles from 10 ml of tissue culture supernatant from two different RD47-derived helper cell lines containing RD47 proviruses with U3-deleted LTRs. The blot was hybridized with a *hygro* probe.

To test whether encapsidation was more efficient, the efficiency of encapsidation of RNAs transcribed from intact step 2 RD47 proviruses was compared by RNA dot blot analysis with that of a retroviral vector (JD214HY [4, 5]) with all *cis*-acting sequences for retroviral replication (Fig. 4). This experiment indicated that RNA of RD47 with the MLV encapsidation sequence was efficiently packaged. The efficiency was about a factor of 100 less that of the JD214HY RNA and thus 100 times more than that of RD17 (4).

Conclusion. Earlier we showed that retroviral proteins encapsidate mRNAs without retroviral *cis*-acting sequences; such RNAs were reverse transcribed and inserted into the genome of infected target cells to result in the formation of cDNA genes (4). To provide further evidence that this cDNA gene formation occurred through an RNA intermediate, we constructed a vector with an intron, pRD35, and followed the experimental protocol described previously (4). Infection of step 2 cells with RD35 resulted in the formation of proviruses with U3-negative LTRs. In such step 2 cells, RNAs are only transcribed from the internal promoters (4). These RNAs contain the intron in the same orientation as the *hygro* gene. Encapsidation of the processed (spliced) RNA into virions and infection of fresh target cells resulted in the formation of a functional cDNA gene without the intron. Thus, these data confirm that the transfer of the hygromycin B phosphotransferase gene occurred through an RNA intermediate, as found in a different system (11). Further, these data rule out the possibility that formation of cDNA genes in our experiments was the result of RNA transcripts originating outside of the provirus in the orientation of the vector. Such RNAs would still contain retrovirus *cis*-acting sequences.

To investigate whether the presence of an encapsidation sequence increased the frequency of cDNA gene formation, we constructed a vector with an additional E sequence, pRD47. We followed the same experimental protocol. The efficiency of transfer of hygro^r was 2 orders of magnitude higher than that of RNAs without encapsidation sequences (4). Recently, it was shown that other sequences in MLV (extending into the *gag* region) further increase packaging efficiency of RNA in MLV vector particles (1). Thus, constructs with such additional sequences may further increase encapsidation efficiency and therefore the efficiency of formation of cDNA genes. JD214HY, a vector with all *cis*-acting sequences, gave titers of about 10^7 CFU/ml at step 3 (4, 5). Thus, transfer of hygro^r by RD47 was 5 orders of magnitude less efficient than transfer by JD214HY. Less efficient packaging of RD47 step 2 RNA accounts for a factor of 100 of the decreased titer. Previously, we estimated that inefficient reverse transcription and integration (as a result of the lack of *cis*-acting viral sequences) reduced cDNA gene formation by 2 and 1 order of magnitudes, respectively (4). Thus, our results with RD47 are consistent with the previous experiments, but with more efficient encapsidation.

We thank S. Hinz, J. Couch, and J. Rein for technical assistance. We thank M. Hannink, B. Sudgen, and A. Panganinban for helpful comments on the manuscript.

This research was supported by Public Health Service research grants CA-22443 and CA-07175 from the National Cancer Institute. R.D. was supported by a fellowship from the Deutsche Forschungsgemeinschaft until June 30. H.M.T. is an American Cancer Society Research Professor.

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