

Covalently Closed Circular DNAs of Murine Type C Retrovirus: Depressed Formation in Cells Treated with Cycloheximide Early After Infection

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Formation of viral closed circular supercoiled DNA duplexes and production of progeny virus were both inhibited in cultured mouse cells treated with cycloheximide in the first 4 h of type C retrovirus infection. With different doses of cycloheximide to cause different degrees of inhibition, the number of viral supercoiled DNA duplexes detected in the cells at 11 h showed an apparent correlation with the amount of progeny virus produced in the 12- to 22-h period of infection. A slight accumulation of the full-genome linear duplex and an open circular duplex of viral DNA intermediate was observed in the cycloheximide-treated cells. Cycloheximide given to the cells during the time of conversion of viral DNA from linear to supercoiled duplex forms (6 to 11 h after virus inoculation) did not inhibit the conversion. These kinetic data suggest that a cycloheximide-sensitive metabolic process, probably early viral protein synthesis, is required for retrovirus replication and supercoiled viral DNA formation in the cell.

Free virus-specific DNA molecules are generally detectable before integration of provirus into the cell genome in the acute phase of retrovirus infection (2, 6, 16, 17, 29). Previous studies on Moloney murine leukemia virus (8, 9, 24, 35), mouse mammary tumor virus (19), and Rous sarcoma virus (12, 22, 27, 28) have demonstrated mainly two physical forms of free viral DNA: double-stranded linear DNA (form III) and double-stranded, covalently closed circular, supercoiled DNA (form I). In the infected cell, form III DNA appears before form I DNA (22, 24, 33). An intermediary pathway of retroviral DNA formation has been constructed in which form I DNA is assigned as the immediate precursor to the integrated proviral DNA and, also, as the derivative of form III DNA (32). Experimental evidence for the precursor-product relationship of form III and form I DNAs was obtained in elegant work on avian sarcoma virus (22, 28) which demonstrated the synthesis of form III DNA in the cytoplasm and its conversion into form I DNAs that were found in the nucleus. The importance of form I DNA for provirus integration into the cell genome was implied by a study on ethidium bromide inhibition of avian sarcoma virus (11). Recently, Fv-1 gene restriction of murine N- and B-tropic retroviruses was found to be related to a reduction of form I DNA in the cell (15, 33). The biochemical mechanism of supercoiled DNA formation by retroviruses, however, is completely unknown.

Here we present experimental results of a study concerning the effect of cycloheximide

treatment on retroviral DNA formation. The study was initiated on the basis of two considerations: (i) treatment of cells with cycloheximide in the early hours can inhibit infection by murine ecotropic type C retroviruses, as originally reported by Salzberg et al. (21); and (ii) the action of this drug apparently takes place during or before the appearance of genome-size free viral DNA intermediates in the cell, as indicated by the kinetic data obtained for these viruses (33). Our results clearly demonstrate that the cycloheximide treatment exerted no obvious effect on the formation of linear form III DNA but markedly depressed its subsequent conversion to supercoiled form I DNA.

MATERIALS AND METHODS

Cells and viruses. We used two mouse culture cell lines, NIH 3T3 and SC-1 cells, and two murine ecotropic type C retroviruses, Gross strain N-tropic and WN1802B B-tropic viruses. Original sources and stock maintenance of these cells and viruses have been described (14). For infection, large pools of 12- to 16-h culture medium of chronically infected SC-1 cells were collected, centrifuged to remove cell debris, and concentrated about 10 times in a membrane filter device (Millipore Corp.). After removal of aggregates by centrifugation at 8,000 rpm for 20 min in a Sorvall centrifuge, the concentrate was stored in equal portions in a liquid N₂ freezer. Virus titers of rapidly thawed concentrate determined by the XC plaque assay (20) using SC-1 cells were found to be 2×10^7 PFU/ml for the N-tropic virus pool and 8×10^6 PFU/ml for the B-tropic virus pool.

Infection procedure. A total of 2×10^6 cells per

100-mm dish (or 3×10^6 cells per 150-mm dish) were plated in Eagle minimal essential medium supplemented with 8% fetal calf serum, antibiotics (100 μg of streptomycin and 100 U of penicillin per ml), and 2 μg of polybrene per ml. Sixteen to 20 h later, with cell numbers approximately doubled, the culture medium was replaced with 8 ml (or 15 ml) of serum-free medium containing 16 μg of polybrene per ml and appropriate infectious units of virus. After 2 h of incubation in a 37°C CO₂ incubator, virus-containing medium was removed from the cells, which were rinsed once and then fed with fresh growth medium.

Cycloheximide treatment. Cycloheximide (Sigma Chemical Co.) was dissolved in 10 mM HCl to make a 5-mg/ml stock solution which was sterilized by membrane filtration, stored at 4°C, and used within 2 weeks after preparation. It was given to the cell by addition in the culture medium (e.g., in the 0- to 4-h treatment, cycloheximide was first included in the virus-containing medium and then in the growth medium for 2 h). After treatment, the cells were rinsed once with balanced salt solution and replenished with the growth medium.

Measurement of virus production. Changes of culture medium were made at 12, 22 (or 24), and 46 h after virus inoculation, and progeny virus released by the cells into the medium in the 12- to 22-h (or 12- to 24-h) and 22- to 46-h intervals were collected. Virus titers of the media were measured either by XC plaque-forming activity (34) in SC-1 cells, as previously described (20), or by viral polymerase activity. For the viral polymerase assay, the medium was centrifuged at 8,000 rpm for 10 min to remove cell debris; virus particles were then pelleted by centrifugation at 50,000 rpm for 60 min in a Spinco 50 Ti rotor. The virus pellet from 8 ml of medium was suspended in 100 μl of a solution containing 0.5% Nonidet P-40 nonionic detergent, 10 mM Tris-chloride, pH 8.0, 500 mM NaCl, 3 mM MgCl₂, 5 mM dithiothreitol, and 10% glycerol. Ten microliters of the virus lysate was included in a 50- μl reaction mixture containing 20 μg of (rA)_n·(dT)₉ template-primer per ml (at a molar ratio of 10:1), 80 μM [³H]dTTP (125 Ci/mol), 0.5 mM MnCl₂, 2 mM MgCl₂, 2 mM dithiothreitol, and 40 mM Tris-chloride, pH 8.0. After incubation at 37°C for 60 min, 40 μl of the reaction mixture was spotted on a filter paper disk for measurement of dTMP incorporation in the cold acid-insoluble state (31).

DNA preparation. Procedures of Hirt (13) were followed with two modifications. First, monolayer cells were rinsed with ice-chilled balanced salt solution (without Mg²⁺ or Ca²⁺) and with an ice-chilled buffer solution of mM Tris-chloride (pH 7.6)–10 mM EDTA and then lysed with 1% sodium dodecyl sulfate (SDS)–10 mM Tris-chloride (pH 7.6)–10 mM EDTA (4 ml per 100-mm dish or 7 ml per 150-mm dish) at 60°C. Second, after separation of cellular chromatin DNA by precipitation in 1 M NaCl, supernatant fluid was digested with pronase (50 $\mu\text{g}/\text{ml}$) and concentrated (e.g., 25 ml to 1 ml) before extraction with a phenol-chloroform mixture. The final DNA preparations were stored as precipitates in 68% ethanol at –20°C. Before electrophoresis, the DNA precipitate was collected by centrifugation, lyophilized to remove residual ethanol, and dissolved in a small volume of electrophoresis

buffer solution which was added 0.5% SDS, 5% glycerol, and bromophenol blue (1 $\mu\text{g}/\text{ml}$).

Agarose gel electrophoresis. Horizontal electrophoresis was performed essentially according to the procedures of McDonnell et al. (18). The gel was made of 0.7% agarose (SeaKem ME, Maine Colloid Corp.) in electrophoresis buffer of 40 mM Tris-chloride (pH 7.8–5 mM sodium acetate–1 mM EDTA). Electrophoresis was performed with the agarose gel submerged about 2 mm under the buffer instead of with 2% agarose wicks. DNA samples were separated by applying a constant voltage in the range of 40 to 80 V until the bromophenol blue dye migrated 16 to 17 cm. Lambda phage DNA fragments, which were prepared by *Hind*III restriction endonuclease digestion and then labeled with [³²P]dCMP in a DNA polymerase reaction, served as molecular weight markers for linear DNA duplexes.

DBM-paper transfer. After electrophoresis, DNAs in the agarose gel were mildly depurinated, alkaline denatured, and then transferred to a diazobenzyloxymethyl (DBM)-paper according to the Southern transfer technique (25) as modified by Wahl et al. (30). Chemical synthesis of nitrobenzyloxymethyl pyridinium chloride and derivatization of nitrobenzyloxymethyl-paper followed the procedures of Alwine et al. (3). Efficiency of DNA transfer from the agarose gel to the DBM-paper, estimated by [³²P]DNA (*Hind*III) fragments, was found to be consistent for DNA of various sizes and was in the range of 30 to 50%.

Molecular hybridization. Virus-specific DNAs transferred to DBM-paper were detected by molecular hybridization according to the dextran sulfate procedures of Wahl et al. (30). Incubation of the paper with ³²P-labeled virus-specific copy DNA (5×10^6 to 8×10^6 cpm or 30 ng) in the hybridization reaction was at 42°C for approximately 40 h. After the incubation, the paper was rinsed five to six times with a solution of 30 mM sodium citrate–300 mM NaCl–0.1% SDS and then washed six times alternatively with this solution and its 10-fold-diluted salt solution (3 mM sodium citrate–30 mM NaCl–0.1% SDS), each for 5 min at 42°C. Finally, the paper was rinsed free of SDS with 1.5 mM sodium citrate–15 mM NaCl solution at room temperature, blotted with filter papers, and air dried. The paper was marked at all four corners with ³²P before autoradiography, which was performed at –80°C, using Kodak X0 R5 X-ray film and DuPont Cronex Lightning-Plus intensifier screens.

Preparation of copy DNA. ³²P-labeled copy DNA of N-tropic virus 70S RNA was prepared essentially according to the procedures of Taylor et al. (26). Virus 70S RNA was prepared from isolated virions by phenol-chloroform extraction and sucrose gradient sedimentation (31). Purified preparations of avian myeloblastosis virus polymerase were kindly supplied by J. Beard (Life Sciences, Inc.). Yields of ³²P-labeled copy DNA from a 0.1-ml reaction mixture, containing 1 μg of viral 70S RNA, 7.5 μM [³²P]dCTP (400 Ci/mmol; Amersham Corp.), and 100- μM concentrations of the other three dNTP's, were approximately 10⁹ cpm, or 0.5 μg of incorporated dNMP's.

Calculation of number of viral DNA molecules. With the X-ray autoradiogram serving as a guide, the

DNA bands in the DBM-paper were located and then cut into squares of 9 by 9 mm. The squares were placed in vials with scintillation fluid for measurement of radioactivity in a scintillation spectrometer. Squares of identical size, cut from regions without a DNA band in the electrophoretic tract of DBM-paper, were used to measure the background radioactivity. With excess probe included in the hybridization mixture to drive the reaction, the complementary DNA (cDNA) radioactivity hybridized to the DNA bands in DBM-paper was found to be directly proportional to the quantity of DNA sample used for electrophoresis. From the hybridized cDNA radioactivity measured by the liquid scintillation counting, the number of viral DNA molecules could be calculated by using the following factors: (i) specific activity of dCTP used for cDNA preparation, including efficiency of liquid scintillation counting, a value of 350 to 400 Ci/mmol, and correction for the ^{32}P decay; (ii) assumed 6×10^6 molecular weight and 0.25 deoxycytidine content of viral DNAs; (iii) efficiency of molecular hybridization, which was estimated to be approximately 0.5 by using viral 35S genomic RNA as a standard; (iv) efficiency of DNA transfer from agarose gel to DBM-paper, which was determined from the 6.4×10^6 -dalton DNA fragment in each experiment and found to be in the range of 0.30 to 0.5; (v) efficiency of DNA extraction, which was determined in each experiment by adding [^3H]-DNA to the cells during lysis in the Hirt procedure and measuring the recovery after DNA isolation and which was in the range of 0.8 to 0.9; and (vi) amount of DNA sample, in terms of cell number, applied to the agarose gel for electrophoresis.

RESULTS

Salzberg et al. (21) first reported an inhibitory effect of cycloheximide in mouse 3T6 cells infected with the Harvey strain of murine sarcoma-leukemia virus. They found that when this drug was used to inhibit protein synthesis of the cells within the first 5 h of infection, it severely depressed the formation of virus-specific RNA as well as the production of infectious virus particles in the next 24 h. The effect appeared to be reversible; 3 days later, virus production by the treated cells approached that of the control cells. A similar phenomenon of cycloheximide effect was observed in NIH 3T3 cells infected with Moloney leukemia virus (23). We have investigated this effect in other mouse cells (e.g., SC-1 cells and embryo cell cultures of BALB/c, C57BL/6, C3H, and DBA/2 mice) infected with other murine ecotropic type C viruses (e.g., WN1802B and N viruses), and also in human HT1080 fibrosarcoma cells infected with feline RD114 or baboon M7 endogenous retrovirus; in all of these cases, treatment of the cells with cycloheximide in the first 4 h of virus inoculation consistently resulted in a marked inhibition of progeny virus production in the next 20 h (data not shown). It is known that cycloheximide treatment may induce the expres-

sion of mouse endogenous type C proviruses (1). We found very low levels of induction in cultured C57BL/6 and DBA/2 mouse cells 2 to 3 days after cycloheximide treatment, but no induction was observed in NIH 3T3, SC-1, or human cells.

Effect on viral DNA formation. NIH 3T3 cells were exposed for 2 h to medium containing Gross strain N-tropic virus (at a multiplicity of infection of 2.6) and cycloheximide ($15 \mu\text{g/ml}$) and then for an additional 2 h to medium containing cycloheximide. The cells were rinsed to remove cycloheximide and replenished with growth medium at 4 h. The control cells received similar treatment of the virus and the medium changes but without cycloheximide. Both the cycloheximide-treated and the control cells were extracted for free viral DNAs at 6, 9, 12, and 24 h, which are suitable times to demonstrate the kinetics of appearance of both linear and circular forms of viral DNA duplexes. The results (Fig. 1 and Table 1) revealed that the cycloheximide treatment in the initial 4 h specifically affected the formation of covalently closed circular supercoiled viral DNA duplexes (form I DNAs) in these NIH 3T3 cells. In the control cells, the viral form III linear DNA duplex was detected as the sole component at 6 h; its level continued to rise at 9 h and reached an apparent plateau at 12 h, whereas the form I supercoiled DNA duplexes were detected 3 to 4 h later and in

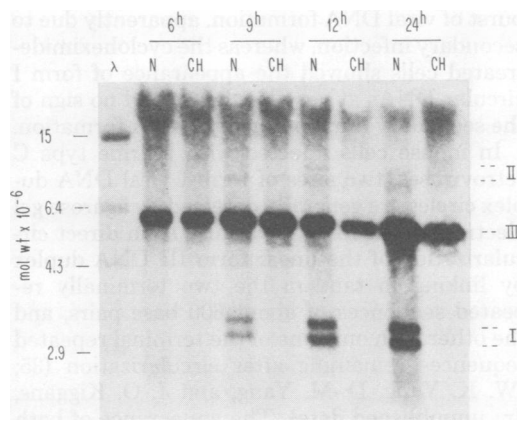


FIG. 1. Autoradiogram showing effects of cycloheximide treatment on the kinetic appearance of various forms of viral DNA duplexes in NIH 3T3 cells inoculated with Gross strain N-tropic type C virus (multiplicity = 2.6). Each electrophoresis lane represents a DNA preparation from one dish of a 6-, 9-, or 12-h cell sample or a half dish of a 24-h cell sample (4.2×10^6 cells per dish at the time of infection). Autoradiography was carried out at -80°C for 4 h. Labeled HindIII fragments of λ DNA served as the molecular weight markers for linear DNA duplex. Abbreviations: N, control cells; CH, cycloheximide-treated cells.

TABLE 1. Effect of cycloheximide treatment on the appearance of linear duplex (III) and closed circular (I) forms of viral DNAs and on progeny virus production in NIH 3T3 cells infected with Gross N-tropic virus

Treatment	DNA form	Viral DNA (molecules/1,000 cells) ^a				Virus production ^b (PFU/ml)
		6 h	9 h	12 h	24 h	
Control (mock infected)	III	1,026	1,297	1,546	2,198	7.8 × 10 ⁴
	I	<1	182	367	570	
Cycloheximide (15 µg/ml)	III	917	1,583	1,543	1,237	5.0
	I	<1	<1	<1	34	

^a Data were derived from the experiment shown in Fig. 1. The large and the small closed circular DNAs were combined in the measurement. For converting the radioactivities into molecules per 1,000 cells, factors determined and used for the calculation were 0.92 (recovery of DNA from cells), 0.42 (efficiency of DNA transfer from the gel to the paper), 0.5 (molecular hybridization of DNA duplexes with the cDNA probe), 0.83 (³²P decay), and 0.43 (counting efficiency). Cell numbers per dish were determined at each time point.

^b Medium (15 ml per 150-mm dish) was changed at 12 h and harvested at 24 h. The XC plaque-forming activity of the medium was measured in SC-1 cells.

lower levels (1/5 to 1/10) than the form III DNA. In the cycloheximide-treated cells, in contrast, no form I viral DNAs were detected in the first 12 h, whereas the linear DNA duplex was present in similar, if not slightly increased, quantities as in the control cells. At 24 h of infection (or 20 h after removal of cycloheximide from the cell medium), the control infected cells showed a burst of viral DNA formation, apparently due to secondary infection, whereas the cycloheximide-treated cells showed the appearance of form I circular DNAs at very low levels and no sign of the secondary burst of form III DNA formation.

In mouse cells infected with murine type C retroviruses, two sizes of form I viral DNA duplex circles are generally detected by agarose gel electrophoresis: one apparently from direct circularization of the linear form III DNA duplex by linking in tandem the two terminally repeated sequences of about 600 base pairs, and the other with only one of the terminal repeated sequences remaining after circularization (35; (W. K. Yang, D.-M. Yang, and J. O. Kiggans, Jr., unpublished data). The appearance of both sizes of form I DNA was equally inhibited by the cycloheximide treatment. In this experiment, the culture media given to the cells at 12 h and collected at 24 h were examined by the XC plaque assay; progeny virus production in this 12-h period was inhibited about 10,000-fold by the cycloheximide treatment. The 4-h treatment with cycloheximide at 15 µg/ml also caused cytotoxic effects. At 0 h, cell count was 4.2 × 10⁶ per 150-mm dish; at 12 h, it was increased to 5.2 × 10⁶ in the control cells but was reduced to 2.9 × 10⁶ in the treated cells (due to cell detach-

ment); at 24 h, values were 8.6 × 10⁶ and 4.2 × 10⁶, respectively.

Different doses of cycloheximide. The severely depressed appearance of form I viral DNAs in cycloheximide-treated cells could have been due to cell killing by the high dose of cycloheximide used in the experiment. Therefore, NIH 3T3 cells were inoculated with another N-tropic murine retrovirus (WN1802N) at a multiplicity of infection of 1.2 in the 0- to 2-h period and also treated with various doses of cycloheximide in the range of 1 to 12 µg/ml in the 0- to 4-h period. The cells were examined for the content of viral DNA duplexes present at 11 h and also for the amount of progeny virus secreted into the culture medium in the 12- to 22-h and the 22- to 46-h periods (Fig. 2, lanes 1 to 5 and 12, and Fig. 3). Four points are evident from this study. First, the cycloheximide dose-response curves of progeny virus production in the 12- to 22-h period and of form I DNA content in the cells at 11 h were very similar in pattern, showing a quantitative and positive correlation between these two indicators of virus replication. Second, the content of form III linear DNA duplex at 11 h was generally higher in the cycloheximide-treated cells than in the control cells (also see the experimental data in Tables 1 and 3). This is consistent with the interpretation that

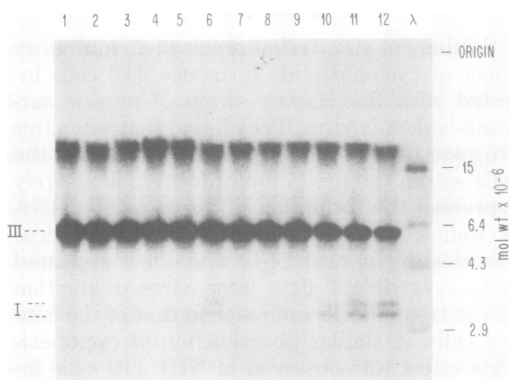


FIG. 2. Autoradiogram showing effects of various cycloheximide doses (lanes 1-5) and various 2-h intervals of cycloheximide treatment (lanes 6-11) on the content of viral DNA duplexes in NIH 3T3 cells after inoculation of WN1802N N-tropic virus (multiplicity = 1.2). DNA extraction was made 11 h after infection. The DNA sample used in each lane was from 3.5 × 10⁶ cells, and the film exposure time for autoradiography was 11 h. Schedules of cycloheximide treatment for the DNA sample of individual lanes are: (1) 1 µg/ml, 0 to 4 h; (2) 2 µg/ml, 0 to 4 h; (3) 4 µg/ml, 0 to 4 h; (4) 8 µg/ml, 0 to 4 h; (5) 12 µg/ml, 0 to 4 h; (6) 10 µg/ml, -2 to 0 h; (7) 10 µg/ml, 0 to 2 h; (8) 10 µg/ml, 2 to 4 h; (9) 10 µg/ml, 4 to 6 h; (10) 10 µg/ml, 6 to 8 h; (11) 10 µg/ml, 8 to 11 h; (12) untreated control.

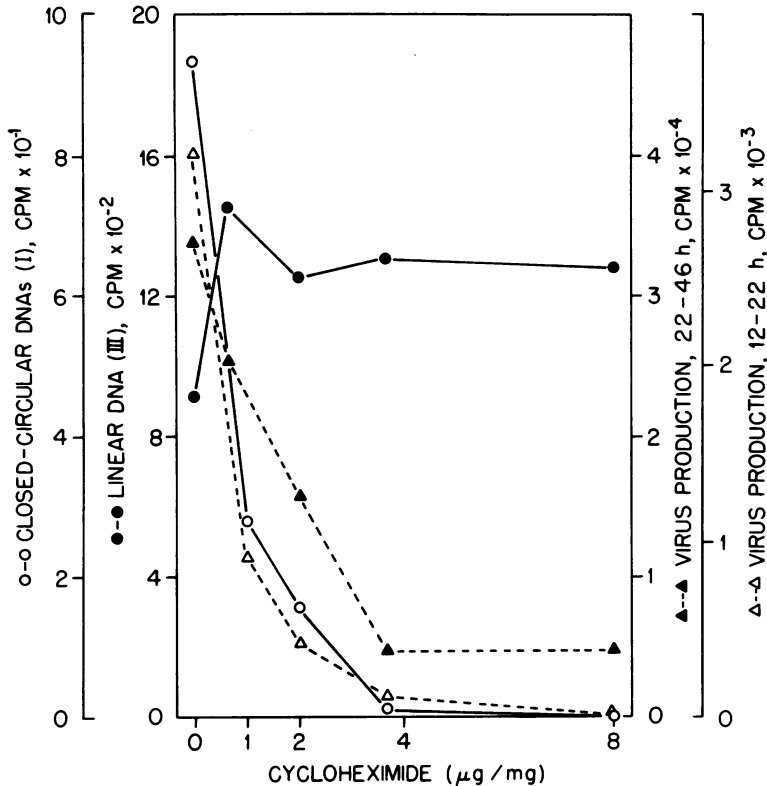


FIG. 3. Cycloheximide dose-response curves of viral DNA content at 11 h and quantities of progeny virus produced in the 12- to 22-h and 22- to 46-h periods after infection. Both the closed circular supercoiled duplex form (I) and the linear duplex form (III) of viral DNA are expressed by radioactivity of hybridized ³²P-labeled copy DNA probes. The virus progeny was expressed as the (rA)_n·(dT)_s-dependent dTMP incorporation activity of the virion polymerase.

conversion of form III DNA into form I DNAs is blocked and the block leads to the accumulation of form III DNA in the cycloheximide-treated cells. Third, at a cycloheximide dose of 4 µg/ml and below, no apparent inhibition of the cell growth pattern was observed. This suggests that the inhibition of virus replication by cycloheximide is not due to a general cytotoxic effect of the drug. Fourth, comparison of progeny virus production at the 12- to 22-h and 22- to 46-h periods demonstrated that virus replication may be restored after the removal of cycloheximide. The restoration of progeny virus production appeared to be slow, presumably because of the required resumption of the form III-to-form I DNA conversion process; the latter recovered slowly after the initial interruption by cycloheximide treatment (see Table 1).

Critical time of cycloheximide sensitivity. To determine the time for cycloheximide to cause maximal effect on the synthesis of viral DNA, an experiment was performed in which NIH 3T3 cells were treated with cycloheximide (10 µg/ml) in 2-h intervals from 2 h before to 10

h after virus inoculation. Results of measurements of the viral DNA duplex content at 11 h and of subsequent progeny virus production are shown in Fig. 2 (lanes 6 to 12) and Table 2. In this experiment, increased accumulation of form III linear DNA duplex was again apparent in the cycloheximide-treated cells. As judged by the inhibitory effect on both form I viral DNA formation and progeny virus production, the first 4 h of virus infection evidently constituted the most sensitive period for cycloheximide inhibition. Cycloheximide given in the 6- to 11-h period showed little or no effect on the two form I DNAs, which appeared normally during this time period, indicating that cycloheximide by itself did not prevent the viral form III-to-form I DNA conversion. Treatment of cells from -2 h to the time (0 h) of virus inoculation also showed only slight inhibition, probably due in part to the cytotoxic effect of the drug at 10 µg/ml. In cells treated with cycloheximide from 8 to 11 h of infection, the appearance of form I DNA circles was not affected, but progeny virus production for the next 35 h was severely inhibited.

TABLE 2. Effects of cycloheximide treatment at different time intervals on viral DNA formation and progeny virus production

Time interval of treatment ^a (h)	Viral DNA (molecules/1,000 cells) ^b		Medium virus polymerase ^c	
	Linear (form III)	Circles (form I)	12-22 h	22-46 h
-2-0	700	30	1,540	17,460
0-2	672	<1	<5	3,176
2-4	672	<1	27	3,949
4-6	737	8	153	12,396
6-8	550	36	341	12,547
8-11	492	63	<5	2,722
None	344	65	4,018	27,325

^a Gross N-tropic virus was given to NIH 3T3 cells in the 0- to 2-h interval at a multiplicity of 1.2. Cell number at 0 h of infection was 3.2×10^6 per 100-mm dish.

^b Data were derived from two separate experiments, including the one shown in Fig. 2 (lanes 6 to 12). Calculation was made as described in Table 1, except that factors determined for DNA recovery and DNA transfer were 0.85 and 0.37, respectively.

^c Expressed as counts per minute of [³H]TMP incorporation into acid-precipitate form in a (rA)_n·(dT)_n-dependent polymerase reaction, using lysed virus particles from 0.6 ml of culture medium (8 ml per 100-mm dish) of the indicated time interval.

It is not known whether this was due to an inhibition of virion protein synthesis, required for progeny virus assembly and secondary infection, or to an interference with other late steps in the virus replication cycle.

Viral DNA intermediates. In our studies of viral DNA intermediates, experimental data have been usually reliable for the form III linear DNA duplex as well as for form I covalently closed circular DNA duplexes, but not for form II open circular DNA duplexes. Form II DNAs migrated more slowly than form III DNA in the agarose gel electrophoresis, and their presence was sometimes confused by the contaminating high-molecular-weight DNA of the cell (Fig. 1). In DNA samples showing two bands of form I DNA circles, two bands of form II DNA circles were generally detected in relatively low amounts, which were presumably generated from form I DNAs by nuclease action during the extraction procedure. However, in some kinetics studies, a distinct species of viral DNA intermediates could be observed; its appearance in the cell was transient, and it occurred later than the form III DNA duplex but slightly earlier than form I DNA duplexes. In agarose gel electrophoresis, it migrated very close to the small species of the form II DNA doublets, and the two together formed a broad band in the region

(Fig. 1, 9- and 24-h samples). The electrophoretic property and the kinetics of appearance would suggest that this DNA intermediate might be the same as the open circular full-genome-length DNA duplex found in the *in vitro* reverse transcription reaction (5, 10; Yang et al., unpublished data), although its molecular identity remains to be shown. This DNA intermediate also showed a tendency to accumulate after cycloheximide treatment in SC-1 cells inoculated with WN1802B virus (Fig. 4) as well as in NIH 3T3 cells inoculated with N-tropic viruses (Fig. 1 and 2). In this aspect, the cycloheximide effect was apparently different from Fv-1-restricted infection of these viruses, in which the formation of this particular DNA intermediate as well as the supercoiled form I DNA intermediates was depressed (33).

DISCUSSION

In this study, we have confirmed and extended an initial finding by Salzberg and co-workers (21, 23) concerning the inhibitory effect of cycloheximide on murine retrovirus infection. This inhibitory effect was observed in all cases of retrovirus infection we examined, which included other murine ecotropic viruses and feline RD114 and baboon M7 endogenous viruses. With the use of a highly sensitive method (30) for analyzing viral DNAs, we were able to demonstrate here that early cycloheximide treatment depressed the formation of covalently closed circular supercoiled form I DNA duplexes and caused an apparently higher than normal accumulation of the linear form III viral DNA intermediate in the infected cells.

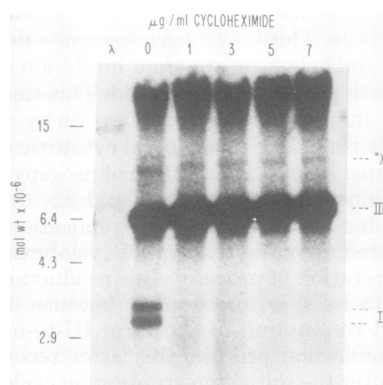


FIG. 4. Autoradiogram showing a distinct viral DNA intermediate (indicated by "X") in SC-1 cells 12 h after infection with WN1802B retrovirus. DNA samples from 4.0×10^6 cells were used in each lane. Cycloheximide treatment was given from 0 to 4 h at concentrations of 0 (lane 1), 1 (lane 2), 3 (lane 3), 5 (lane 4), and 7 (lane 5) $\mu\text{g/ml}$.

A particularly interesting feature is the lag between the most effective time of cycloheximide treatment and the time when the inhibitory effect is manifested in the viral DNAs. When cells were exposed to the drug in the 0- to 4-h period, the form III viral DNA intermediate was synthesized in normal amounts, first appearing at 4 to 5 h, and yet the appearance of form I DNAs, normally occurring at 7 to 8 h, was inhibited. According to a study by Shank and Varmus on avian sarcoma virus (22), viral DNA is first synthesized as form III in the cytoplasm and converted into form I during or after entering the nucleus. Our kinetic analyses of DNA formation in cells infected with murine retroviruses also suggest a precursor-product relationship of form III and form I viral DNAs (33). In the present study, we observed that the depressed appearance of form I was generally associated with a corresponding increased accumulation of form III viral DNA in cycloheximide-treated cells. These kinetic observations would suggest that a cycloheximide-sensitive metabolic process, which takes place in the first 4 h of retrovirus infection, is essential for subsequent conversion of viral DNA from linear duplex to covalently closed circular forms.

The effect of cycloheximide is likely mediated through inhibition of protein synthesis, the best-known characteristic of this drug. Our unpublished data indicated that other inhibitors of protein synthesis, such as puromycin and sodium fluoride, given to the cell during the first 4 h of virus inoculation, also caused a similar depressed appearance of form I DNA. Synthesis of early proteins has not been known for retroviruses, and many workers consider that the virions of retroviruses themselves carry protein molecules required for early functions (e.g., reverse transcription). However, a few studies have recently suggested that virion genomic RNAs with 3' polyadenylate and 5' cap structures may serve as mRNA's in the early stage of infection (4, 7, 21, 23). Bassin and co-workers (4) found that heat-inactivated virions of N-tropic murine leukemia viruses could effectively abrogate the Fv-1^b restriction mechanism of mouse cells; this observation was considered to be due to a messenger function of viral genomic RNA expressed in the cells. Also of particular interest is the demonstration that genomic RNA of murine retroviruses, upon entering the cell, became associated with polysomes and that cycloheximide treatment prevented the occurrence of this association (21, 23). Thus, one speculation is that a virus gene-coded early protein(s) synthesized in the first few hours postinfection would be involved in the viral DNA superhelix formation for the purpose of gene integration. This is

compatible with our observation that relatively low inhibition was achieved when cycloheximide was given 2 h before and removed from cells at the time of virus inoculation (Fig. 2, lane 6, and Table 2). However, all of these experimental data are only circumstantial evidence for the possible existence of a virus-specific function in the form III-to-form I DNA conversion. Alternatively, the cycloheximide effect could be due to inhibition of cellular proteins, which were induced by the virus infection (or had a rapid metabolic turnover) and were required for viral DNA maturation, or cycloheximide treatment could affect intracellular traffic and compartmentation of retroviral replication complexes. These various alternatives remain to be investigated.

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