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Restricted Infectivity of Ecotropic Type C Retroviruses in Mouse Teratocarcinoma Cells: Studies on Viral DNA Intermediates

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Replication of Gross strain N-tropic type C retrovirus was markedly restricted in a pluripotential undifferentiated embryonal cell line (PCC_4) of murine teratocarcinoma, whereas the same virus could cause productive infection in a myoblast-derived differentiated line (PCD_1) of the same tumor origin. To investigate the restriction mechanism, we compared the initial viral DNA formation in these two cell lines. Analyses by means of a modified Hirt extraction procedure and a modified Southern gel transfer method indicated that PCC_4 and PCD_1 cells supported the synthesis of viral DNA intermediates after inoculation of the Gross virus. In both cells, a linear DNA duplex (form III viral DNA) appeared at 4 hr, reached a maximal level at 8–9 hr, and declined rapidly thereafter, while two closed-circular supercoiled DNA duplexes (form I viral DNA) showed their appearance, increase and decline in the 8–24 hr period. During the period from 34 to 78 hr after virus inoculation, another burst of viral DNA synthesis occurred in PCD_1 cells, presumably due to secondary virus infection, while at this period both form III and form I viral DNAs became undetectable in PCC_4 cells. The Hirt supernatant DNAs prepared from PCD_1 and PCC_4 cells 10 hr after virus inoculation were equally infectious for NIH3T3 cells in a DNA transfection assay. Both PCD_1 and PCC_4 cells were very poor recipients for DNA transfection, although one positive result with PCD_1 cells might suggest a difference between the two cell types in this aspect. These results indicate that restriction of type C retrovirus in undifferentiated embryonal carcinoma cells occurs at a step subsequent to formation and maturation of viral DNA intermediates.

Key words: retroviruses, embryonal carcinoma, viral DNA forms, transfection, diazobenzyloxymethyl-paper transfer

Studies on mouse teratocarcinomas in the animal [1–3] as well as in cell culture [4, 5] have indicated that the oncogenic potential of these tumors is associated with their undifferentiated cell components, which resemble early mouse embryo cells in the capacity

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to undergo ontogenetic development. Lines of cultured teratocarcinoma cells representing different developmental and differentiating states have been established in vitro and serve as a useful model for studying the differentiation process and the neoplastic property. One particular study has been the interaction between teratocarcinoma cells and tumor viruses. It has been shown that undifferentiated embryonal carcinoma cells are refractory to infection with polyoma and simian virus 40 (SV40) viruses, whereas differentiated cells of the same tumor origin are permissive to these viruses [6, 7]. A similar phenomenon indicating the effect of cell differentiation on viral infection has been found with murine ecotropic type C retroviruses [8, 9]. Restriction of the replication of these DNA and RNA tumor viruses apparently takes place intracellularly in the undifferentiated cells [9–11]. Cell-virus interaction has been investigated at the molecular level mainly with DNA tumor viruses [eg, 12–14], as reviewed by Lehman [15] and Khoury [16].

Here we report a comparative study regarding the restriction mechanism of retrovirus replication in two cell culture lines of teratocarcinomas, myoblast-derived PCD_1 cells and undifferentiated pluripotential PCC_4, which are permissive and restrictive, respectively, to productive infection with Gross strain of murine type C retrovirus. Three particular questions were asked: 1) Does reverse transcription of genomic RNA of the incoming Gross virus occur in PCC_4 cells as well as in PCD_1 cells to generate free viral DNA intermediates, which are required for gene integration? This was analyzed biochemically by using a modified Hirt's extraction procedure [22] and a modified Southern's gel transfer method [23] to detect linear and covalently closed circular forms of viral DNA. 2) Are viral DNA intermediates, if formed in PCC_4 cells, biologically active? This was determined by a DNA transfection assay. 3) Are PCC_4 cells, while not susceptible to virion infection, also refractory to transfection with infectious viral DNA? This was examined by using these cells as recipients of a known infectious DNA preparation in DNA transfection experiments. Our results indicate that productive infection by murine ecotropic type C retrovirus is restricted in undifferentiated teratocarcinoma cells at a stage subsequent to the synthesis of complete viral DNA intermediates.

METHODS

Cells and Viruses

A pluripotential undifferentiated cell line, PCC_4, and a myoblast-derived differentiated cell line, PCD_1, were originally derived from testicular teratocarcinomas of 129/J mouse; in vitro cell cultures and biological characterization of these two lines have been described [8, 17]. The sources of Gross strain N-tropic type C retroviruses, SC-1 cells and NIH3T3 cells were as previously described [18, 19]. Growth medium used for all cell cultures was Eagle's minimal essential medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin; all cell cultures used were free of mycoplasma contamination, as ascertained by regular examination. For infection purposes, medium was harvested at 12-hr intervals from SC-1 cells chronically infected with Gross strain N-tropic virus, centrifuged to remove cell debris, concentrated in a Millipore membrane filter device, and stored at −80°C. One large lot of concentrated N-tropic virus preparation used for this study contained 2×10^7 plaque-forming units per ml, as determined by the XC plaque assay [20].

Infection Procedure

Newly confluent cell cultures were trypsinized and plated at 1.5×10^6 cells per 100-mm dish in growth medium containing 2 μg/ml polybrene. Sixteen hours later, with

cell numbers determined and known to be approximately doubled, the cells were inoculated with 8 ml per dish of serum-free medium containing 16 μg/ml polybrene and appropriate amounts of virus concentrate to give a multiplicity of infection in the range of 1 to 3. After 2-hr incubation in a 37°C CO_2 incubator, the virus-containing medium was removed and the cells were replenished with the growth medium.

DNA Extraction

Free viral DNA molecules were prepared from cells by a method modified from Hirt [21], as previously described [22]. Briefly, the method included lysis of cells at 60°C, removal of cellular DNA by precipitation in 1 M NaCl, pronase digestion and concentration of the supernatant, deproteination of the concentrated supernatant by phenol-chloroform extraction, and recovery of nucleic acids in 68% ethanol. DNA preparations without complete removal of RNA were used directly for electrophoretic analysis, since an alkaline treatment step was included in the DNA transfer procedure. For use in DNA transfection studies, ribonuclease digestion of the supernatant DNA preparations was performed [18]. A preparation of DNA was made from SC-1 cells chronically infected with Gross strain N-tropic virus [18], and this preparation was used to test the susceptibility of teratocarcinoma cells to DNA transfection.

Electrophoresis and Molecular Hybridization

Viral DNA forms were analyzed by a method of agarose gel electrophoresis/diazobenzyloxymethyl-paper transfer/molecular hybridization, as described before [22, 23]. Briefly, free viral DNA from the cells were separated by horizontal electrophoresis in 0.7% agarose gel, partially depurinated by acid treatment, denatured by alkaline treatment, transferred from the gel to a sheet of diazobenzyloxymethyl-paper, reacted with [^{32}P]-labeled copy DNA of virus genomic RNA in a molecular hybridization mixture containing dextran sulfate, located in the paper by autoradiography at −70°C with the use of an x-ray film and intensifier screens, and quantitated by direct measurement of hybridized cDNA radioactivity in the paper. [^{32}P]-labeled cDNA was prepared by using 70S RNA of Gross strain N-tropic virus as template, essentially according to the calf-thymus oligodeoxynucleotide procedure of Taylor et al [24]. Virus was isolated from freshly harvested 3-hr medium of chronically infected SC-1 cells and used directly for preparing 70S RNA by phenol-chloroform extraction and sucrose gradient sedimentation procedures [25]. Nitrobenzyloxymethyl pyridinium chloride and diazobyenzyloxymethyl-paper were synthesized following exactly the procedures described by Alwine et al [26]. Numbers of retroviral DNA molecules were calculated from radioactivity of the hybridized cDNA by using factors to correct for efficiency of DNA extraction from the cell, efficiency of molecular hybridization, specific activity of cDNA, and [^{32}P] decay – all of which were determined for individual experiments, as previously described [22].

DNA Transfection

Detailed procedures for performing DNA transfection in cultured mouse cells have been published [18, 27]. In essence, the calcium precipitation method of Graham and van der Eb [28] was used for DNA administration to the cell. The DNA recipient cells were co-cultivated with indicator cells (eg, SC-1 cells) and observed up to 6 cell passages for virus production by the XC plaque assay. As before, proper shearing of DNA preparations and inclusion of hydrocortisone/insulin/polybrene in the growth medium were employed to increase the sensitivity of the DNA transfection method [18]. Each DNA dose

TABLE I. Titration of a Concentrated Preparation of Gross N-Tropic Virus in SC-1, NIH3T3, PCD_1, and PCC_4 Cells by the XC Plaque-Forming Assay

	XC Plaque number in log_{10} dilutions of concentrate									
Cells	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.6	6.0	6.5
SC-1	–	–	–	–	TNTC	TNTC	TNTC	68 ± 4	21 ± 2	8 ± 2
NIH3T3	–	–	–	–	TNTC	TNTC	32 ± 4	12 ± 4	3 ± 2	0
PCD_1	–	–	TNTC	TNTC	41 ± 5	18 ± 4	5 ± 2	2 ± 2	0	0
PCC_4	2 ± 2	0	0	0	0	0	–	–	–	–

was tested in triplicate or hexaplicate; DNA infectious activity was expressed as the number of XC-plaque-positive dishes per total number of dishes tested.

RESULTS

Infectivity of Gross N-Tropic Virus

Table I shows the XC plaque-forming activity of Gross strain N-tropic virus in PCC_4, PCD_1, and two known susceptible cell lines, NIH3T3 ($Fv\text{-}1^n$) and SC-1 ($Fv\text{-}1^-$). The numbers of XC plaques formed in SC-1, NIH3T3, and PCD_1 cells were apparently proportional to the amount of the virus given, indicating the one-hit pattern of virus infection. This is consistent with the fact that PCD_1 cells were originated from the 129/J mouse, which contains $Fv\text{-}1^n$ allele and is susceptible to infection with N-tropic type C viruses [29]. The virus titers determined as plaque-forming units per ml of the virus concentrate were 2×10^7 for SC-1, 3×10^6 for NIH3T3, 4×10^5 for PCD_1 and 2×10^2 or less for PCC_4 cells. The virus infectivity in these cells was also determined by immunofluorescence in one experiment. Forty-two hours after inoculation of this virus at a multiplicity of infection of 2 (on the basis of XC titer in SC-1 cells), the percent of cells showing positive virus-antigen production was 95% for SC-1 cells, 88% for N3T3 cells, 63% for PCD_1 cells, and less than 1% for PCC_4 cells. These results indicate that Gross strain N-tropic virus is strongly restricted in undifferentiated teratocarcinoma cells by a mechanism apparently distinct from that of Fv-1 gene.

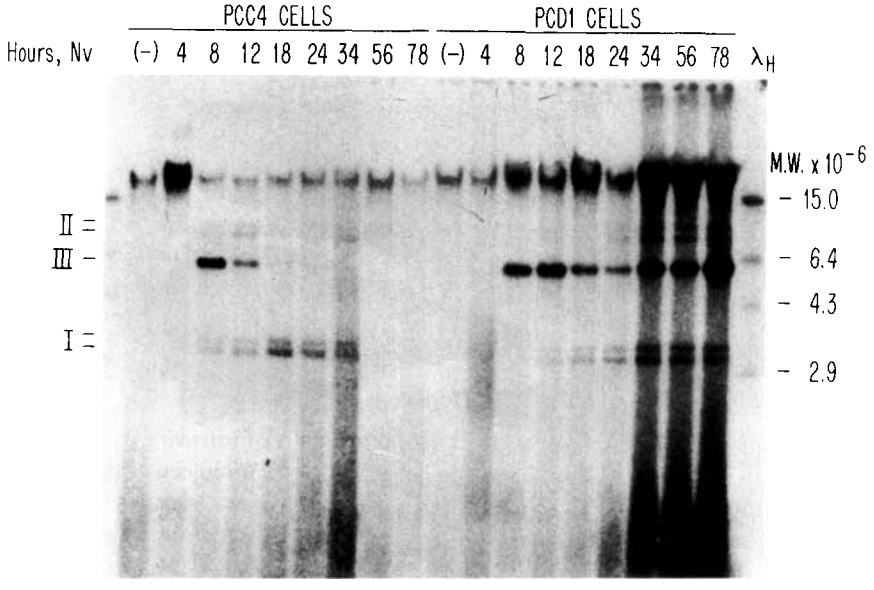

Fig. 1. Autoradiograms of the kinetic analysis of the appearance of linear DNA duplex form (III), 2 open-circular DNA forms (II), and 2 covalently closed-circular supercoiled DNA forms (I) of Gross strain N-tropic virus in undifferentiated PCC_4 and differentiated PCD_1 teratocarcinoma cells inoculated with the virus at a multiplicity of infection of 1.2. Hind III fragments of λ phage DNA (λ H) served as the molecular weight markers for linear DNA duplexes.

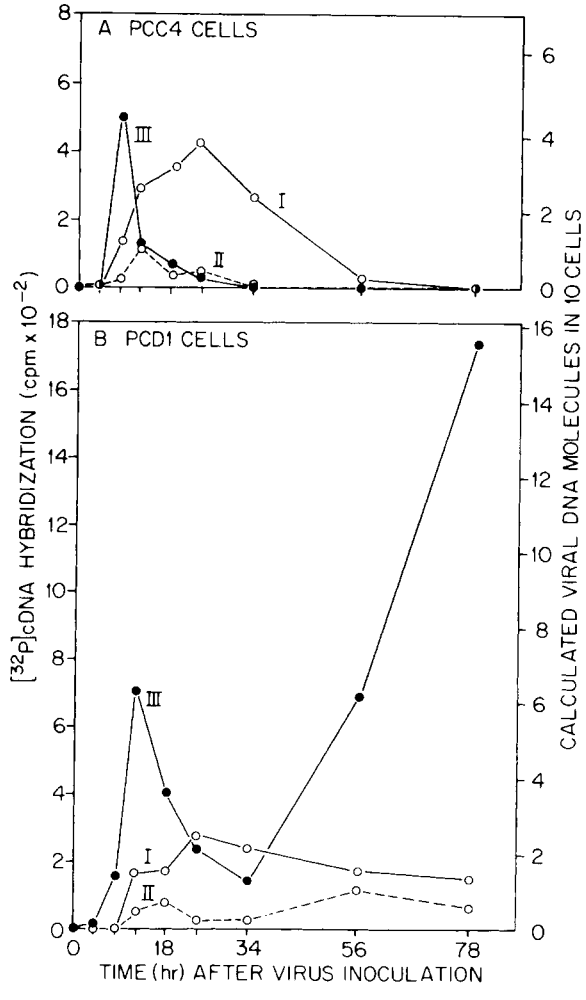

Fig. 2. Kinetic curves of viral DNA formation. The diazobenzyloxylmethyl-paper sheets from the experiments of Figure 1 were used for direct measurement of hybridized cDNA radioactivity by liquid scintillation counting. The radioactivity is expressed as cpm per 10^7 cells, using the cell count at the time of infection.

Kinetics of the Formation of Viral DNA Duplexes

After entering the permissive cells, the genomic RNA of retroviruses is reversely transcribed into a DNA duplex which is processed further into molecular form(s) suitable for gene integration [30]. With the use of modified Hirt extraction/agarose gel electrophoresis/diazobenzyloxymethyl-paper transfer/molecular hybridization procedures, the appearance of the full-length linear duplex form (III) and then the covalently closed circular supercoiled forms (I) of retroviral DNA in the cell can be quantitatively analyzed [22]. Such experiments were made in PCC_4 and PCD cells and the representative results are shown in Figures 1 and 2. The linear duplex (form III), a large and a small open-circular duplex (form II), and a large and a small covalently closed-circular supercoiled duplex (form I) of Gross N-tropic virus DNA migrated in 0.7% agarose gel electrophoresis re-

spectively to 5.9, 8.5, 10.0, 3.1, and 3.5 megadalton regions (with linear fragments of Hind III digested λ phage DNA serving as standards). In the undifferentiated PCC_4 cells, the linear duplex form of viral DNA was first detected 4–5 hr after virus inoculation, rapidly rose to a maximal level at 8–9 hr, declined precipitously, and was not detectable at 34 hr and thereafter; the two covalently closed-circular supercoiled forms first appeared at 8 hr, rose to a maximal level at around 24 hr, and then decreased gradually to an undetectable level at 78 hr; the two open-circular forms were detected at lower levels than the other DNA forms and appeared kinetically between form III and form I DNA. Initial kinetic patterns of the appearance of the three viral DNA forms in PCD_1 cells were similar to those in PCC_4 cells; however, in the 24–78-hr period, the differentiated teratocarcinoma cells always showed another burst of viral DNA synthesis while the viral DNA intermediates in PCC_4 cells were disappearing. Compared to NIH3T3 and SC-1 cells, which are highly permissive for Gross N-tropic virus, both teratocarcinoma cell lines showed more marked rise and decline of form III DNA as well as a more evident indication of the precursor-product relationship between viral form III and form I DNAs; however, with the same virus dose, the maximal levels of form III DNA in the first 12-hr period were 3- to 5-fold lower in the teratocarcinoma cells than in the NIH3T3 cells and SC-1 cells. In addition, NIH3T3 and SC-1 cells always showed the secondary burst of viral DNA synthesis [22], which were probably the result of secondary infection by virus progeny. These results suggest that the undifferentiated PCC_4 and the differentiated PCD_1 cells are similar in the initial synthesis as well as in the biochemical process of viral DNA circularization; the lack of a secondary burst of viral DNA synthesis in PCC_4 cells presumably reflects the lack of progeny virus production.

Infectious Activity of Unintegrated Viral DNAs

Although electrophoretic analyses revealed no difference in gross kinetics of viral DNA formation, there remained a possibility that subtle and minor molecular defects might be present in the Gross virus DNA molecules synthesized in PCC_4 cells. This possibility was ruled out by subsequent experiments in which infectious activity of viral DNA preparations was measured by means of an DNA transfection assay (Table II). NIH3T3 cells are competent for transfection with murine type C virus DNA preparations [18]; they were tested with the Hirt supernatant DNA preparations from PCC_4 and PCD_1 cells 10 hr and 48 hr after inoculation with Gross N-tropic virus. The results indicated that 10 hr after virus inoculation, PCC_4 and PCD_1 cells produced viral DNAs of apparently

TABLE II. Infectious Activities of Unintegrated Viral DNA Preparations Isolated From Undifferentiated PCC_4 and Differentiated PCD_1 Teratocarcinoma Cells for 10 hr and 48 hr After Gross N-Tropic Virus Inoculation

Hirt extraction		DNA doses (μg)		
Time after virus	Cells	0.13	0.25	0.5
10 hr	PCC_4	1/6[a]	1/6	3/6
	PCD_1	0/6	1/6	2/6
48 hr	PCC_4	0/6	0/6	0/6
	PCD_1	2/6	4/6	3/6

[a]DNA transfection was carried out in NIH3T3 cells which, after DNA inoculation, were examined for virus production up to 5 subcultivations by using the XC plaque assay [20]. Numbers indicate dishes showing positive XC plaque per total dishes tested.

TABLE III. DNA Transfection of Mouse PCC_4, PCD_1, and NIH3T3 Cells With SC-1 Cell DNA Containing Integrated Gross N-Tropic MuLV Genome (Number of Positive XC/Numbers of Dishes Tested in 4 Experiments)

Recipient[a]		DNA dose (μg)		
Cells	Subcultures	0.5	1.0	2.0
PCC_4[b]	1	0/18	0/18	0/18
	2	0/18	0/18	0/18
	3	0/18	0/18	0/18
	4	0/18	0/18	0/18
PCD_1	1	0/18	0/18	0/18
	2	0/18	0/18	0/18
	3	0/18	0/18	0/18
	4	1/18	1/18	0/18
NIH3T3	1	1/12	3/12	5/12
	2	1/12	4/12	6/12
	3	1/12	5/12	8/12
	4	2/12	7/12	9/12

[a]After DNA inoculation, the recipient cells were grown for 48 hr, trypsinized, and co-cultivated with SC-1 cells. At every subcultivation, the cultures were analyzed for virus production by XC plaque assay.

[b]These cells grew rapidly and after 3 passages the co-cultivated SC-1 cells became minor population in the dish.

equal infectious activity for NIH3T3 cells. The marked difference observed with the 48-hr DNA preparations was probably due to the fact that viral DNAs were present in high levels in PCD_1 cells but disappeared to undetectable levels in PCC_4 cells (Figs. 1 and 2). Since the linear duplex form of retroviral DNA shows more infectious activity than the covalently closed circular forms in the DNA transfection assay, these results indicate that the linear DNA duplex of Gross virus formed in PCC_4 cells is biologically active.

Susceptibility (or Competence) to DNA Transfection

One experimental approach to determine the site of restriction in the retrovirus replication cycle is to test by DNA transfection. If infection of a cell with RNA genome-containing virion particles is restricted, whereas infection with infectious retroviral DNA is not, the site of restriction in this cell may be at or prior to the formation of active viral DNA. Therefore, PCC_4, PCD_1, and the control competent NIH3T3 cells were tested as recipients for a DNA preparation from chronically infected SC-1 cells containing the integrated Gross N-tropic virus genome. Virus production resulting from inoculation was determined by co-cultivation of the cells with SC-1 cells, which nonselectively amplify the progeny viruses for the XC plaque assay [18, 27]. A total of four such DNA transfection experiments were performed. Persistently negative results were obtained with both PCC_4 and PCD_1 cells, except in one experiment one positive transfection result was obtained with PCD_1 cells but not with PCC_4 cells (Table III). Because of the complication that the two teratocarcinoma cell lines were both much less competent than NIH3T3 cells, this result was only suggestive that the differentiated teratocarcinoma cells may be more capable than undifferentiated PCC_4 cells in expressing the inoculated DNA of N-tropic virus.

DISCUSSION

Teratocarcinoma cells present an interesting system for studying retrovirus replication and expression. To exogenous infection with murine type C retroviruses, teratocarcinoma cells are susceptible when they are in the differentiated state but refractory when they are in the undifferentiated state [8, 9]. DNA of the mouse germ-line cell is known to harbor type C retroviral genes [31]; according to a recent study [32], expression of the endogenous retroviral genes were more apparent in the undifferentiated than in the differentiated teratocarcinoma cells. The present study compared the initial viral DNA formation in cells of the differentiated PCD_1 line and the undifferentiated PCC_4 line after inoculation of Gross N-tropic virus. Analyses of unintegrated viral DNAs by electrophoresis/molecular hybridization and by DNA transfection both revealed that both cells had equal capacity for supporting the synthesis of retroviral DNA as well as the formation of supercoiled DNA.

After penetration and uncoating processes, and prior to gene integration, the genome of retroviruses undergoes a series of biochemical reactions including reverse transcription and DNA maturation processes [30]. Viral RNA is reverse-transcribed to generate the linear DNA duplex of full genome size with terminal repeated sequences of about 600 base pairs [33]; this process occurs in the cytoplasm [34]. The covalently closed circular supercoiled DNA forms (I), generated in the nucleus from the linear duplex form (III) [34], are presumably essential intermediates required for gene integration in the retrovirus replication cycle. These processes may be subject to genetic and metabolic control by the host cell. For example, Fv-1 gene restriction [22] and inhibition of early protein synthesis have been shown to depress the formation of retroviral supercoiled DNA duplexes by apparently different biochemical mechanisms [35]. However, the results of this study clearly demonstrated that PCC_4 cells behaved normally in all these biochemical processes, although subsequent progeny virus production and hence the secondary burst of viral DNA synthesis were blocked.

In this regard, it is interesting to compare our results with those of Gautsch in a cell fusion study [36]. According to his study, two cell lines of embryonal carcinoma were shown to be refractory to infection with Moloney leukemia virus; however, if in the 8–12-hr period after the virus inoculation these cells were fused with permissive SC-1 cells, the heterokaryons showed positive virus production. According to our study, the 8–12-hr period represents the time when the linear DNA duplex form produced by the inoculated retrovirus is at its maximal level in the cell. Thus the linear DNA duplex formed in the cytoplasm of undifferentiated teratocarcinoma cells might enter the nucleus of SC-1 cells and continue to complete the replication cycle. At 24 hr, almost all the viral DNAs were in covalently closed-circular supercoiled forms and presumably only present in the nucleus of undifferentiated teratocarcinoma cells; fusion with SC-1 cells at this time apparently did not cause the transfer of viral DNA into the nucleus of SC-1 cells and hence failed to obtain progeny virus production. Our results of DNA transfection experiments (Table II) are consistent with this interpretation.

The precise mechanism of type C retrovirus restriction in undifferentiated teratocarcinoma cells remains to be elucidated. The candidate sites of the restriction are the process of gene integration, the different sites of integration in cellular DNA, and the transcriptional and post-transcriptional mechanisms involved in expression of the inte-

grated viral genome. Further studies of molecular interaction between type C retroviruses and teratocarcinoma cells may help the understanding of these processes.

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