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 vitro system to demonstrate that the rate of retroviral recombination decreases
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 BstBI site at position 624 and the mutated gfp was designated gfp-BstBI. To introduce a frame-shift mutation into the gfp gene, the gfp-BstBI gene sequence was digested with BstBI, followed by repair with Klenow fragment. BstBI 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 NcoI site at position 169. This resulting gfp gene was designated gfp-NcoI. Gfp-NcoI was digested with NcoI followed by repair with Klenow fragment and ligation. NcoI 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 NdeI–BanHI 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 BanHI–BglII fragment (from positions 1631 to 2150) fragment of gfp-BstBI was derived from pEGFP and the frame-shift mutation was located at the 3’ end of the gfp gene. The 0.7 kb BgII–NolI fragment (from positions 2151 to 3190) fragment of gfp-NcoI1 was also derived from pEGFP and the frame-shift mutation was located at the 5’ end of the gfp gene. The 0.6 kb NolI–NdeI 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’ and Hyg’ 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
Table 1. Recombination rate determined by microscopic analysis of cells infected with JZ481

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<th>Recomb. rate (%)</th>
<th>Green</th>
<th>Clear</th>
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*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.
Retroviral recombinations are temperature dependent

Resistance gene (Neo) for JZ442+3′ Hyg. Visible colonies were observed with the mutated gfp gene when observed under a fluorescence microscope.

When infected, the cells were selected for neomycin resistance of the cells at higher or lower temperature. Twenty-four hours post-infection, the cells were selected for neomycin resistance. 

Table 2. Recombination rate determined by microscopic analysis of cells infected with JZ442+3′ Hyg

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 clear colonies to the number of total colonies.

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<th>Clone*</th>
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*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<sup>+</sup> and Hyg<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 NcoI and hybridized with 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 EcoRV and hybridized with a hyg probe. EcoRV 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 alteration 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.

We would like to thank William Bargmann, Kathleen Boris-Lawrie, Rick Parks and Christy Sapp for helpful comments on the manuscript. This research was supported by Public Health Service research grants CA70407 from the National Institutes of Health.

**References**


Received 6 December 2000; Accepted 6 February 2001