Synthesis of Proviral DNA in Inbred Mouse-derived Clones of Cells Expressing Different Fv-1 Phenotypes

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SUMMARY

Formation of proviral DNAs by B-tropic murine leukaemia viruses (MLVs) was examined in N-type and dually permissive mutant cells derived from two inbred mouse strains, DDD and G, both of which are N-type. In the N-type cells, formation of circular proviral DNA was strongly suppressed relative to that of linear DNA. Mutation resulting in loss of the N-type Fv-1 restriction resulted in efficient formation of circular DNA by the previously restricted B-tropic MLV. This showed that Fv-1 restriction and inhibition of closed circular DNA formation were controlled by the same gene. The efficiency of formation of circular proviral DNA by the defective Kirsten murine sarcoma virus was determined by the tropism of the helper virus.

The RNA form of the retrovirus genome of murine leukaemia virus (MLV) is transcribed into linear and circular DNA duplexes after infection, and then integrated into the host chromosome. However, it is still unclear which of the free forms of viral DNA is the final precursor of the integrated form (Varmus, 1983). Study of the restriction exercised by the Fv-1 locus of mice has been found appropriate for studying this problem. The formation of circular but not linear DNA was suppressed in Fv-1 restrictive (N-type) cells (Jolicoeur & Rassart, 1980; Yang et al., 1980; Chinsky & Soeiro, 1981), and hence circular DNA was considered to be the proximate precursor. However, these experiments are not entirely free of criticism. One argument is that the genetic backgrounds of the pairs of N-type and B-type mouse cells used are widely different. Even in the case of congenic mice obtained by eight serial backcrosses (Axelrad, 1968), at least $6 \times 10^5$ to $1 \times 10^6$ base pairs around the Fv-1 locus are still of donor origin (assuming that each mouse chromosome has $5 \times 10^8$ to $1 \times 10^9$ base pairs and that the DNA of the donor origin is diluted twofold at each backcross). In addition, the formation of the circular duplex was not prevented in some Fv-1 restrictive hosts (Chinsky & Soeiro, 1981). Thus, the problem has not been settled. The first criticism could be answered by experiments with the Fv-1 mutant cells obtained following somatic mutation in culture.

D8bl and D3hl are N-type and dually permissive clonally related cell lines derived from a litter of inbred strain DDD mice (Yoshikura et al., 1982), and G33k5 and G66m2 are N-type and dually permissive cells similarly derived from a litter of inbred strain G mice (Yoshikura et al., 1978). Both pairs of N-type and dually permissive cells were obtained by repeated colony isolations, selecting clones that were strongly restrictive to B-tropic virus but fully permissive to N-tropic virus, or those which were permissive to both N-tropic and B-tropic viruses. D8bl cells are about $10^3$-fold less sensitive to B-tropic WNB2N6 virus than D3hl cells, whereas both cell lines are equally sensitive to N-tropic virus (Yoshikura et al., 1982; Fig. 1a). In addition, cultivation of B-tropic virus in D8bl cells resulted in the conversion of B-tropic virus to NB-tropic virus, while in D3hl cells no such host range conversion of the virus was detected (Fig. 1a, c). Cultivation of N-tropic FN2N7 virus in both types of cell did not affect the tropism of the virus (Fig. 1b). Thus, D8bl cells are considered to be truly N-type, and D3hl are truly dually permissive.

Using these pairs of N-type and dually permissive cells, synthesis of MLV provirus was...
examined. Cells seeded at a density of $2.0 \times 10^6$ per 100 mm dish were infected with virus at a multiplicity of 0.1 for MLV and 0.03 for murine sarcoma virus (MSV) in the presence of Polybrene (10 µg/ml). The viruses were Kirsten (Ki) MSV enveloped in N-tropic FN2N7 or B-tropic WNB2N6 virus coats (Yoshikura & Yoshida, 1978). These were obtained from the culture medium of Ki-NRK cells infected with the respective MLV helper. The medium of the infected cultures was renewed 6 h later. At 6 or 24 h after inoculation, the cells were harvested for analysis of unintegrated DNA in the Hirt supernatant (Hirt, 1967), extracted according to the modified method of Yang et al. (1980). DNA was analysed by horizontal agarose gel electrophoresis, one-half of the DNA extracted from each dish having been applied to each lane, transferred to nitrocellulose sheets (Southern, 1975), baked, and then hybridized either with the whole genome of Moloney (Mo) MLV inserted at the HindIII site of pBR322 (P8.2) (Berns et al., 1980) or with the HincII-HincII fragment of Ki-MSV which includes the Ki-ras gene (Tsuchida et al., 1982). The Mo-MLV probe mainly detected MLV (9-6 kb), while the Ki-ras probe detected Ki-MSV DNA (7-0 kb) only.

Linear DNAs of N-tropic and B-tropic viruses were synthesized to an equal extent in D8b1 and D3h1 cells (Fig. 2a). In the N-type G strain-derived G33k5 cells, linear DNAs of both N-tropic and B-tropic viruses were formed less efficiently than in the other cells. This was due to slower growth of G33k5 cells relative to other cells [note that MLV infection is dependent upon cell growth (Yoshikura, 1968)]. Formation of circular DNA compared to that of linear DNA by N-tropic FN2N7 virus was equally efficient in D8b1, D3h1, G33k5 and G66m2 cells (Fig. 2a, b). But the formation of circular DNA by B-tropic MLV was lower in N-type D8b1 or G33k5 cells relative to dually permissive cells (Fig. 2a, b). This was confirmed by measuring the
Fig. 2. Proviral DNA formed in the inbred mouse-derived clonal cells. Cells (2 x 10^6/dish) were infected with Ki-MSV (FN2N7) (1.2 x 10^5 p.f.u. and 0.7 x 10^5 f.f.u. per dish) or with Ki-MSV (WNB2N6) (1.2 x 10^5 p.f.u. and 0.7 x 10^5 f.f.u. per dish). DNA in the Hirt supernatant was extracted at 6 and 24 h. One-half of the DNA from each dish was applied to each lane and electrophoresed. (a, b, c) Hybridization with a MLV probe; (d, e, f) hybridization with a Ki-ras probe. The viruses used for infection, cells and the time of harvest are indicated at the top of the figure. L, Linear proviral DNA; C, closed circular DNA. Molecular sizes are marked (kb).

intensity of the bands in the X-ray film with a Shimazu CS-910 scanner. The intensities of the circular (C)DNAs [with either one or two long terminal repeat sequences (LTR)] and that of linear (L) DNA were measured, and the ratio C/L was calculated. Then, the C/L ratio obtained in N-type cells was divided by that from dually permissive cells of the same origin to obtain the N/D ratio. The N/D ratios were 0.79 to 0.87 for N-tropic MLV and 0 to 0.43 for B-tropic MLV (Table 1). A control experiment (Fig. 2c) showed that circular DNA of B-tropic virus was produced in D3h1 cells as efficiently as in B-type BALB/c strain-derived BE cells.

Thus, in infection of N-type cells by B-tropic virus, although the formation of linear DNA was not restricted that of circular DNA was inhibited. In infection of dually permissive mutant cells by B-tropic virus, the restriction of circular DNA was absent. As the two types of cells were obtained by somatic mutation in culture, the gene controlling the sensitivity to B-tropic virus
Table 1. Formation of proviral DNA in N-type and dually permissive cells: photodensitometric data*

<table>
<thead>
<tr>
<th>Infecting virus</th>
<th>Hybridization probe</th>
<th>DDD origin</th>
<th>G origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D8b1 (N)†</td>
<td>D3h1 (D)†</td>
</tr>
<tr>
<td>Ki-MSV (FN2N7)</td>
<td>MLV</td>
<td>0.13</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Ki-ras</td>
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<td>0.41</td>
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<tr>
<td>Ki-MSV (WNB2N6)</td>
<td>MLV</td>
<td>0.13</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Ki-ras</td>
<td>0.17</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* The X-ray film shown in Fig. 2 was analysed using a Shimazu scanner. Data at 24 h were used.
† C/L ratio.
‡ C/L ratio of N-type cells divided by C/L ratio of dually permissive cells.

and that controlling the formation of circular DNA must be the same. If these two phenotypes were controlled by two independent genes, and assuming that the spontaneous mutation rate per locus was $10^{-7}$, the probability of occurrence of a double mutant would be $10^{-14}$. This figure is too low to allow detection in cultured cells. In addition, the same genetic correlation was observed with pairs of cell mutants which were established independently from two different mouse strains.

One interpretation of our data is that the immediate precursor of the integrated form of DNA is the circular DNA duplex and not linear DNA, as virus susceptibility was correlated with formation of circular molecules and not with linear molecules. Another interpretation is that correlation of restriction of viral replication and that of circular DNA formation is a coincidence (in this case, the linear molecule would be the immediate precursor for integration); this could arise by the existence of a common process in the circularization of linear DNA and integration of the linear or circular provirus.

In speculating on the mechanism of Fv-1 restriction, it should be borne in mind that circular DNAs with one LTR and those with two LTRs were both restricted in the restrictive cells (Fig. 2). The circular molecules with one LTR must be produced by homologous recombination in the LTR sequence, whereas those with two LTRs must be the product of blunt-end ligation (Varmus, 1983). How could one cellular mutation result in prevention of both types of recombination which must be different in mechanism? One possibility is that one of the DNA duplexes is the precursor of the other, and that the Fv-1 gene product inhibits the formation of the precursor form. However, transition from either of the DNA forms to the other has not been reported.

In our experiments, the restriction of formation of circular DNA of Ki-MSV was determined entirely by the tropism of the helper virus used for rescue (Fig. 2d, e). The N/D ratio was 0.67 to 0.87 for Ki-MSV rescued by N-tropic virus and 0.38 to 0.49 for Ki-MSV rescued by B-tropic virus (Table 1). This observation is consistent with previous results establishing the Fv-1 determinant as a viral structural protein (p30) (Faller & Hopkins, 1977; Hopkins et al., 1977; Gautsch et al., 1978; Rommelaere et al., 1979; Rassart et al., 1981; Ou et al., 1983; DesGroseillers & Jolicoeur, 1983) and confirms the phenotypic mixing reported by Rein et al. (1976).

The titration data in Fig. 1(a) were obtained with culture fluid of D8b1 or D3h1 cells which had been infected with WNB2N6 virus for 2 months, and 100% of the cells were virus producers (estimated by infectious centre assay). The B tropic virus was released from restrictive D8b1 cells to the same extent as from non-restrictive D3h1 cells. (The NB-tropic virus present in the medium of D8b1 cultures was about 1%, with the majority of virus remaining B-tropic.) This shows that Fv-1 restriction is exclusively an early event in MLV infection.

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