MOLECULAR AND CELLULAR BIOLOGY, Jan. 1984, p. 151–159
0270-7306/84/010151-09$02.00/0

Vol. 4, No. 1

An X-Linked Gene Affecting Mouse Cell DNA Synthesis Also Affects Production of Unintegrated Linear and Supercoiled DNA of Murine Leukemia Virus

ANDREA RICHTER,[1] HARVEY L. OZER,[2] LUC DESGROSEILLERS,[1] AND PAUL JOLICOEUR[1,3]*

Institut de Recherches Cliniques de Montréal, Montreal, Quebec, Canada H2W 1R7[1]; Department of Biological Sciences, Hunter College, City University of New York, New York, New York 10021[2]; and Département de Microbiologie et d'Immunologie, Université de Montréal, Montreal, Quebec, Canada H3E 3J7[3]

Received 18 May 1983/Accepted 19 October 1983

To identify specific cellular factors which could be required during the synthesis of retroviral DNA, we have studied the replication of murine leukemia virus in mouse cells temperature sensitive for cell DNA synthesis (M. L. Slater and H. L. Ozer, Cell 7:289–295, 1976) and in several of their revertants. This mutation has previously been mapped on the X chromosome. We found that a short incubation of mutant cells at a nonpermissive temperature (39°C) during the early part of the virus cycle (between 0- to 20-h postinfection) greatly inhibited virus production. This effect was not observed in revertant or wild-type cells. Molecular studies by the Southern transfer procedure of the unintegrated viral DNA synthesized in these cells at a permissive (33°C) or nonpermissive temperature revealed that the levels of linear double-stranded viral DNA (8.8 kilobase pairs) were nearly identical in mutant or revertant cells incubated at 33 or 39°C. However, the levels of two species of supercoiled viral DNA (with one or two long terminal repeats) were significantly lower in mutant cells incubated at 39°C than in mutant cells incubated at 33°C or in revertant cells incubated at 39°C. Pulse-chase experiments showed that linear viral DNA made at 39°C could not be converted into supercoiled viral DNA in mutant cells after a shift down to 33°C. In contrast, such conversion was observed in revertant cells. Restriction endonuclease analysis did not detect differences in the structure of linear viral DNA made at 39°C in mutant cells as compared to linear viral DNA isolated from the same cells at 33°C. However, linear viral DNA made at 39°C in mutant cells was poorly infectious in transfection assays. Taken together, these results strongly suggest that this X-linked gene, affecting mouse cell DNA synthesis, is operating in the early phase of murine leukemia virus replication. It seems to affect the level of production of unintegrated linear viral DNA only slightly while greatly reducing the infectivity of these molecules. In contrast, the accumulation of supercoiled viral DNA and subsequent progeny virus production are greatly reduced. Our pulse-chase experiments suggest that the apparent, but not yet identified, defect in linear viral DNA molecules might be responsible for their subsequent impaired circularization.

The replication of the RNA genome of retroviruses by reverse transcriptase is a complex process. Linear double-stranded DNA molecules with segmented (+) strand are first synthesized with a redundant sequence at each end, the long terminal repeats (LTR) (for review, see reference 36). LTR are composed of a sequence derived from the 3′ end and another one from the 5′ end of the RNA genome. The linear DNA molecules mature as nonsegmented, fully double-stranded molecules and appear to be converted in the nucleus into two species of covalently closed circular viral DNAs (20, 32), one shorter species harboring one LTR copy and the larger species harboring two LTR copies (31, 42). Several of these steps seem to be carried out by reverse transcriptase alone or possibly in combination with other virion proteins. Indeed, infectious double-stranded linear viral DNA, as well as circular viral DNA with one LTR copy can be synthesized in vitro by purified virions (1, 2, 5, 6, 10). This in vitro reaction, however, does not yield closed circular viral DNA with two LTR copies. Most importantly, the infectivity of viral DNA made in vitro is several orders of magnitude lower than that of viral DNA made early during infection (10, 23, 28). This suggests that cell factors are required during the reverse transcription process. Other experiments also indicate that the cell provides essential factors during some steps of reverse transcription. Serum starvation has been shown to markedly affect the production of unintegrated viral DNA in newly infected avian (7, 8, 37) or murine (14) cells. Cycloheximide added early after infection prevented the formation of both species of supercoiled viral DNA, suggesting that newly synthesized proteins are required to complete this step (41). The treatment of newly infected murine (4, 13) or avian (16) cells with aphidicolin, an inhibitor of cellular DNA polymerase α (17) and δ (24), also prevented the formation of supercoiled viral DNA, suggesting that cellular DNA polymerases may be involved at this step of the virus cycle.

Although very suggestive, these experiments do not permit one to distinguish between a number of factors possibly affected by the inhibitors. Also, it is not clear whether the effect of these treatments is directly on retroviral DNA synthesis or only indirectly influencing it. It would therefore be helpful to identify the cellular factors required in the reverse transcription process by using cell mutants. This approach has been quite successful in procaryotic systems (22). Mutants involved in cell DNA synthesis would be the most likely candidates to affect the synthesis of retroviral DNA. Very few such mutants have been isolated in mammalian cells. One cell line, the mouse *ts*2 line (34) derived from BALB/3T3 cells, would appear appropriate. It is temperature sensitive (*ts*) for cell DNA synthesis, and at the nonper-

* Corresponding author.

missive temperature this mutation also prevents the synthesis of polyomaviral DNA (34). Genetic analysis has revealed that the mutation is X linked and that it could be complemented by human or mouse sequences (18). Several revertant cell lines are also available (18). In contrast to *ts* mutants derived from mouse L cells, which are retrovirus producers (unpublished observation), the cell lines used in this study do not shed viruses and are readily infectable with retroviruses. These factors make the *ts* cell line derived from BALB/3T3 cells an attractive system for the study of the replication of murine leukemia virus (MuLV).

We studied the replication of MuLV in these mutant and revertant cells. We found that this mutation markedly inhibited virus production when cells were shifted up to the nonpermissive temperature (39°C) early during the virus cycle. Levels of unintegrated linear viral DNA were not markedly affected when cells were incubated at 39°C, but the levels of supercoiled viral DNA decreased significantly. Although our analysis did not reveal any structural modifications of the linear viral DNA molecules made at nonpermissive temperature, these molecules had poor infectivity in transfection assays.

MATERIALS AND METHODS

Cells and viruses. The isolation and characterization of the mutant, revertant, and hybrid cell lines used in this study have been described previously (18, 34). Cell mutants were all originally derived from BALB/3T3 cells. All cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum (GIBCO Diagnostics, Madison, Wis.) and antibiotics (50 μg of streptomycin and 50 U of penicillin per ml) at 33°C. The origin of the B-tropic Friend virus has been given elsewhere (19). The virus was produced by chronically infected BALB/3T3 cells. Titers of 1×10^7 to 1.5×10^7 PFU/ml as determined in a standard XC assay on BALB/3T3 cells (29) were routinely obtained. The Moloney MuLV (clone 1) (33) was produced by chronically infected NIH/3T3 cells. The virus was grown to a titer of 1×10^6 to 2×10^6 PFU/ml as determined in a standard XC test on BALB/3T3 cells.

For infection, cells were seeded at a density of 6×10^3 to 1×10^4 cells per cm^2 in petri dishes and allowed to grow overnight at 33°C. They were then infected at a multiplicity of infection (MOI) of 1 or 2 at 33°C for 1 h in the presence of polybrene (8 μg/ml) (Aldrich Chemical Co., Milwaukee, Wis.). After infection, the virus was aspirated, and the cells were washed once with phosphate-buffered saline and then refed with medium. Mock-infected cultures were treated the same way except the virus suspension was substituted by medium. Infected and control cultures, were incubated at 33 or 39°C for the indicated time.

Reverse transcriptase assay for virus production. The presence of virus in the supernatant of infected cells was determined by the reverse transcriptase assay as previously described (19).

Extraction of unintegrated viral DNA. Unintegrated viral DNA was prepared from the Hirt supernatant (15) of newly infected cells at the time indicated after infection as described earlier (19). Supercoiled and linear viral DNAs were either separated on propidium iodide-cesium chloride gradients or on agarose gels.

Agarose gel electrophoresis and transfer of DNA. Agarose gel electrophoresis and DNA transfer onto nitrocellulose membranes were done by the technique of Southern (35) as described previously (19). Virus-specific DNA fragments transferred to nitrocellulose membranes were detected by hybridization with ^{32}P-labeled cDNA (19) or ^{32}P-labeled cloned Moloney MuLV DNA as previously described (25). Cloned DNA was labeled by nick translation as described before (25, 27). The specific activity of the probes varied between 2×10^8 and 4×10^8 cpm/μg.

BUdR labeling and thymidine chase. Unintegrated viral DNA was labeled with 5-bromodeoxyuridine (BUdR) by infecting the cells with virus suspensions, followed by exposure to BUdR (5 μg/ml) at the indicated times postinfection for the periods described in the figure legends as described before (20). For the thymidine chase, BUdR-containing medium was removed from the infected cells; cells were washed twice with phosphate-buffered saline and then incubated in medium containing 10 μg of thymidine per ml.

CsCl density gradient. BUdR-substituted viral DNA was fractionated by equilibrium banding in density gradients of cesium chloride as previously described (20). DNAs extracted from Hirt supernatants were suspended in 6.0 ml of 0.01 M Tris-hydrochloride (pH 7.5)–0.01 M EDTA and added to 8.2 g of CsCl. The gradients were spun to equilibrium at 33,000 rpm for 40 to 60 h at 22°C in a type 50 or 75 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Covalently closed circular viral DNA was separated from linear and nicked circular viral DNA by banding in propidium iodide-cesium chloride gradients as previously described (19).

Sucrose gradient sedimentation. DNA extracted from the Hirt supernatant was suspended in 0.01 M Tris-hydrochloride (pH 7.5) and layered over 15 to 30% (wt/vol) sucrose gradients containing 0.01 M Tris-hydrochloride (pH 7.5), 0.1 M NaCl, and 5 mM EDTA. The gradients were centrifuged at 23,000 rpm for 16 h at 20°C in an SW41 Spinco rotor. After centrifugation, fractions of 0.4 ml were collected from the bottom of the tube as described previously (19).

Restriction endonuclease cleavages. Restriction endonuclease digestions were performed as recommended by the supplier (Boehringer Mannheim Corp., Montréal, Canada; New England Biolabs, Beverly, Mass.) as previously described (25).

Transfection assays. Unintegrated linear viral DNA extracted by the Hirt procedure was either purified on propidium iodide-cesium chloride gradients, by preparative agarose gel electrophoresis with the glass powder procedure (38), or on neutral sucrose gradients. The levels of the enriched linear viral DNA of different groups were compared by hybridization by the agarose gel-Southern transfer procedure. Equal amounts of hybridizing linear viral DNA (8.8 kilobase pairs [kbp]) from experimental groups were used for transfection either by the calcium phosphate procedure or by the microinjection technique. A modification (39) of the original calcium phosphate precipitation method (11) was used to transfect 10^6 NIH/3T3 cells in a 100-mm petri dish. The transfected cells were passaged for 10 to 14 days. Virus production was measured by the reverse transcriptase assay or the XC test. For the microinjection procedure, the enriched linear viral DNAs were suspended in 1% KCl and microinjected into 500-1,000 NIH/3T3 cells as described previously (25). Cells were passaged for up to 4 weeks, and virus production in the supernatant was measured weekly by the reverse transcriptase assay.

RESULTS

Replication of MuLV in *ts*2 cells. We used the *ts*2 cell line, a mutant line derived from mouse BALB/3T3 cells, for our initial studies on the replication of MuLV. When these cells are incubated at the nonpermissive temperature (39°C), DNA synthesis and cell growth stop, whereas RNA and

protein synthesis are not affected (34). Cells were infected with MuLV at 33°C (permissive temperature) for 1 h to limit our study on the effect of this *ts* mutation on MuLV replication to postpenetration events. After infection, *ts*2 cells were shifted to the nonpermissive temperature (39°C) for defined time periods before being returned to the permissive temperature. The replication of MuLV was monitored 48 h postinfection by measuring virus production in the culture supernatant by the reverse transcriptase assay. As the time of incubation of *ts*2 cells at 39°C early during the virus cycle was extended, a progressive decline of virus production was observed (Table 1). Incubation at 39°C for 24 h decreased virus production to 0.5% of the control value. We have confirmed the previous findings by Slater and Ozer (34) that the effect of this mutation on cell DNA synthesis was reversible within 36 h after a 20 h shift up to 39°C (data not shown). Therefore, these results show that the mutation affecting *ts*2 cell DNA synthesis also has a profound effect early in the virus cycle such that virus production is markedly reduced.

Replication of MuLV in several mutant and revertant cell lines. To establish that the inhibitory effect on MuLV replication observed at the nonpermissive temperature was not restricted to *ts*2 cells, but rather reflected the specific effect of this X-linked mutation, we studied MuLV replication in other related cell lines. Two of these cell lines (*ts*20 and *ts*22), *ts* for DNA synthesis, were isolated independently. They do not complement *ts*2 cells and likely harbor a mutation in the same X-linked gene as *ts*2 cells. *ts*2-TG-31 and *ts*2-TGO cell lines are, respectively, thioguanine- and thioguanine-ouabain-resistant cell lines derived from *ts*2 cells. *ts*2-TGOR and *ts*2R are non-*ts* revertants isolated from *ts*2-TGO and *ts*2 cell lines, respectively. The other cell lines studied were hybrids between *ts*2 cell derivatives and non-*ts* mouse or human fibroblasts. In each case for the latter, hybrids were also used which were back selected in thioguanine, resulting in loss of the human X chromosome and reappearance of the *ts* phenotype. The replication of MuLV was measured in each of these cell lines at the permissive and nonpermissive temperature. MuLV replication was inhibited at 39°C in all cell lines *ts* for cell DNA synthesis (Table 2). Virus replication at 39°C was unaffected in all cell lines which were wild type for cell DNA synthesis. Thus, a perfect correlation between cellular DNA synthesis and virus replication was observed in 15 different cell lines. These results suggest that the same gene product which appears to be required for cellular DNA synthesis is also required for the completion of the early part of the retrovirus cycle.

TABLE 1. MuLV replication in *ts*2 cells

Time at 39°C postinfection[a] (h)	Friend MuLV		Moloney MuLV
	^{3}H-labeled reverse transcriptase (cpm)[b]	Relative virus production	Relative virus production
0–5	9.3×10^4	0.775	
0–9	3.5×10^4	0.292	
0–14	0.59×10^4	0.049	
0–20			0.037[c]
0–24	0.053×10^4	0.005	
0 (control, 33°C)	12.0×10^4	1.000	1.00

[a] *ts*2 cells (2×10^5) were seeded in 60-mm petri dishes and incubated at 33°C. They were infected 24 h later with B-Friend or Moloney MuLV at an MOI of 1 in the presence of 8 μg of polybrene per ml at 33°C for 1 h. Infected cells were then incubated at 39°C for the indicated times and subsequently shifted down to 33°C for 48 h. Control cultures were kept at 33°C. Medium was changed 24 h postinfection and collected 24 h later (48 h postinfection). Virus production in the culture supernatant was measured by the reverse transcriptase assay.

[b] Activity detected in 0.05 ml of the concentrated virus suspension (0.5 ml).

[c] Mean value of 12 experiments.

Analysis of unintegrated viral DNA made in *ts*2 cells. Since this gene product appears to act early during the virus cycle, we studied the main step of the early part of the virus cycle, namely, the production of viral DNA by the reverse transcription process. It has been shown before that unintegrated linear double-stranded and supercoiled (form 1) viral DNA could be detected in murine cells in the first 24 h after infection with MuLV (9). To determine whether accumulation of these two species of viral DNA is affected by this mutation, *ts*2 cells were infected with MuLV for 1 h at 33°C to allow penetration at the permissive temperature and then incubated for 20 h at the permissive or nonpermissive temperature. Unintegrated viral DNA was extracted by the Hirt procedure from both groups and analyzed by the agarose gel-Southern transfer procedure with a virus-specific probe. Most of the viral DNA made at the permissive or nonpermissive temperature that was detected by this technique migrated as 8.8-kbp linear double-stranded molecules as found previously in other murine cells (42). No aberrant molecular forms were detected in cells incubated at 33 or 39°C. The levels of linear DNA, which forms the predominant species, were nearly identical in both groups (Fig. 1, lanes a and b). Quantitative analysis by end-point dilution and by densitometry (not shown) revealed that the levels of linear viral DNA in cells incubated in nonpermissive conditions were only two- to threefold lower than in cells incubated at the permissive temperature (Fig. 1, lanes c through j). Interestingly, the levels of the two species of supercoiled viral DNA were low in this cell line compared with wild-type BALB/3T3 cells incubated at the same temperature (33°C) (data not shown), but they were about 10-fold higher than the levels detected at 39°C as estimated by end-point dilution (Fig. 1) and by densitometry (data not shown). In this experiment, virus production at the nonpermissive temperature (measured by the reverse transcriptase assay) was 200-fold lower than that at the permissive temperature. Therefore, these results indicated that this mutant gene product did not prevent the formation of complete linear double-stranded DNA molecules, but rather more strongly inhibited the accumulation of both forms of supercoiled viral DNA and most strongly affected virus production.

To determine whether the nearly identical levels of linear double-stranded viral DNAs found at the permissive or nonpermissive temperature 20 h postinfection reflected equal production of these molecules, we measured their rate of synthesis by a short pulse with BUdR. We have previously shown that unintegrated viral DNA made early after infection of murine cells could be labeled adequately with BUdR and quantitatively recovered as fully substituted molecules (HH) on CsCl gradients (20). *ts*2 cells were infected at 33°C and then incubated at 33 or 39°C. At different times postinfection the medium was changed, and cells were pulsed in the presence of BUdR (5 μg/ml) for 6.5 h. The fully substituted (HH) unintegrated viral DNA molecules were first purified on CsCl density gradients and then analyzed by agarose gel-Southern transfer procedure. The accumulation of linear viral DNA was nearly equal at the

TABLE 2. Correlation of MuLV replication with DNA phenotype

Origin and cell line	DNA phenotype	Virus production[b] 39°C/33°C
BALB/3T3		
*ts*2	*ts*	0.010
*ts*20	*ts*	0.024
*ts*22	*ts*	0.040
*ts*2-TG-31	*ts*	0.009
*ts*2-TGO	*ts*	0.011
*ts*2-TGOR	non-*ts*	0.780
*ts*2-R	non-*ts*	1.368
Hybrid-1		
2TGO/HF	non-*ts*	4.621
2TGO/HF-TG-1	*ts*	0.069
2TGO/HF-TG-2	*ts*	0.006
Hybrid-2		
2/HF clone 11 (early passage)	non-*ts*	1.488
2/HF clone 11-TG-4	*ts*	0.027
2/HF clone 11-TG-5	*ts*	0.127
2/HF clone 11 (late passage)	non-*ts*	4.891
Hybrid-3		
2/THO	non-*ts*	1.265

[a] Hybrid-1, mouse *ts*2-TGO × normal human diploid fibroblast (HS74BM); hybrid-2, mouse *ts*2-TG-31 × normal human diploid fibroblast (HS74BM); hybrid-3, mouse *ts*2 × mouse non-*ts* BALB/3T3 (THO).

[b] Ratio of reverse transcriptase activity from cells infected with B-Friend MuLV (MOI, 1) as described in Table 1, footnote *a*.

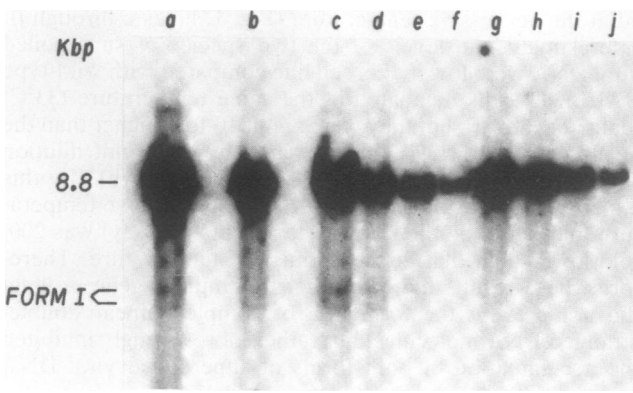

FIG. 1. Agarose gel electrophoresis of unintegrated viral DNA from *ts*2 cells incubated at permissive or nonpermissive temperatures. Subconfluent *ts*2 cells (1.4×10^8 cells) were infected with Moloney MuLV (MOI, 1) in the presence of polybrene (8 μg/ml) for 1 h. Medium was added, and cells, which were divided into two groups, were either incubated at 33 or 39°C for 20 h. After incubation, DNA extracted by the Hirt procedure was separated on 1% agarose gels, transferred to nitrocellulose filters, and hybridized with [^{32}P]cDNA. Virus-specific DNA was detected by autoradiography. The medium harvested 48 h postinfection from daughter plates (8×10^5 cells) incubated at 33 or 39°C for 20 h and then for 28 h at 33°C gave, respectively, 816,970 and 4,390 cpm of reverse transcriptase activity. Samples from *ts*2 cells incubated at 33°C (lanes a and c through f) were undiluted (1/10 of the total extract) (lane a) and diluted at 1:2.5 (lane c), 1:5 (lane d), 1:10 (lane e), and 1:30 (lane f). Samples from *ts*2 cells incubated at 39°C (lanes b and g through j) were undiluted (1/10 of the total extract) (lane b) and diluted at 1:2.5 (lane g), 1:5 (lane h), 1:10 (lane i), and 1:30 (lane j).

permissive or nonpermissive temperatures early after infection (Fig. 2). At later times, slightly less linear viral DNA accumulated (three- to fivefold less) at 39°C. Therefore, a total measure of unintegrated viral DNA at 20 h postinfection appears to reflect the rate of accumulation of these molecules at different times postinfection. Because of the short BUdR pulse (6.5 h) performed, supercoiled viral DNA was barely detectable in this experiment.

Analysis of unintegrated viral DNA made in various *ts* or revertant cell lines. Our biological experiments presented in Table 2 indicated a strong correlation between the presence of this *ts* mutation affecting cell DNA synthesis and the inhibition of MuLV replication. To determine whether this mutation had the specific effect of reducing the levels of circular viral DNA only in *ts*2 cells incubated at the nonpermissive temperature, we measured the levels of linear and circular viral DNA made at 39°C in a series of *ts* and non-*ts* derivatives of *ts*2 and in wild-type BALB/3T3 cells. The hybrid cell lines are particularly relevant since the *ts* derivative (2/HF clone 11-TG-5) is derived directly from the non-*ts*

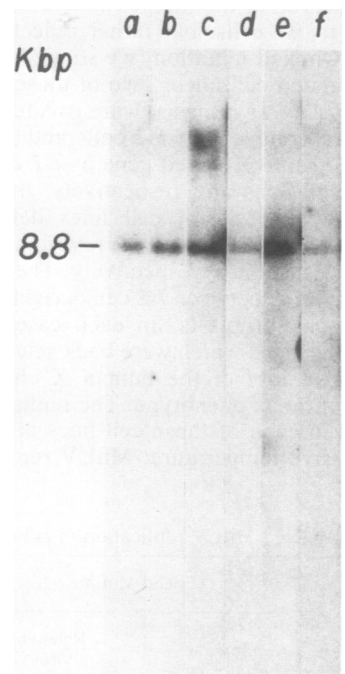

FIG. 2. Kinetics of viral DNA synthesis in *ts*2 cells incubated at permissive or nonpermissive temperatures. Subconfluent *ts*2 cells (1.28×10^8 cells) were infected with B-Friend MuLV (MOI, 1) in the presence of polybrene (8 μg/ml) for 1 h. Half of the cells were then incubated at 33°C, and the other half were incubated at 39°C. Cells were divided into three new subgroups which were incubated with BUdR (5 μg/ml) from 0 to 6.5 h (lanes a and b), from 6.5 to 13 h (lanes c and d), and from 13 to 19.5 h (lanes e and f). After each BUdR pulse, DNA was extracted by the Hirt procedure and banded in CsCl density gradients. DNA banding at a density above 1.76 g/cm^3 was processed for the agarose gel electrophoresis-DNA transfer procedure, and virus-specific DNA was detected with [^{32}P] cDNA as described in the legend to Fig. 1. Fully substituted viral DNA from cells incubated at 33°C (lanes a, c, and e) or 39°C (lanes b, d, and f) in the presence of BUdR from 0 to 6.5 h (lanes a and b), from 6.5 to 13 h (lanes c and d), and from 13 to 19.5 h (lanes e and f) postinfection. The medium harvested at 48 h postinfection from sister plates (8×10^6 cells) incubated for 19.5 h at 33 or 39°C and then for 28 h at 33°C give, respectively, 412,790 and 20,940 cpm of reverse transcriptase activity.

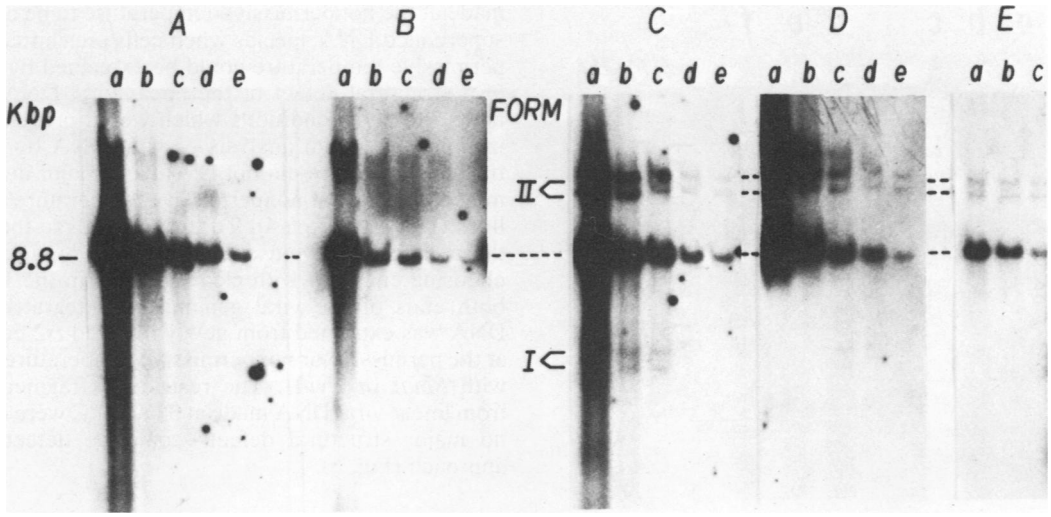

FIG. 3. Agarose gel electrophoresis of unintegrated viral DNA from other mutant, revertant, or wild-type cell lines. Subconfluent cells (2×10^7 cells from each cell line) were infected simultaneously with Moloney MuLV (MOI, 1) in the presence of polybrene (8 μg/ml) for 1 h. Medium was added and cells were then incubated at 39°C for 20 h. After infection, DNA was extracted by the Hirt procedure, analyzed by the agarose gel electrophoresis-DNA transfer procedure, and virus-specific DNA was detected with [^{32}P]-labeled cloned Moloney MuLV viral DNA, as described in the legend to Fig. 1. The progeny viruses in the culture supernatant harvested at 48 h postinfection from sister plates (8×10^5 cells) incubated for 20 h at 39°C and then for 28 h at 33°C were measured by the reverse transcriptase assay. The results were 3,210 cpm for *ts*2-TG-31, 4,940 cpm for 2/HF clone 11-TG-5, 222,030 cpm for *ts*2R, 214,920 cpm for 2/HF clone 11 (early passage), and 141,880 cpm for BALB/3T3 cells. Samples presented in panels A to D were run, hybridized, and exposed simultaneously. The experiment with BALB/3T3 cells (with appropriate control *ts*2 cells) was done a different day. A, Viral DNA from *ts*2-TG-31 cells. Lanes: a, undiluted (1/10 of the total extract); b, diluted 1:5; c, diluted 1:10; d, diluted 1:20; and e, diluted 1:60. B, Viral DNA from 2/HF clone 11-TG-5 cells. Lanes: a, undiluted (1/10 of the total extract); b, diluted 1:5; c, diluted 1:10; d, diluted 1:20; and e, diluted 1:60. C, Viral DNA from *ts*2R cells. Lanes: a, undiluted (1/10 of the total extract); b, diluted 1:5; c, diluted 1:10; d, diluted 1:20; and e, diluted 1:60. D, Viral DNA from 2/HF clone 11 (early passage). Lanes: a, undiluted (1/10 of the total extract); b, diluted 1:5; c, diluted 1:10; d, diluted 1:20; and e, diluted 1:60. E, Viral DNA from BALB/3T3 cells. Lanes: a, undiluted (1/40 of the total extract); b, diluted 1:2.5; and c, diluted 1:5. The hybridizing material seen in the upper part of panel B is cell DNA and not form II viral DNA as dilutions failed to reveal discrete bands and as confirmed in other experiments. An artefact in the lower left part of panel D (lanes a and b) obscures the presence of form I viral DNA.

parental hybrid (2/HF clone 11) by selection against a linked gene. Levels of linear viral DNA (8.8 kbp) were found to be almost identical in all of these cell lines (Fig. 3), confirming our previous results. However, the amount of circular viral DNA (form I and its derivative nicked form II generated during preparation of the samples) varied markedly from one cell line to another. They were undetectable in the two cell lines *ts* for cell DNA synthesis (*ts*2-TG-31 and 2/HF clone 11-TG-5) (Fig. 3A and B) but were easily detected in the two revertant cell lines which were not *ts* for cell DNA synthesis (*ts*2R and 2/HF clone 11) and in wild-type BALB/3T3 cells (Fig. 3C through E). By end-point dilution, we estimated that levels of circular viral DNA were decreased 20- to 60-fold in cells *ts* for cell DNA synthesis as compared with levels in wild-type or revertant cells. The MuLV replication was inhibited 30- to 60-fold at the nonpermissive temperature in these mutant cells as compared with the permissive temperature. These results extended our previous data obtained with *ts*2 cells and indicated that the presence of this X-linked *ts* mutation prevented the formation of circular viral DNA without greatly affecting the accumulation of linear double-stranded viral DNA. This apparent perturbation could lead to an abortive virus cycle.

Pulse-chase experiments of BUdR-labeled viral DNA made at 39°C in mutant and in revertant cell lines infected with MuLV. The low levels of supercoiled viral DNA detected in mutant cells incubated at the nonpermissive temperature could result from a direct block of the circularization process or from the synthesis of a faulty linear viral DNA whose defect would prevent its circularization. To distinguish between these two possibilities, we first studied whether the linear viral DNA molecules made at the nonpermissive temperature were able to circularize when cells were shifted down to 33°C. To follow the fate of these linear viral DNA molecules made at 39°C, a BUdR-thymidine pulse-chase experiment was performed.

Mutant (*ts*2-TG-31) and revertant (*ts*2R) cells were infected at 33°C with Moloney MuLV (MOI, 1) for 1 h and then incubated at 39°C for 12 h. At this time, cells were pulsed with BUdR (5 μg/ml) for 8 h at 39°C. At the end of the pulse, one-third of the *ts*2-TG-31 and *ts*2R cells were extracted by the Hirt procedure to recover unintegrated viral DNA. The remaining cells were incubated with thymidine (10 μg/ml) at 33°C for chase periods of 6 and 12 h at permissive temperature. Unintegrated viral DNA was extracted from each group, and the fully substituted molecules (HH), recovered on CSCl density gradients, were analyzed by the agarose gel-Southern transfer procedure. The results of this experiment are shown in Fig. 4. At the end of the BUdR pulse period, linear viral DNA could be detected in both cell lines (Fig. 4, lanes a and d) despite the 60-fold reduction of progeny viruses in *ts*2-TG-31 cells. Levels of linear viral DNA were reduced by 10-fold in this cell line as compared with those found in *ts*2R cells. This is in agreement with the experiment shown in Fig. 2 in which a lower accumulation of linear viral DNA was detected late after infection in mutant cells incubated at 39°C, as compared with 33°C. In both cell lines, levels of 8.8-kbp linear viral DNA were relatively stable

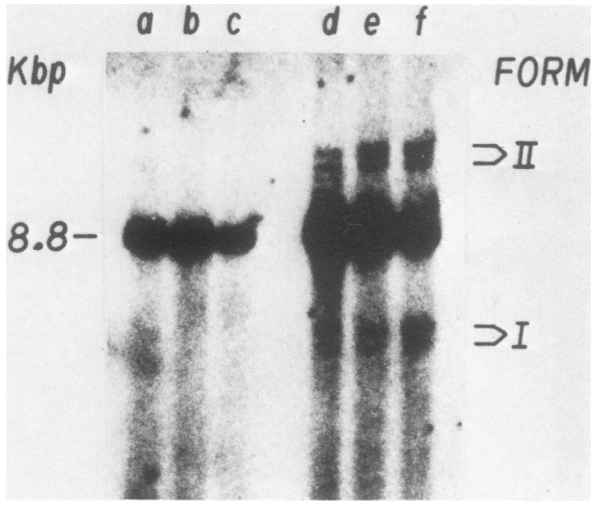

FIG. 4. Pulse-chase studies on viral DNA made at the nonpermissive temperature in mutant and revertant cell lines. Subconfluent *ts*2-TG-31 and *ts*2R cells (each 6×10^7 cells) were infected with Moloney MuLV (MOI, 1) in the presence of polybrene (8 μg/ml) for 1 h. Medium was added, and cells were then incubated at 39°C. At 12 h postinfection BUdR (5 μg/ml) was added, and incubation at 39°C continued for 8 h. At the end of this BUdR pulse (20 h postinfection) 2×10^7 cells from each line were extracted by the Hirt procedure. The remaining cells were washed and subjected to a chase procedure at the permissive temperature (33°C) by incubation with medium containing thymidine (10 μg/ml) for 6 and 12 h. After each chase period, 2×10^7 cells from each cell line were extracted by the Hirt procedure. The DNA was purified from the Hirt supernatants and centrifuged on CsCl gradients. DNA in fractions with a density above 1.76 g/cm^3 was subjected to 1% agarose gel electrophoresis-Southern transfer procedure as described in the legend to Fig. 1. Virus-specific DNA was detected with ^{32}P-labeled cloned Moloney MuLV viral DNA. The medium harvested at 48 h postinfection from sister plates (8×10^5 cells), treated similarly with BUdR, incubated for 20 h at 39°C, and then for 28 h at 33°C gave 383,280 and 6,090 of reverse transcriptase activity for *ts*2R cells and *ts*2-TG-31 cells, respectively. Viral DNA from *ts*2-TG-31 (lanes a through c) or *ts*2R (lanes d through f) cells pulsed with BUdR at 39°C and then chased at 33°C for 0 (lanes a and d), 6 (lanes b and e), and 12 h (lanes c and f) in the presence of thymidine. The viral DNAs from *ts*2R cells (lanes d through f) have been diluted fivefold compared with the DNA from *ts*2-TG-31 (lanes a through c).

during the chase period and decreased only slightly (Fig. 4, lanes b, c, e, and f). In agreement with our previous results, levels of circular viral DNA were higher in *ts*2R cells at the end of the pulse period (Fig. 4, lanes a and d). Levels of the two species (with one or two LTR copies) of circular viral DNA increased relative to the level of 8.8-kbp linear species during the chase period in *ts*2R cells (Fig. 4, lanes e and f), suggesting that some linear viral DNA was converted into the two species of covalently closed circular DNA. Such a conversion of linear viral DNA was not observed in *ts*2-TG-31 mutant cells during the chase.

This result indicated that linear viral DNA made under nonpermissive conditions was not converted into supercoiled viral DNA when cells were incubated under permissive conditions for up to 12 h.

Restriction endonuclease analysis of linear viral DNA isolated from mutant or revertant cells incubated at permissive or nonpermissive temperature. The lower level of circular viral DNA detected in mutant cells incubated at the nonpermissive temperature and the inability of the linear viral DNA made at the nonpermissive temperature to be converted into supercoiled DNA species when cells are shifted down to the permissive temperature could be explained by the presence of a structural defect of the linear viral DNA made under nonpermissive conditions which would prevent its circularization. Our initial analysis of viral DNA by the Southern transfer procedure did not reveal any major defects in these molecules made at nonpermissive temperature in mutant cell lines (Fig. 1 through 4). To further analyze the structure of these molecules, we used restriction endonuclease analysis, choosing enzymes with cleavage sites in the LTR to study both ends of the viral genome. Unintegrated linear viral DNA was extracted from newly infected *ts*2 cells incubated at the permissive or nonpermissive temperature and digested with *Sma*I or *Pvu*II. The restriction fragments generated from linear viral DNA made at 33 or 39°C were identical, and no major structural defects could be detected with this approach (Fig. 5).

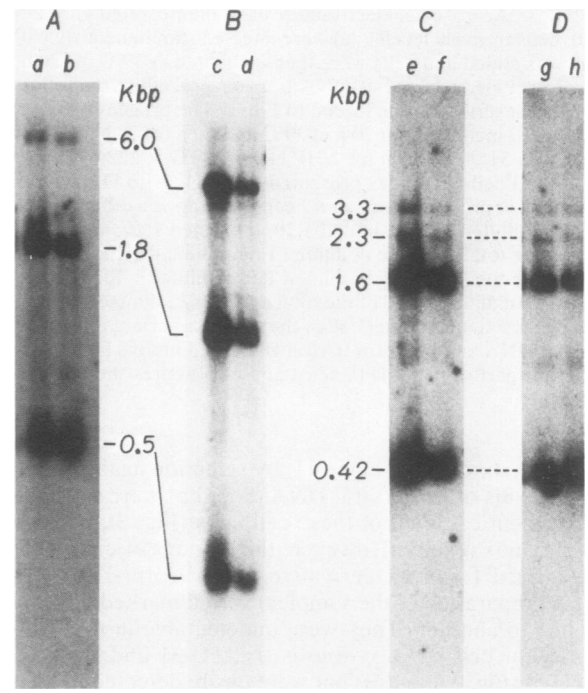

FIG. 5. Restriction endonuclease digestions of linear viral DNA extracted from mutant cell lines incubated at permissive and nonpermissive temperatures. Unintegrated linear viral DNA was extracted 20 h postinfection by the Hirt procedure from mutant cell lines infected with Moloney MuLV (MOI, 1) and digested with different restriction endonucleases. After digestion, DNAs were electrophoresed on 1.5% (panel B) or 2% (paneis A, C, and D) agarose gels, and the fragments were transferred onto a nitrocellulose membrane and annealed to ^{32}P-labeled cloned Moloney MuLV DNA and ^{32}P-labeled cloned Moloney MuLV LTR DNA as described in the text. Fragment size was estimated by running ^{32}P-labeled *Hin*dIII-digested lambda DNA and *Hpa*II-digested pBR322 DNA as markers. Viral DNA extracted from *ts*2-TG-31 (panels A and C) or *ts*2 (panels B and D) cells incubated at 33°C (lanes a, c, e, and g) or 39°C (lanes b, d, f, and h) digested with *Sma*I (panels A and B) and *Pvu*II (panels C and D). Material shown in panels A and C was from the same experiment described in the legend to Fig. 6. Material shown in B was from the same experiment described in Table 3, experiment 3. Material shown in D was from 0.6×10^8 *ts*2 cells in each group. Progeny viruses collected from sister plates (8×10^5 cells) incubated at 33 or 39°C gave, respectively, 163,900 and 24,300 cpm of reverse transcriptase activity.

Infectivity of linear viral DNA isolated from mutant cells incubated at the permissive or nonpermissive temperature. Our restriction analysis did not reveal structural changes in linear viral DNA made in mutant cells incubated at the permissive or nonpermissive temperatures. To reveal possible structural differences affecting infectivity, we used transfection assays to measure the biological activity of the two DNAs.

Unintegrated 8.8-kbp linear viral DNA was extracted from newly infected *ts*2 cells incubated at 33 or 39°C. These viral DNAs, purified either on sucrose density gradients, on propidium iodide-cesium chloride gradients, or on agarose gels, were then quantitated by molecular hybridization, and their concentration was adjusted. Not more than two- to threefold variations could be observed between the levels found in cells incubated at 33 or 39°C, in concordance with our previous results. These DNAs were then transfected into NIH/3T3 cells by the calcium phosphate procedure or were microinjected into NIH/3T3 cells. Their biological competence was measured by their ability to generate production of viral particles in the culture supernatant of the transfected NIH/3T3 cells. These progeny viruses were measured by the reverse transcriptase assay. Linear viral DNA made in *ts*2 cells incubated at the nonpermissive temperature for 20 h postinfection was reproducibly much less infectious than viral DNA made at the permissive temperature (Table 3).

To extend these results, a similar experiment was performed with the cell line *ts*2-TG-31. In this case as well, linear viral DNA synthesized in *ts*2-TG-31 cells incubated at 39°C for 20 h was less infectious than viral DNA synthesized at 33°C (Fig. 6). In this experiment, the low amount of virus initially detected in cells microinjected with viral DNA made at 39°C spread, as expected, very efficiently through the culture. Experimental variations (cell type, DNA purification method, or transfection efficiency) could explain the differences observed between the relative amount of viruses detected in this experiment and in the experiments presented in Table 3. The results of these four experiments indicated that the linear viral DNA (8.8 kbp) made at the nonpermissive temperature in mutant cell lines is poorly infectious, suggesting that it may be structurally impaired.

TABLE 3. Biological activity of viral DNA made at permissive or nonpermissive temperature in *ts*2 cells

Transfection procedure	Temp[a] (°C)	Relative virus production[b]	Reverse transcriptase activity (cpm) at day posttransfection		
			7	9	13
Calcium phosphate	33	1.0	22,000		
(expt 1)	39	0.0056	620		
Calcium phosphate	33	1.0		6,100	
(expt 2)	39	0.0128		300	
Microinjection	33	1.0	800	24,000	262,000
(expt 3)	39	0.031	850	1,300	750

[a] Unintegrated linear viral DNA extracted from 5×10^7 to 7×10^7 *ts*2 cells incubated for 20 h at 33 or 39°C was purified either on preparative agarose gels (expt 1), on propidium iodide-cesium chloride gradients (expt 2), or on sucrose gradients (expt 3). Transfections of NIH/3T3 cells were done with part (80%) of these enriched viral DNAs by the calcium phosphate procedure (expt 1 and 2). Alternatively (expt 3), part (66%) of the enriched viral DNA from each group was suspended in 0.014 ml of 1% KCl, and this solution was used for microinjection into NIH/3T3 cells as described in the text.

[b] The medium harvested at 48 h postinfection from sister plates of *ts*2 cells (8×10^5 cells) incubated at 33 or 39°C for 20 h and then for 28 h at 33°C was assayed for reverse transcriptase activity. The ratio of the activity (cpm) obtained for each group is shown as in Table 1.

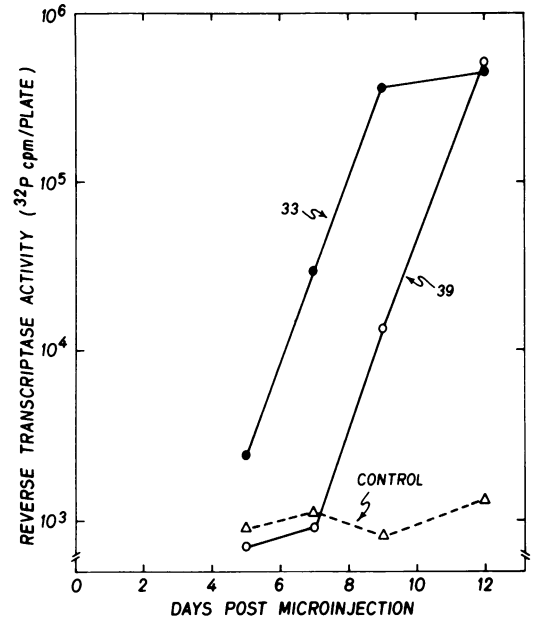

FIG. 6. Biological activity of viral DNA made at permissive or nonpermissive temperature in *ts*2-TG-31 cells. Subconfluent *ts*2-TG-31 cells (8×10^7 cells) were infected with Moloney MuLV (MOI, 1) in the presence of polybrene (8 μg/ml) for 1 h. Medium was then added, and half of the cells were incubated at 33°C and the other half were incubated at 39°C for 20 h. After incubation, cells were extracted by the Hirt procedure and linear viral DNA was enriched by sucrose gradient centrifugation as described in the text. The medium harvested 48 h postinfection from daughter plates (8×10^5 cells) of *ts*2-TG-31 cells incubated at 33 or 39°C for 20 h and then for 28 h at 33°C gave, respectively, 768,100 and 22,070 cpm of reverse transcriptase activity. Part (54%) of the enriched linear viral DNA from each group was suspended in 0.018 ml of 1% KCl and this solution was used for microinjection into 700 to 800 NIH/3T3 cells as described in the text. Cells were passaged on the second day postmicroinjection, and 2×10^4 cells were reseeded. Progeny viruses from microinjected NIH/3T3 cells were measured by the reverse transcriptase activity of a 24-h harvest medium at the indicated time. Progeny viruses were from uninfected NIH/3T3 cells (△) or from NIH/3T3 cells microinjected with linear viral DNA made in *ts*2-TG-31 cells at 33 (●) or 39°C (○).

DISCUSSION

We studied the replication of MuLV in mouse cells *ts* for cell DNA synthesis. Several of the mutant or revertant cell lines analyzed were derived from *ts*2 cells, whereas two other mutants (*ts*20 and *ts*22) were isolated independently from *ts*2 (18). These last two mutants did not complement each other, nor did they complement *ts*2, suggesting that the same gene is altered in these three different mutants (18). This gene has previously been mapped on the long arm of the X chromosome between the PGK and HPRT loci (18). Our results suggest that this gene (as defined by a *ts* mutation) codes for a product which is acting early during the retrovirus cycle.

Our characterization of the molecular defects responsible for the low virus production in mutant cells incubated at the nonpermissive temperature revealed one major anomaly in the reverse transcription process, a marked decrease in the levels of the two species of supercoiled viral DNA. In most experiments this decrease was of the same order of magni-

tude as the inhibition of virus production. Both phenomena could have a common cause, or the low levels of supercoiled DNA could explain the low virus production. Our data do not allow us to choose among these possibilities.

Surprisingly, the levels of linear double-stranded viral DNA in mutant cells incubated at the nonpermissive temperature were only slightly affected compared with the levels found in the same cells incubated at 33°C. Our restriction analysis revealed that these molecules appear normal and had no major structural defects. However, this analysis is rather insensitive and would not allow the detection of several important defects such as single strandedness at the ends, point mutations, abnormal gaps, etc. In fact, some defects are likely to be present since linear viral DNA made under nonpermissive conditions in mutant cells was found to be poorly infectious. From our pulse-chase experiments (Fig. 4), it appears that the structural changes in linear DNA molecules made at the nonpermissive temperature prevent conversion of the linear molecules into supercoiled molecules even at the permissive temperature. This suggests that these molecules cannot be repaired and are irreversibly altered once made at 39°C. It has previously been shown in avian (32) and murine (20) cells that linear viral DNA is the precursor of both species of supercoiled viral DNA. Since the larger species of supercoiled viral DNA (with two LTR copies) may be made by blunt-end ligation of linear DNA (33), our data suggest that the ends of the linear DNA molecules might have a defect which could prevent their circularization and reduce their infectivity, implicating an X-linked gene product in the completion of the LTR. This model would explain both the lower infectivity of the linear DNA made at 39°C and the low levels of supercoiled viral DNA detected. Although this unifying model appears attractive, we have not yet been able to detect structural defects in linear DNA because of the limitations of the techniques. The gene product could also be required at two different and independent steps of the reverse transcription process, namely, in the production of infectious linear viral DNA and in the circularization process itself. As in our first biological experiments on virus production after a short incubation at the nonpermissive temperature (Tables 1 and 2), the absence of conversion of linear DNA made at 39°C into supercoiled DNA after shift down to 33°C seems to reflect the presence of an irreversible lesion. However, the extent of this phenomenon could not be assessed properly because of the low levels of supercoiled DNA made at 39°C during the pulse period and the inefficient conversion of linear DNA into supercoiled DNA at 33°C in the control group. An alternate interpretation is that the defect which makes linear viral DNA molecules synthesized at 39°C poorly infectious may not be at the ends; the inability of the cells to convert linear viral DNA made at 39°C to supercoiled molecules during the short (6- to 12-h) chase period at 33°C may reflect merely the slow reversibility of the mutation on cell metabolism. We consider this less likely, however, since the effect on cell DNA synthesis is quite rapidly reversible (34). In any case our data clearly demonstrate that both linear and supercoiled viral DNA were altered in these mutant cells incubated at 39°C. To our knowledge, this is the first time that a gene product which appears to be required for cell DNA synthesis has been shown to participate in the production of unintegrated retroviral DNA.

What is the role of this X-linked gene product in the production of fully infectious viral DNA? The product of this gene being unknown, we cannot directly answer this question at the moment. It appears that the total cellular dNTP pools are not affected in these mutant cells (N. Khan and H. L. Ozer, unpublished data). The most striking molecular anomaly, a decrease in the levels of supercoiled viral DNA under nonpermissive conditions, has been seen previously under different experimental conditions. We first described it as the main feature of the *Fv-1* gene restriction (19), and it was later confirmed by others (3, 40). The *Fv-1* gene maps on mouse chromosome 4 (30) and has been shown to require the presence of a viral determinant to operate (21, 26). A selective decrease of supercoiled viral DNA has also been observed in newly infected cells treated with ethidium bromide (12), cycloheximide (41), or aphidicolin (13, 16), suggesting that newly synthesized protein(s), possibly DNA polymerase α or δ or both, are required for the formation of these DNA molecules. If all of these conditions affect a single factor, then the most likely candidate would appear to be DNA polymerase α or δ or an associated factor. We could postulate that the X-linked gene codes for DNA polymerase α or δ or an associated factor and that the *Fv-1* gene product, after its interaction with the viral determinant, inhibits it. Alternatively, all of these experimental conditions could affect different cellular factors which interact as a multifactor complex involving cellular and viral proteins to produce retroviral DNA. Whenever one of these factors in the complex is functionally inactive (missing, inhibited, or altered), the same molecular phenotype would be seen, i.e., a decrease in the levels of supercoiled viral DNA.

The use of mammalian cells mutated in a gene involved in DNA synthesis has allowed us to establish that this X-linked gene product of murine or human cells is required during the early phase of MuLV replication. The use of a similar approach with other DNA^- mutants which can complement *ts2* cells would probably help to unravel the various cell factors required for the production of fully infectious retroviral DNA.

ACKNOWLEDGMENTS

We are grateful to Eric Rassart for technical help during the initial phase of this study.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada to P.J. and by grant CA 23002 from the National Cancer Institute to H.L.O. A.R. is a recipient of a Fellowship from the Cancer Research Society Inc., Montreal, Canada, and L.D.G. is a recipient of a Fellowship from the Medical Research Council of Canada.

LITERATURE CITED

1. **Benz, E. W., and D. Dina.** 1979. Moloney murine sarcoma virions synthesize full-genome length double-stranded DNA in vitro. Proc. Natl. Acad. Sci. U.S.A. **76**:3294–3298.
2. **Boone, L. R., and A. M. Skalka.** 1981. Viral DNA synthesized in vitro by avian retrovirus particles permeabilized with melittin. II. Evidence for a strand displacement mechanism in plus-strand synthesis. J. Virol. **37**:117–126.
3. **Chinsky, J., and R. Soeiro.** 1981. *Fv-1* host restriction of Friend leukemia virus: analysis of unintegrated proviral DNA. J. Virol. **40**:45–55.
4. **Chinsky, J., and R. Soeiro.** 1982. Studies with aphidicolin on the *Fv-1* host restriction of Friend murine leukemia virus. J. Virol. **43**:182–190.
5. **DesGroseillers, L., E. Rassart, M. Zollinger, and P. Jolicoeur.** 1982. Synthesis of murine leukemia viral DNA in vitro: Evidence for plus-strand DNA synthesis at both ends of the genome. J. Virol. **42**:326–330.
6. **Dina, D., and E. W. Benz, Jr.** 1980. Structure of murine sarcoma virus DNA replicative intermediates synthesized in vitro. J. Virol. **33**:377–389.
7. **Fristch, E., and H. M. Temin.** 1977. Formation and structure of

infectious DNA of spleen necrosis virus. J. Virol. **21**:119–130.
8. **Fristch, E. F., and H. M. Temin.** 1977. Inhibition of viral DNA synthesis in stationary chicken embryo fibroblasts infected with avian retroviruses. J. Virol **24**:461–469.
9. **Gianni, A. M., D. Smotkin, and R. A. Weinberg.** 1975. Murine leukemia virus: detection of unintegrated double-stranded DNA forms of the provirus. Proc. Natl. Acad. Sci. U.S.A. **72**:447–451.
10. **Gilboa, E., S. Goff, A. Shields, F. Yoshimura, S. Mitra, and D. Baltimore.** 1979. In vitro synthesis of a 9 kbp terminally redundant DNA carrying the infectivity of a Moloney murine leukemia virus. Cell **16**:863–874.
11. **Graham, R., and A. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
12. **Guntaka, R. V., B. W. J. Mahy, J. M. Bishop, and H. E. Varmus.** 1975. Ethidium bromide inhibits appearance of closed circular viral DNA and integration virus-specific DNA in duck cells infected by avian sarcoma virus. Nature (London) **253**:507–511.
13. **Hagino-Yamagishi, K., K. Kano, and Y. Mano.** 1981. Effects of aphidicolin on retrovirus DNA synthesis in vivo. Biochem. Biophys. Res. Commun. **102**:1372–1378.
14. **Harel, J., E. Rassart, and P. Jolicoeur.** 1981. Cell cycle dependence of synthesis of unintegrated viral DNA in mouse cells newly infected with murine leukemia virus. Virology **110**:202–207.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. **26**:365–369.
16. **Hsu, T. W., and J. M. Taylor.** 1982. Effect of aphidicolin on avian sarcoma virus replication. J. Virol. **44**:493–498.
17. **Huberman, J. A.** 1981. New views of the biochemistry of eucaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α. Cell **23**:647–648.
18. **Jha, K. K., M. Siniscalco, and H. L. Ozer.** 1980. Temperature-sensitive mutants of BALB/3T3 cells. III. Hybrids between ts2 and other mouse mutant cells affected in DNA synthesis and correction of ts2 defect by human X chromosome. Somatic Cell Genet. **6**:603–614.
19. **Jolicoeur, P., and E. Rassart.** 1980. Effect of *Fv-1* gene product on synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. J. Virol. **33**:183–195.
20. **Jolicoeur, P., and E. Rassart.** 1981. Fate of unintegrated viral DNA in *Fv-1* permissive and resistant mouse cells infected with murine leukemia virus. J. Virol. **37**:609–619.
21. **Kashmiri, S. V. S., A. Rein, R. H. Bassin, B. I. Gerwin, and S. Gisselbrecht.** 1977. Donation of N- or B-tropic phenotype to NB-tropic murine leukemia virus during mixed infections. J. Virol. **22**:626–633.
22. **Kornberg, A.** 1980. DNA replication. W. H. Freeman and Co., San Francisco.
23. **Lai, M. H. T., and I. M. Verma.** 1980. Genome organization of retrovirus. VII. Infection by double-stranded DNA synthesized in vitro from Moloney murine leukemia virus generates a virus indistinguishable from the original virus used in reverse transcription. Virology **100**:194–198.
24. **Lee, M. Y. W. T., C. K. Tan, K. M. Downey, and A. G. So.** 1981. Structural and functional properties of calf thymus DNA polymerase δ. Prog. Nucleic Acid Res. Mol. Biol. **26**:83–96.
25. **Rassart, E., P. Sankar-Mistry, G. Lemay, L. DesGroseillers, and P. Jolicoeur.** 1983. New class of leukemogenic ecotropic recombinant murine leukemia virus isolated from radiation-induced thymomas of C57BL/6 mouse. J. Virol. **45**:565–575.
26. **Rein, A., S. V. S. Kashmiri, R. H. Bassin, B. I. Gerwin, and G. Duran-Troise.** 1976. Phenotypic mixing between N- and B-tropic murine leukemia viruses: infectious particles with dual sensitivity to Fv-1 restriction. Cell **7**:373–379.
27. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–257.
28. **Rothenberg, E., D. Smotkin, D. Baltimore, and R. A. Weinberg.** 1977. In vitro synthesis of infectious DNA of murine leukemia virus. Nature (London) **269**:122–126.
29. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
30. **Rowe, W. P., and H. Sato.** 1973. Genetic mapping of the Fv-1 locus of the mouse. Science **180**:640–641.
31. **Shank, P. R., S. H. Hughes, H. J. Kung, J. E. Majors, N. Quintrell, R. V. Guntaka, J. M. Bishop, and H. E. Varmus.** 1978. Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two species of circular DNA. Cell **15**:1383–1395.
32. **Shank, P. R., and H. E. Varmus.** 1978. Virus-specific DNA in the cytoplasm of avian sarcoma virus-infected cells is a precursor to covalently closed circular viral DNA in the nucleus. J. Virol. **25**:104–114.
33. **Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. U.S.A. **77**:3932–3936.
34. **Slater, M. L., and H. L. Ozer.** 1976. Temperature-sensitive mutants of BALB/3T3 cells: description of a mutant affected in cellular and polyoma virus DNA synthesis. Cell **7**:289–295.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **Varmus, H. E.** 1982. Form and function of retroviral proviruses. Science **216**:812–820.
37. **Varmus, H. E., T. Padgett, S. Heasley, G. Simon, and J. M. Bishop.** 1977. Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. Cell **11**:307–318.
38. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
39. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.
40. **Yang, W. K., J. O. Kiggans, D. M. Yang, C. Y. Ou, R. W. Tennant, A. Brown, and R. H. Bassin.** 1980. Synthesis and circulation of N- and B-tropic retroviral DNA in Fv-1 permissive and restrictive mouse cells. Proc. Natl. Acad. Sci. U.S.A. **77**:2994-2998.
41. **Yang, W. K., D. M. Yang, and J. O. Kiggans, Jr.** 1980. Covalently closed circular DNAs of murine type C retrovirus: depressed formation in cells treated with cycloheximide early after infection. J. Virol. **36**:181–188.
42. **Yoshimura, F. K., and R. A. Weinberg.** 1979. Restriction endonuclease cleavage of linear and closed circular murine leukemia viral DNAs: discovery of a smaller circular form. Cell **16**:323–332.