

Retroviral recombination is temperature dependent

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Two conflicting *in vitro* observations suggest that retroviral recombinations are temperature dependent. Ouhammouch & Brody (*Nucleic Acids Research* 20, 5443–5450, 1992) suggested that retroviral recombination rates should increase as temperature increases. However, Shimomaye & Salvato (*Gene Analysis Techniques* 6, 25–28, 1989) and Brooks *et al.* (*Biotechniques* 19, 806–812, 814–815, 1985) found that at low temperature the tightly folded structure of RNAs may hinder reverse transcription proceeding along the RNA template, which increases its chance of dissociating from the template; therefore, raising the reaction temperature was the simplest way to overcome template secondary structure and prevent premature termination of cDNA synthesis. In this report, two vectors based on murine leukaemia virus (MLV) were constructed. The first contained two mutated *gfp* genes in tandem positions. The upstream *gfp* gene encoded a mutation at its 3' end, while the downstream *gfp* gene encoded a mutation at its 5' end. The recombination that occurred between the two mutated *gfp* genes restored a functional *gfp* gene. The cells that contained the functional *gfp* gene were green when observed under a fluorescence microscope. The second MLV vector contained a functional *gfp* gene with two identical sequences flanking either end. A recombination that occurred between the two identical sequences resulted in deletion of the *gfp* gene. Cells containing the vector with the *gfp* deletion were colourless or clear when observed under the microscope. Using these two vectors, we have demonstrated that retroviral recombination is temperature dependent and the rate of recombination decreases as temperature is raised from 31 to 43 °C.

Introduction

Retroviruses are a family of animal viruses that use RNA as their viral genetic material and DNA as their cellular genetic material. The parental virus attaches via its envelope protein to a specific receptor on the surface of a susceptible cell, leading to fusion and entry of the core. After entering the cell, the reverse transcriptase within the virion transcribes the RNA genome into double-stranded DNA. Following DNA synthesis, which takes place asynchronously within the first 4–8 h after infection, the viral DNA becomes tightly associated with or within the nucleus. Then the double-stranded DNA integrates into the host chromosomal DNA. Integrated viral DNAs are called proviruses.

Retroviral recombination plays an important role in retroviral carcinogenesis and in the AIDS epidemic (Coffin *et*

al., 1997). Most retroviral recombinations occur during minus-strand DNA synthesis (Zhang *et al.*, 2000). Two conflicting *in vitro* observations suggest that retroviral recombinations are temperature dependent. Ouhammouch & Brody (1992) demonstrated that during *in vitro* cDNA synthesis, the avian myeloblastosis virus (AMV) reverse transcriptase could switch from one template to another in a temperature-dependent manner. Chimeric cDNA molecules were generated with an increasing efficiency from lower temperature to higher temperature. This result suggested that retroviral recombination rates should increase as temperature increases. However, Shimomaye & Salvato (1989) and Brooks *et al.* (1995) found that raising the reaction temperature was the simplest way to overcome template secondary structure to prevent premature termination of cDNA synthesis. Their results suggested that the recombination rate should decrease as temperature increases.

In this report, we have established an *in vivo* system to demonstrate that the rate of retroviral recombination decreases

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as the temperature of reverse transcription is increased from 31 to 43 °C.

Methods

■ **Nomenclature.** Plasmids are designated, for example, pJZ481, pJZ442 + 3' Hyg; viruses made from these plasmids are designated, for example, JZ481, JZ442 + 3' Hyg.

■ **Vector constructions.** All recombinant techniques were carried out by conventional procedures (Sambrook *et al.*, 1989). All vector sequences are available upon request.

(i) **Introduction of restriction enzyme sites into the *gfp* gene.** The *gfp* gene was from the jellyfish *Aequorea victoria* (pEGFP; Clontech) (Chalfie *et al.*, 1994). Introduction of frame-shift mutations has been described previously (Zhang *et al.*, 2000). Briefly, the *gfp* gene was mutated by PCR to create a *Bst*BI site at position 624 and the mutated *gfp* was designated *gfp-Bst*BI. To introduce a frame-shift mutation into the *gfp* gene, the *gfp-Bst*BI gene sequence was digested with *Bst*BI, followed by repair with Klenow fragment. *Bst*BI digestion created two DNA ends that contained a two-base overhang. When the overhangs were repaired by the Klenow fragment two blunt ends were created. Ligation of these two blunt ends with T4 ligase created a 2 bp insertion, which shifted the *gfp* open reading frame by two (+2).

The *gfp* gene was also mutated so that it contained a *Nco*I site at position 169. This resulting *gfp* gene was designated *gfp-Nco*I. *Gfp-Nco*I was digested with *Nco*I followed by repair with Klenow fragment and ligation. *Nco*I digestion created two DNA ends that contained a four-base overhang. As a result, this *gfp* open reading frame was shifted by one (+1) (Zhang *et al.*, 2000).

(ii) **Construction of pJZ481.** pJZ481 (Fig. 1A) is an MLV vector which contains two mutated *gfp* genes and a neomycin resistance gene (*neo*)

(Li & Zhang, 2000). The pJZ481 construct, from 5' to 3', was assembled as follows. The 5.4 kb *Nde*I–*Bam*HI fragment (from positions 3810 to 1630), which contained the *neo* gene and the two MLV long terminal repeats (LTRs), was isolated from pLN (Miller & Rosman, 1989). The 0.7 kb *Bam*HI–*Bgl*II (from positions 1631 to 2150) fragment of *gfp-Bst*BI⁻ was derived from pEGFP and the frame-shift mutation was located at the 3' end of the *gfp* gene. The 0.7 kb *Bgl*II–*Not*I (from positions 2151 to 3190) fragment of *gfp-Nco*I⁻ was also derived from pEGFP and the frame-shift mutation was located at the 5' end of the *gfp* gene. The 0.6 kb *Not*I–*Nde*I fragment (from positions 3191 to 3809) was isolated from pCITE-1 (Novagen) and contained the internal ribosome entry segment (IRES) sequence.

(iii) **Construction of pJZ442 + 3' Hyg.** The pJZ442 + 3' Hyg (Fig. 1B) construct was described previously (Zhang & Sapp, 1999). Briefly, this MLV-based construct carries the hygromycin resistance gene (*hyg*) and the *gfp* gene, along with an IRES sequence between the two genes. This vector also includes the insertion of a sequence homologous to the 290 bp sequence of the 3' *hyg* gene into the 3' untranslated portion of the *gfp* gene.

■ **Introduction of JZ481 and JZ442+3' Hyg into helper cell line PG13.** Plasmid DNAs of pJZ481 and pJZ442 + 3' Hyg were transfected into an MLV amphotropic helper cell line PA317 (Miller & Buttimore, 1986). The supernatant media containing the viruses were collected and designated STEP 1 virus. The STEP 1 virus was used to infect the MLV xenotropic helper cell line PG13 (Miller *et al.*, 1991). The viruses released from the infected PG13 cells were unable to infect NIH 3T3 derivatives – including PG13 (Miller *et al.*, 1991). This procedure ensured that the infection of D17 target cells with viruses collected from PG13 cells represented only a single round of infection. Infected PG13 cells were selected for Neo^r and Hyg^r for JZ481 and JZ442 + 3' Hyg respectively. Visible colonies appeared after 10 days of selection. The cells of well-separated clear colonies were isolated and designated STEP 2 cells for JZ481. Green colonies were isolated as JZ442 + 3' Hyg STEP

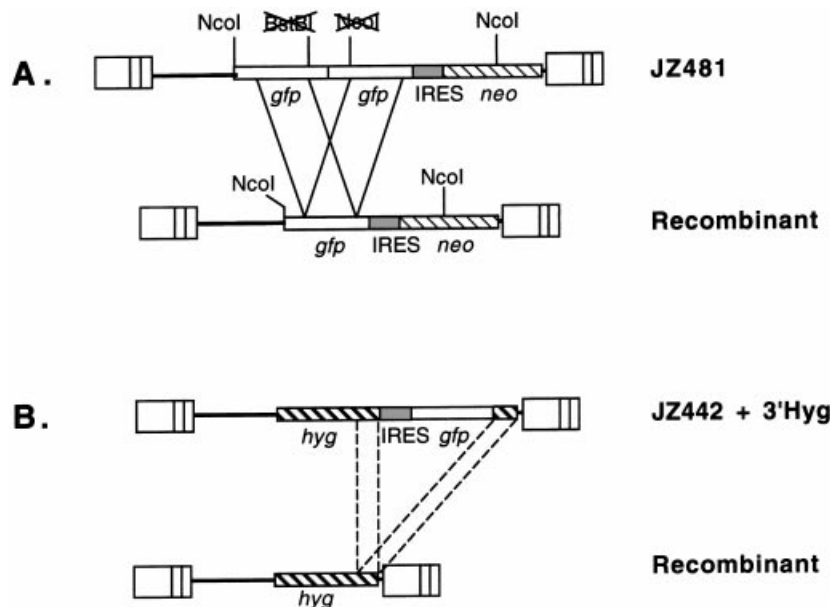


Fig. 1. Structures of retroviral vectors used for determination of the recombination rate. (A) Structure of the retrovirus vector containing nonfunctional *gfp* genes (two mutated *gfp* genes in tandem positions) and a *neo* gene. The *gfp* gene is expressed from the 5' MLV LTR, and the *neo* gene is expressed from an encephalomyocarditis virus IRES. The upstream *gfp* gene encodes a frame-shift mutation at its 3' end while the downstream *gfp* gene encodes another frame-shift mutation at its 5' end. Recombination between the 450 bp identical sequences results in a functional *gfp* gene. (B) Structure of the retroviral vectors containing two identical sequences flanking a functional *gfp* gene. JZ442 + 3' Hyg carries a functional *gfp* gene and a *hyg* gene with an IRES sequence between them. In addition, it also contains 290 bp of the 3' *hyg* sequence downstream of the *gfp* gene. After one round of replication, the downstream 3' *hyg* gene sequence will recombine with the identical upstream *hyg* gene sequence which results in the deletion of the *gfp* gene. The broken lines between the vectors and recombinant proviruses indicate identical sequences.

2 cells. Viruses harvested from STEP 2 cells were designated STEP 2 viruses.

Infection of target D17 cells. STEP 2 viruses were incubated with D17 cells for 5 min at 37 °C. The cells were washed with TD three times to remove viruses that did not attach to the cells. Fresh medium was added and the cells were incubated at 24, 31, 37 and 43 °C. Nine hours after infection cells were transferred to 37 °C. Medium with hygromycin for JZ442 + 3' Hyg or medium with G418 for JZ481 was added 24 h after infection. Visible colonies were observed 10–12 days after infection and designated STEP 3 cells.

Cells, transfection and infection. The processing of D17 cells (ATCC CRL-8468), PA317 helper cells (ATCC CRL-9078), PG13 helper cells (ATCC CRL-10686), DNA transfections, virus harvesting and virus infections were as previously described (Zhang & Temin, 1993).

Fluorescence microscopy. A fluorescence inverted microscope (Zeiss Axiovert 25) with a mercury arc lamp (100 W) and a fluorescence filter set (CZ909) consisting of a 470/40 nm exciter, a 515 nm emitter, and a 500 nm beam splitter was used to detect green fluorescent protein in living cells.

Results

Construction of vectors for determination of retroviral recombination

Previous studies of retroviral recombination compared titres of two vectors with two different drug resistance genes (Hu & Temin, 1990; Zhang & Temin, 1993). A system has been developed to determine the recombination between two identical sequences within the same retroviral vector (Zhang & Sapp, 1999; Li & Zhang, 2000). Those vectors encoded only one drug resistance gene and the recombination rates were determined by screening an unselected colour reporter gene. Compared to the data determined by drug selections, the deviation of the rates was found to be smaller when using vectors that rely on the unselected colour report gene for quantification of recombination frequency. Two vectors with identical sequences were tested in this study. The first vector, JZ481 (Fig. 1A), contained nonfunctional *gfp* genes (two mutated *gfp* genes in tandem positions) and a *neo* gene. The upstream *gfp* gene encoded a frame-shift mutation at its 3' end whereas the downstream *gfp* gene encoded another frame-shift mutation at its 5' end. Therefore, the *gfp* genes in this vector were not functional. The two mutated *gfp* genes were followed by an IRES sequence and a drug resistance gene (*neo*) (Li & Zhang, 2000). The *neo* gene allows for measurement of viral titre independently upon recombination. The IRES sequence of the encephalomyocarditis virus origin allows the ribosome to bind to the internal AUG that initiates translation of the second gene independently of the upstream gene(s) (Adam *et al.*, 1991; Boris-Lawrie & Temin, 1993). The rates of backward frame-shift mutations of these two mutated *gfp* genes were less than 10⁻⁵ per replication cycle (Zhang *et al.*, 2000). Recombination between the two identical sequences of 450 kb in length resulted in a functional *gfp* gene, in which the two frame-shift mutations were deleted (Fig. 1A). Consequently, cells containing the functional *gfp* gene were green while cells

Table 1. Recombination rate determined by microscopic analysis of cells infected with JZ481

Green, no. of green colonies observed by fluorescence microscopy; clear, no. of clear colonies observed by fluorescence microscopy; total, no. of green + clear colonies observed by fluorescence microscopy. The rate of recombination was determined by the ratio of the number of green colonies to the number of total colonies.

Clone*	24 °C			31 °C			37 °C			43 °C		
	Green	Clear	Total	Green	Clear	Total	Green	Clear	Total	Green	Clear	Total
Clone 1												
E1	42	66	108	42	50	92	41	45	86	13	22	35
E2	33	53	86	32	38	70	46	70	116	11	36	47
E3	37	48	85	33	33	66	43	72	115	9	28	37
E4	33	47	80	41	42	83	43	50	93	17	37	54
Clone 2												
E1	100	93	193	39	31	70	77	74	151	46	86	132
E2	70	88	158	68	52	120	68	74	142	60	81	141
E3	101	101	202	66	70	136	56	90	146	35	63	98
E4				65	74	139	46.8					
				44.0 ± 5.2†			49.8 ± 4.3					
										44.0 ± 5.4		
												32.8 ± 6.9

*Numbers of virus clones used in this study. For each clone, four individual experiments were carried out and designated E1, E2, E3 and E4.
† Mean ± standard deviation.

represented a single round of viral infection. Neo^r and Hyg^r colonies were analysed by fluorescence microscopy. For D17 cells infected with JZ481, green cells represented recombinants carrying a functional *gfp* gene (Fig. 1A), whereas clear cells contained parental mutated *gfp* genes. The rate of recombination was determined by the ratio of the number of green colonies to the number of total Neo^r colonies (green plus clear colonies) (Table 1 and Fig. 2). For D17 cells infected with JZ442 + 3' Hyg (Fig. 1B), the rate of recombination was determined by the ratio of the number of clear colonies over the number of total Hyg^r colonies (Table 2 and Fig. 2). As indicated in Fig. 2, the recombination rate decreased as the temperature of reverse transcription increased from 31 to 43 °C. A slight decrease in the recombination rate at 24 °C was observed.

To determine the nature of the recombinants of JZ481, cellular genomic DNA from green and clear colonies was digested with *Nco*I and hybridized with a *neo* probe. The parental provirus produced a 2.7 kb fragment, while the recombinant provirus with a functional *gfp* gene produced a 1.9 kb fragment (Fig. 1A). DNA from green cell clones formed a distinct 1.9 kb band, indicating that functional *gfp* genes in green cells resulted from recombination between the two tandem mutated *gfp* genes (Li & Zhang, 2000).

To determine the nature of the recombinants of JZ442 + 3' Hyg, DNAs from clear and green colonies were digested with *Eco*RV and hybridized with a *hyg* probe. *Eco*RV digested within the two LTRs of both parental and recombinant proviruses (Fig. 1B). The parental proviruses from green colonies produced a 4.2 kb fragment and the recombinant proviruses from clear colonies produced a 2.5 kb fragment (Zhang & Sapp, 1999). The Southern analysis indicated that the phenotype of most clear colonies resulted from deletion between the two identical 290 bp sequences of 3' *hyg*.

Discussion

A cell culture system has been established to evaluate the effect of temperature on retroviral recombination. Ouhammouch & Brody (1992) reported that in an *in vitro* system, efficiency of template switch increased when temperature increased. In another *in vitro* system, Shimomaye & Salvato (1989) and Brooks *et al.* (1995) reported that the efficiency of template switch decreased with increased temperature. We demonstrated that the recombination rate decreased as the temperature of reverse transcription increased from 31 to 43 °C *in vivo*.

Retroviral RNA molecules usually form numerous secondary structures (Leis *et al.*, 1993; Coffin *et al.*, 1997). One possible explanation for such an observation is that temperature change induces alternation in the secondary structure of RNA molecules. At higher temperature, the looser conformation of RNA molecules tends to form, which may facilitate the processing of reverse transcription. Since most

retroviral recombinations occur during minus-strand DNA synthesis (Zhang *et al.*, 2000), at low temperature these tightly folded structures may hinder reverse transcription proceeding along the RNA template, which increases its chance of dissociating from the template (Mikkelsen *et al.*, 2000; Pathak & Temin, 1992). *In vitro* studies have shown that pausing of retroviral reverse transcriptase enhances strand transfer (Wu *et al.*, 1995, 1996; Kim *et al.*, 1997). The rate of recombination did not decrease much when the temperature increased from 31 to 43 °C in this system, probably because the secondary structures within the *gfp* gene or the *hyg* gene sequences were not strong. A more dramatic change of rates is expected if recombination occurs between two sequences with tighter secondary structures. In addition, the temperature range in this report was limited due to tissue culture conditions, which were different from *in vitro* experiments (Shimomaye & Salvato, 1989; Brooks *et al.*, 1995).

Retroviral recombination may involve three steps: (1) pausing of reverse transcription; (2) the reverse transcription growing point leaving the original template; and (3) the growing point landing on the target/alternate template. In addition to affecting RNA secondary structures and the pausing of reverse transcriptase (step 1), higher temperature may also promote the dissociation of reverse transcriptase from its template (step 2), which increases the change of template switches. However, our *in vivo* results suggest that step 1 (pausing) is more crucial than step 2 (leaving) in retroviral recombination when reverse transcriptions are between 31 and 43 °C.

Another possibility is that decreasing temperature reduced elongation rates of reverse transcriptase, which also results in the pausing of reverse transcription. The pausing increased the chance of template switching, thereby increased the rate of recombination at lower temperature. It is also possible that decreasing temperature might have an effect upon the core particle structure, or it might have a direct effect upon reverse transcriptase as well as other unforeseen effect(s), which in turn affects viral recombinations.

Our data for 24 °C showed a slight decrease in the recombination rate compared to that for 31 °C. Two factors might have contributed to this observation. First, at temperatures lower than 31 °C RNA molecules might undergo little change as temperature altered, or secondly, the frequency of dissociation of the reverse transcriptase from its template was reduced.

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