Studies with Aphidicolin on the *Fv-1* Host Restriction of Friend Murine Leukemia Virus

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The murine gene *Fv-1* exerts a major control over the replication of Friend murine leukemia virus (F-MuLV). An effect of the gene product has been determined to be at the level of accumulation and integration of viral DNA. Aphidicolin, an inhibitor of eucaryotic DNA polymerase α , was studied in murine cells infected either permissively or nonpermissively with regard to the *Fv-1* genotype. Results indicated that inhibition of DNA polymerase α did not affect the accumulation of form III viral DNA in either permissive or nonpermissive cells. However, the normal accumulation of circular form I DNA in permissive cells was inhibited. The block in the accumulation of form I DNA resembled that occurring in some F-MuLV *Fv-1*-nonpermissive infections. Additionally, aphidicolin treatment resulted in the accumulation of novel low-molecular-weight viral DNA species, normally detectable in a nonpermissive infection of NIH cells with B-tropic F-MuLV. These data suggest that the *Fv-1* gene product may interact with host DNA polymerase α to prevent viral replication.

The murine gene *Fv-1* has been demonstrated to encode for a factor which inhibits the establishment of an infectious center by murine leukemia viruses (5, 14; for review, see references 3 and 7). The gene exists in at least two allelic forms, *n* and *b*, and all naturally occurring ecotropic murine leukemia viruses can be classified as either N tropic or B tropic. Tropism denotes the relative ability to replicate successfully in prototypic *Fv-1^{n/n}* or *Fv-1^{b/b}* mouse strains. N-tropic viruses are inhibited in their replication in *Fv-1^{b/b}* mouse cells, and reciprocally, B-tropic viruses grow poorly in *Fv-1^{n/n}* strains.

A central event in the replication of retroviruses is the synthesis and integration into the host chromosome of the DNA copy of the viral genome. The action of the virus-encoded enzyme reverse transcriptase is responsible for copying the viral genome into double-stranded DNA (20). At least two major forms of viral DNA are observed after acute infection of permissive host cells (as reviewed in reference 22). Both a linear double-stranded form and two covalently closed circular forms of viral DNA accumulate (16, 25). It has been demonstrated that the linear form is a precursor to the circular species (17). However, the immediate precursor to the integrated form of viral DNA has yet to be identified.

We and others have shown that unintegrated viral DNA species which accumulate in *Fv-1*-restrictive hosts manifest a decrease in circular

DNA forms (2, 9, 23). The degree of decreased accumulation of form I DNA that we have observed in certain restrictive infections (three- to four-fold), is difficult to correlate directly with the 100- to 500-fold decrease in viral yield observed in nonpermissively infected cell cultures. Therefore, we have suggested a model in which the *Fv-1* effect may be at the integration step itself either alone or in addition to the observed decrease in accumulation of viral form I DNA (2).

In this study, we investigated the effect of the tetracyclic diterpenoid aphidicolin on viral DNA metabolism (1). This drug, which has been shown to inhibit eucaryotic DNA polymerase α specifically (6, 12), demonstrates no inhibition *in vitro* of reverse transcriptase-mediated viral DNA synthesis (13). However, in the presence of aphidicolin, avian retroviruses synthesize linear (form III) viral DNA, they fail to accumulate form I circles, and viral DNA integration does not occur (T. Hsu and J. Taylor, personal communication). The action of this drug therefore mimics the results of an infection of *Fv-1*-restrictive murine cells. The effect of aphidicolin on Friend murine leukemia virus (F-MuLV) infection of permissive and nonpermissive cells was investigated to evaluate the potential role of DNA polymerase α in these events. Our results demonstrate that the accumulation of viral DNA in *Fv-1*-restrictive and aphidicolin-treated cells is very similar. This suggests that the *Fv-1* effect could be mediated through an inhibition of a

DNA polymerase α function which appears to be required for the viral cycle to be completed.

(The data reported here are from a thesis to be submitted by J.C. to the Albert Einstein College of Medicine, Yeshiva University, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Cells and viruses. NIH, BALB/c, and XC cells were obtained from J. Hartley and W. Rowe. Secondary tail fibroblast cells from DBA/2, NIH, and C57L animals were the generous gift of R. Gieb and F. Lilly. N-, B-, and NB-tropic F-MuLV have previously been described (2, 3).

Preparation of viral DNA. Viral stocks, routinely titered by direct XC assay (15), were used to infect 2×10^6 to 5×10^6 cells grown on T150 flasks (Corning Glass Works) in minimal essential medium containing polybrene (5 $\mu\text{g/ml}$). At appropriate times, Hirt supernatant DNA was prepared as previously described (2). After phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, DNA samples were subjected to electrophoresis in 0.8% agarose (Seakem) for 14 to 20 h at 55 V. Subsequent blotting to nitrocellulose and hybridization to nick-translated ^{32}P -labeled cloned F-MuLV DNA (the gift of A. Oliff) has previously been described (2).

Aphidicolin treatment. Aphidicolin was the generous gift of M. Horwitz of this institution. Cells were routinely treated with 10 μM aphidicolin added directly to the growth medium (either minimal essential medium or Dulbecco-modified Eagle medium with 10% fetal calf serum). To remove the drug, cells were washed twice with phosphate-buffered saline (GIBCO Laboratories) and twice with growth medium, the final wash being 5 to 10 min at 37°C. Fresh medium was then added to the cells, and incubation continued.

^3H thymidine incorporation. ^3H thymidine (40 to 60 Ci/mmol; Amersham Corp.) was added to the growth medium at 10 $\mu\text{Ci/ml}$. After incubation at 37°C, cells were washed with phosphate-buffered saline and lysed in the presence of 0.2 N NaOH at 37°C for 15 min. After neutralization, samples were analyzed by trichloroacetic acid precipitation onto GF/C filters

(Whatman), and the incorporated radioactivity was determined by scintillation counting.

Microdensitometry. The band intensities of the autoradiograms were measured by using a double-beam recording microdensitometer (CMKIII C; Joyce, Loeb and Co., Ltd., England). Peak areas representing either form III or form I DNA were cut from the tracing and weighed on an analytical balance (Mettler) as described (2).

RESULTS

Effect of aphidicolin on cellular DNA synthesis and viral progeny formation. As part of our preliminary studies of the effect of aphidicolin on the replication of F-MuLV, we studied the kinetics of drug effects on cellular DNA synthesis. The single concentration of drug used (10 μM) is that known to inhibit reversibly 90% of DNA synthesis of either HeLa or CHO cells (11). Murine fibroblast cells growing as a monolayer were treated with drug in otherwise normal growth medium. After 15 min of drug treatment, ^3H thymidine was added to the cultures, and the effect of the drug on incorporation of label into DNA was measured. Within minutes of addition, and for periods of up to 12 h, approximately 90% inhibition of thymidine incorporation was observed (Table 1). No effect of the drug on soluble radioactivity was found, suggesting that the 90% inhibition of thymidine incorporation was not due to a pool effect (data not shown). In one set of experiments, cells were treated for 12 h, and the result of drug treatment was measured by 15-min pulse incorporation of thymidine. At this time, a 93% inhibition was observed. After removal of drug and washing of the cell monolayer, the 15-min incorporation of thymidine was measured immediately and at 30 min, 90 min, and 6 h after drug reversal. The results, normalized to the control culture, indicated that by 30 min after drug removal, incorporation had attained 65% of con-

TABLE 1. Effect of aphidicolin on cellular DNA synthesis^a

Time of treatment	Control (cpm)	Aphidicolin (cpm)	Inhibition (%)
(1) 15 min	116,190	13,498 (11.6)	88.4
(2) a. 12 h	37,335	2,588 (6.9)	93.1
b. 12 h + 30-min reversal		24,397 (65.3)	34.7
(3) a. 12 h	58,967	6,970 (11.8)	88.2
b. 12 h + 90-min reversal		58,093 (98.5)	1.5
c. 12 h + 6-h reversal		87,915 (>100.0)	0.0

^a Mouse fibroblast monolayer cells were treated with aphidicolin (10 μM) in normal minimal essential medium for 15 min or 12 h. At the end of these times, ^3H thymidine (10 $\mu\text{Ci/ml}$) was added to the medium (see text), and incorporation was measured for a pulse of 15 min. In experiments 2 and 3, a series of parallel cultures were treated with aphidicolin for 12 h and then rinsed to remove the drug. These cells and their mock-treated controls were then incubated in minimal essential medium for 0 min (2a and 3a), 30 min (2b), 90 min (3b), and 6 h (3c) before a 15-min thymidine incorporation was measured. The results are expressed in percentage of control mock-treated cultures.

trol, and that by 90 min it was at control levels (Table 1). These data indicated that even after prolonged (12 h) treatment of cells, DNA synthesis returned to control levels quickly. Longer cell treatment resulted in significant cell death, and reversibility of DNA synthesis after 13 h was not measured.

Virus progeny can be measured reliably as early as 24 h postinfection. This yield, although lower in titer than that of a fully infected culture, represents virus released from cells produced solely from the first round of infection. We took advantage of our ability to measure first-round virus yield to assay the reversibility of the effects of the drug. We compared the titer of virus released at 24 h from permissively infected cells treated with aphidicolin either continuously or for only the first 11 h of infection. As controls, permissively infected non-drug-treated cells released virus at 10^4 PFU/ml. By comparison, an *Fv-I*-restrictive infection yielded only 10^2 PFU/ml, the expected 100-fold decrease in titer. Permissively infected cells treated with drug only during the first 11 h of infection yielded (at 24 h) 10^3 PFU/ml, a 10-fold decrease from the control untreated cell culture whereas infection in the continuous presence of drug yielded no detectable progeny. The 10-fold-reduced virus yield resulting after the removal of aphidicolin suggested that the drug effects were reversible, and that drug present during the first 11 h imposed a lag on virus progeny production. After 35 h, both control and drug-treated cells were releasing 10^5 PFU/ml, indicating that the lag in production of infectious virus induced by early drug treatment was totally reversed. These data suggested that drug treatment during the early phase of infection blocked an essential step in virus replication. However, the effect was reversible as it imposed a lag in the production of progeny virus which was evident only during the first round of infection.

An evaluation of long-term reversal of drug treatment was tested by means of the XC assay. Both B- and NB-tropic F-MuLV were used to infect cells either permissively or nonpermissively according to *Fv-I* type. During the first 16 h of infection, one set of cultures was treated as above with aphidicolin, and control cells were mock treated. At the end of this period, both control and drug-treated cultures were rinsed to remove the drug, and the XC assay was carried out as usual. The titer of virus in both permissive and nonpermissive infection was unchanged by the presence of drug during the first 16 h of infection (Table 2). Second, there was no change in the *Fv-I* host restriction pattern; neither an increase nor a decrease in the *Fv-I* effect was observed. These results indicated that treatment with aphidicolin during the initial phase of infec-

tion did not alter virus restriction due to the *Fv-I* gene. Furthermore, if the drug was removed after 16 h and time for cell recovery was allowed, there was no gross alteration of long-term viral growth kinetics as measured by the XC assay.

Accumulation of unintegrated viral DNA. After acute infection of susceptible cells, virus-specific DNA molecules accumulate in the host cell before integration. Two major viral DNA forms are found: a linear double-stranded DNA (form III) and covalently closed circular DNA (form I) (16, 25). Two sizes of the MuLV form I DNA are observed representing molecules containing either one or two copies of an approximately 600-base-pair terminally repeated sequence known as a long terminal repeat (25). The long terminal repeat sequences are also normally present at each end of the MuLV form III linear DNA (25). These two major forms of unintegrated viral DNA are easily identified by standard molecular hybridization procedures (2, 18).

To investigate the effect of aphidicolin, B-tropic F-MuLV was used to infect permissive BALB/c (Fig. 1, lanes 1 and 2) and nonpermissive NIH cells (Fig. 1, lanes 3 and 4) in the presence and absence of the drug. Hirt supernatant DNA was extracted, separated by electrophoresis, and transferred to nitrocellulose by the Southern procedure (18). Form III, linear full-length DNA, represented the predominant form at this time (13 h) of infection, and the two bands of form I were noted in this control permissive infection. By comparison, the *Fv-I*-nonpermissive cell seen in lane 3 also showed a prominent form III, but no form I DNA was present. These data, which confirm our earlier studies, also revealed (lane 3) a less distinct band(s) which

TABLE 2. Aphidicolin effect on virus titration^a

<i>Fv-I</i>	Virus tropism	Cell type	Titer (PFU/ml)	
			Control	Aphidicolin
P	B	B	4.0×10^6	3.8×10^6
NP	B	N	2.0×10^4	1.5×10^4
P	NB	B	1.0×10^7	1.1×10^7
P	NB	N	0.9×10^7	1.0×10^7

^a A series of monolayer cultures using cells of BALB/c or NIH origin were plated as for a routine XC assay (18) in minimal essential medium. Serial dilutions of virus (B- or NB-tropic F-MuLV) were added to the cell medium either with aphidicolin (10 μ M) or without (control). After 16 h, medium containing drug and any unabsorbed virus was removed, the cells were rinsed, and the medium was replenished. Control cells were similarly treated. The cells were then allowed to grow to confluence and were subjected to UV irradiation before the addition of XC cells as in the normal titration assay. Permissive (P) and nonpermissive (NP) virus cell combinations were compared.

FIG. 1. Autoradiogram of filter blot hybridization analysis of the effect of aphidicolin on viral DNA. Each lane represents the Hirt supernatant DNA extracted from between 2×10^6 and 4×10^6 cells infected with B-tropic F-MuLV at multiplicity of 1. DNA samples were electrophoresed through 0.8% agarose for 20 h at 55 V (2 V/cm), transferred to nitrocellulose, and hybridized to nick-translated ^{32}P -labeled F-MuLV DNA. Lanes 1 and 2 represent a permissive 13-h infection of BALB/c cells in the absence (lane 1) or presence (lane 2) of aphidicolin (10 μM). Lanes 3 and 4 represent nonpermissive infections of NIH cells without (lane 3) or with (lane 4) the drug. Lanes 5 through 7 represent 17-h infections of secondary tail fibroblast cultures derived from mouse strains C57L (lane 5), NIH (lane 6), and DBA/2 (lane 7). Lanes 8 and 9 represent 17-h infections with NB-tropic F-MuLV (multiplicity of 1) of DBA/2 (lane 8) and C57L (lane 9) tail fibroblasts (all *Fv-I^{h/n}*). No aphidicolin was included in infections in lanes 5 through 9. Material at the origin is host DNA (endogenous viral genomes) which contaminates Hirt supernatant extracts.

migrated just faster than form I in the gel. The same two infections, carried out in the continuous presence of aphidicolin, are shown in lanes 2 and 4, respectively. In the permissive infection (lane 2), no accumulation of form I DNA was seen, resembling the nonpermissive infection carried out in the absence of the drug (lane 3). Only the linear form could be seen in the drug-treated nonpermissive infection (lane 4). In both drug-treated cultures, however, a new, rapidly migrating band of DNA (denoted "i'") was now apparent (see arrow, lanes 2 and 4). Lane 2 additionally showed the accumulation of two very faint bands migrating just ahead of form I DNA. These bands, which we have called "i," were also seen in the nonpermissive infection in the absence of drug treatment (lane 3) and have previously been described by this laboratory (2).

The accumulation of these species is therefore assumed to be due to effects of the drug, which exaggerates a normal event which we have observed in permissive infections only with viruses which replicate to very high titer. NB-tropic F-MuLV, in addition to the expected forms III and

I, accumulated other viral DNA species after acute infection of fibroblast cell lines. In Fig. 1, lane 9, is shown an example of an NB-tropic F-MuLV infection of secondary fibroblast cultures obtained from tails of C57L mice. These animals are N type (*Fv-I^{h/n}*) in terms of host restriction of ecotropic viruses. Both the most rapidly migrating band (arrow) as well as an "i'" band migrating just ahead of form I were seen in this non-drug-treated 13-h infection. We have tentatively named the less rapidly migrating band "i" on the assumption that this represents a normal intermediate in the synthesis of viral DNA. We have not, however, shown it to be a true intermediate in the pathway of synthesis of circular or integrated viral DNA. Rather, it may represent the product of a dead-end pathway seen only because this NB-tropic virus replicates to very high titer. Low-molecular-weight viral DNA has also been observed in avian sarcoma virus-infected duck cells (21). The structure and significance of these molecules have not yet been defined. However, because these DNA species were seen in the presence of aphidicolin, a drug which blocks accumulation of form I DNA, we assume that they are linear in configuration and that their appearance could be due to premature terminations or "strong stops" in DNA synthesis. The most rapidly migrating species ("i'") has been shown to anneal both to *env* and to whole genomic probes (data not shown). We are currently investigating the genomic location of these sequences.

We compared the accumulation of DNA species in secondary tail fibroblast cultures to determine whether this accumulation was specific to the cells used, that is, cell lines versus secondary fibroblast cultures. By comparison with the growth in C57L fibroblasts (Fig. 1, lane 9), this same NB-tropic virus, although unrestricted in *Fv-I^{h/n}*-type DBA/2 cells, accumulated markedly less form I DNA, consistent with our earlier studies in this cell type (2), and little if any of the two rapidly migrating species were seen (Fig. 1, lane 8). To study the accumulation of low-molecular-weight viral DNA species in *Fv-I*-nonpermissive infections, we used B-tropic virus, which was restricted in tail fibroblast cells of both C57L (lane 5) and DBA/2 (lane 7) as well as those of NIH mice (lane 6). In none of these restrictive infected cells was any apparent form I accumulated. In the NIH tail fibroblast infection (lane 6), the "i'" species was easily seen, but was only minimally accumulated in the other *Fv-I^{h/n}* cell types. In this infection of NIH tail fibroblast cells, the "i" species, easily detectable in an infection of a permanent line of NIH cells (lane 3), was not found. However, the "i'" species, which was easily detected in the NIH tail fibroblast cell (lane 6), was not seen in the

NIH fibroblast cell line (lane 3). We have not resolved whether these differences in accumulation of viral DNA species are age or cell line specific. However, our observations again demonstrate, as in our earlier work, that the accumulation of unintegrated intracellular viral DNA species depends both on the virus and on the cell type, and that differences occur between cells even of the same apparent *Fv-1* type.

In conclusion, the effects of aphidicolin on accumulation of viral DNA were seen to mimic those observed during *Fv-1*-nonpermissive infections. Not only was there a block in the accumulation of closed supercoiled form I DNA, but there was also the appearance of novel lower-molecular-weight forms. These more rapidly migrating forms were observed in the absence of drug either in very high-titer nonrestrictive infections (Fig. 1, lane 9) or in *Fv-1*-blocked infections (lanes 3, 5, 6, and 7). We therefore have assumed that the species which appear after drug treatment are "normal" products whose accumulation is not usually observed. Whether these are normal intermediates or abortive products of side pathways cannot be determined by these results.

Fate of viral DNA on removal of aphidicolin. To study further the relationships of form III and the rapidly migrating DNA species seen with aphidicolin treatment, we followed the fate of these species on removal of the drug. We used both permissive and nonpermissive infections to follow the fate of these species under conditions in which the *Fv-1* gene effect determined the accumulation of form I DNA. In a nonpermissive infection of B-tropic F-MuLV in NIH cells after 13 h, we again (Fig. 2) saw the accumulation of the "i" DNA species (see arrow, lane 1). For comparison, lane 5 represents DNA accumulation without drug, and neither form I nor the "i" species was present. At the end of 13 h, the cells were rinsed free from drug, and parallel cultures were incubated for 0.75 (lane 2), 1.5 (lane 3), and 3 (lane 4) h, respectively. Within 30 to 45 min after drug removal, the "i" species had disappeared. However, the doublet "i'" species neither disappeared nor accumulated over the ensuing 3 h of chase. Form III linear DNA remained essentially unchanged, and no form I appeared due to the *Fv-1* block. Lanes 6 and 7 of Fig. 2 show, respectively, a permissive and a nonpermissive infection with this same B-tropic virus after only 4.5 h of infection. Lane 7 clearly shows accumulation of the nonchasing "i" species and indicates that this DNA accumulated in B-virus-infected NIH cells very rapidly, even before any significant accumulation of form I could be seen (lane 6) in a permissive infection. For controls, two permissive infections are shown in lanes 8 and 9 after 14 h of

infection, indicating both the relative accumulation and the migration of forms III and I DNA.

This experiment, then, showed that form "i" accumulated very early in nonpermissive infections in the absence (lane 7) or presence (lane 1) of aphidicolin. This species neither accumulated nor disappeared after drug removal (lanes 2 through 4) and suggested a dead-end product. The "i'" intermediate (see arrow lane 1) disappeared rapidly after drug removal. Form III linear DNA also failed to "turn over" after drug removal, but could not chase into form I DNA because of the *Fv-1* effect (lanes 2 through 4).

Since this was an *Fv-1*-blocked infection, we did not anticipate the appearance of form I DNA even after drug removal. We therefore turned to a permissive infection for further investigation of the fate of linear viral DNA formed in the presence of the drug. Figure 3 shows the accumulation of NB-tropic viral DNA (after 13 h of infection) in the presence (lane 1) and absence

FIG. 2. Autoradiogram of filter blot hybridization analysis of viral DNA: aphidicolin chase study. Each lane represents the total Hirt supernatant DNA extraction of 5×10^6 infected cells. DNA samples were separated in 0.8% agarose by electrophoresis for 15 h at 55 V (2 V/cm). Lane 1 represents the viral DNA extracted from a nonpermissive infection of NIH cells with B-tropic F-MuLV for 13 h in the presence of aphidicolin (lane 1). Lanes 2 through 4 represent infection of these same cells incubated after removal of drug for 0.75 (lane 2), 1.5 (lane 3), and 3 (lane 4) h. Lane 5 represents a nonpermissive 16-h infection of BALB/c cells without drug treatment. Lanes 6 and 7 represent early infection (4.5 h) of permissive BALB/c (lane 6) and nonpermissive NIH (lane 7) cell lines without drug treatment. Lanes 8 and 9 represent late (14 h) permissive infections (also without drug) of BALB/c cells with NB-tropic F-MuLV (lane 8) or B-tropic F-MuLV (lane 9) to indicate the position of true form I DNA.

FIG. 3. Analysis of viral DNA accumulated in *Fv-1* permissive cells after removal of aphidicolin. Lanes 1 and 2 represent an autoradiogram of the filter blot hybridization of Hirt supernatant DNA formed on NB-tropic F-MuLV infection (multiplicity of 1) of NIH cells for 13 h in the presence (+) or absence (-) of aphidicolin (10 μ M). Microdensitometry tracings of lanes 1 and 2, seen in sections a and d, respectively, are shown for comparison. Form I and "i" species are indicated below their peaks. Species "i" (lane 2) are not included in the tracing. After 13 h of infection, the drug was removed, and the cultures were incubated for 6 (b) and 9 (c) h before harvest of Hirt supernatant DNA. Cells grown in the presence of aphidicolin, and similarly rinsed at 13 h, were infected at the time of drug removal for 6 (e) and 9 (f) h, respectively, before DNA extraction.

(lane 2) of drug in permissively infected NIH cells. Microdensitometry tracings of these autoradiograms are shown as sections a (lane 1) and d (lane 2), respectively. The prominent "i" band is included and shown to be approximately equal in each infection. The two more rapidly migrating species "i'" are not included in the tracing. Approximately equal accumulations of form III could be seen, but only in the drug-free infection was form I found. The reason for the absence of the "i'" DNA species seen in drug-treated cells above is unclear. Sections b and c represent the results of incubation of parallel infected cells for 6 and 9 h after removal of the drug. In each case, a small accumulation of closed circular molecules was seen (see form I arrows, sections b and c). However, during this period, 6 to 9 h after reversal, a time at which DNA synthesis (thymidine incorporation) had returned to control levels (Table 1), the amount of linear form III had changed little if at all, and there was no evidence for any progressive accumulation of circular DNA. This result suggested that little of

the viral DNA accumulated in the presence of aphidicolin could chase into form I DNA in an *Fv-1*-permissive infection after removal of the drug. As a control for the reversibility of the drug effects, and to determine whether the appearance of form I DNA after drug removal represented new synthesis rather than conversion of form III molecules, we treated two uninfected cultures with aphidicolin in an identical manner. In this case, we infected these cultures only at the time of drug removal. After 6 and 9 h of an infection which started only at the time of drug removal, large amounts of linear DNA were found, and progressively increasing amounts of both form I species were detected (see Fig. 3, sections e and f). Furthermore, the accumulation of forms I and III DNA in a parallel non-drug-treated culture after 6 h of infection (data not shown) was very similar to that found after infection of cells after removal of the drug.

These results indicated that the amount of circular viral DNA accumulated 6 and 9 h after

drug removal from 13-h infections was similar to that found after 6 to 9 h of a new infection of either drug-reversed or control cells. Slight differences in amounts of viral DNA from control cultures might be attributed to the approximately 10 to 20% cell death that occurred after 13 h of drug treatment. These data suggest further the possibility that all form I DNA that accumulated after reversal of drug effects could represent synthesis of new DNA formed only after the drug was removed or to asynchronous DNA synthesis which began only after the effects of the drug had dissipated. However, we are able to conclude that the majority, if not all, of the linear DNA formed in the presence of aphidicolin does not chase into circular DNA after drug removal, even in *Fv-1*-permissive cells.

DISCUSSION

Studies of F-MuLV infection of *Fv-1*-nonpermissive cells demonstrate that there is a block in the normal cycle of viral DNA accumulation. This is manifested usually as a decrease in the accumulation of form I circular viral DNA and the appearance of levels of linear DNA similar to that found in permissive cells (2, 9, 23). In some virus-cell combinations which result in more severe restriction of viral progeny, the accumulation of linear viral DNA is severely decreased and may be the primary site of an *Fv-1* effect (2). Nonpermissive infection of *Fv-1*-restrictive cells in both circumstances results in a failure of integration of new viral DNA (8, 19). Whether the primary block of the *Fv-1* gene product occurs at the formation of linear or circular DNA is not resolved. As an alternative model, the integration step itself may be affected either alone or in addition to the effects on viral DNA synthesis or circularization.

This report deals with the effects of an inhibitor of eucaryotic cellular DNA polymerase α on the DNA steps in murine retrovirus infection. The results indicate that at a concentration of drug which inhibits cellular DNA synthesis by 90%, the synthesis of form III viral DNA appears unaffected. These data confirm the *in vitro* studies of Pedrali-Noy and Spadari on the reverse transcriptase of avian myeloblastosis virus (13). Hsu and Taylor (personal communication) have demonstrated, in tissue culture infection with avian retroviruses, that although aphidicolin has no effect on the accumulation of linear viral DNA, neither circular nor integrated viral DNA is formed, suggesting a role for DNA polymerase α in these events.

Our data confirm their experiments and compare the results of the block of accumulation of viral DNA forms resulting from drug treatment and that due to the *Fv-1* effect. In each case, the

accumulation of linear viral DNA appears to be unaffected by either the drug or the *Fv-1* gene product. However, in each case, form I is affected. In the absence of aphidicolin, in some *Fv-1*-nonpermissive infections such as N-tropic infection of BALB/c cells, or B-tropic infection of DBA/2 cell lines, reduced but easily detectable form I DNA accumulates (2). However, in B-tropic infection of NIH cells, no circular forms are seen. Interestingly, secondary fibroblast cells derived from the tail of 10- to 20-day-old C57L, NFS (NIH), or DBA/2 mice all show a strong block in circular DNA accumulation (Fig. 1, lanes 5 through 7). The effects of aphidicolin on viral DNA synthesis most closely mimic the effects of these latter viral-cell combinations. That is, essentially normal amounts of linear DNA accumulate, but no form I species are detected. Additionally, in the drug-treated cells, novel species of viral DNA accumulate. These species are seen in the absence of the drug with NB-tropic F-MuLV-permissive infection of C57L or NIH cells. Because of this observation, we assume these species to be normal products of virus infection which accumulate to detectable levels only in certain permissive high-titer infections, in some *Fv-1*-nonpermissive infections, or in the presence of aphidicolin.

One such species, which migrates as a doublet just ahead of normal circular DNA, is seen best in Fig. 2. The "i" putative intermediate is formed very early after infection (lane 7) and appears as a doublet in lanes 1 through 4 of cells infected in the presence of drug. Lane 5 of this experiment reveals this doublet to be present without drug treatment in NIH cells nonpermissively infected with B-tropic virus, as we have previously reported (2). Since aphidicolin appears to block the circularization of linear DNA, these data suggest strongly that this doublet, which accumulates in the presence of the drug, represents a form of linear DNA. By analogy, the "i" species which accumulate in B-tropic F-MuLV infection of NIH cells due to the *Fv-1* effect are assumed to be linear in conformation. We have also found (data not shown) that denaturation of unintegrated viral DNA by formamide results in the disappearance of form III DNA. Simultaneously, bands of viral DNA resembling "i" species are found migrating just ahead of native form I. These findings taken together suggest that the "i" species may be linear single-stranded molecules. It is possible that under conditions of nonpermissive infection, a less stable, nonintegrated viral DNA complex is formed which is, when extracted, more easily denatured than the same structure formed in permissively infected cells. This possibility is currently being investigated.

Lastly, the data show that the overwhelming

majority, if not all, of the linear viral DNA formed in the presence of aphidicolin does not chase into circular DNA in either *Fv-1*-nonpermissive (Fig. 2) or *Fv-1*-permissive cells (Fig. 3). Three explanations for the failure of the linear DNA formed in the presence of the drug to undergo the expected conversion to circular DNA on drug removal suggest themselves. One is that the linear forms accumulated during drug treatment are abnormal and therefore cannot circularize. Second, there may be a residual drug effect on cells which prevents this conversion. Our data indicate that the linear and circular DNA formed on infection of cells after drug removal is very similar to that of a control infection (Fig. 3). Also, the rate of thymidine incorporation approximates normal values within 90 min after drug removal (Table 1). These results are both inconsistent with a residual drug effect. Lastly, these data suggest that in all infections, the vast amount of linear DNA formed may not participate in the formation of circular species. This conclusion would not contradict the studies of Shank and Varmus (17), who showed the precursor-product relationship between linear and circular species. Since there is no estimate as to what fraction of linear DNA might participate in circularization, integration, or both, it is possible that in all retrovirus infections only a very minor percentage undergoes such a conversion.

In conclusion, these studies have shown that the drug aphidicolin affects a block in the normal viral DNA events occurring in retrovirus-infected cells. Similar effects have been described for cycloheximide (24) as well as for ethidium bromide (4). This block resembles that imposed by the *Fv-1* gene product during infection with a restricted virus. The results therefore show a formal similarity between the pharmacological and the biological block. These data suggest that the *Fv-1* gene product may function in a manner similar to that of the drug, that is, by inhibiting a necessary DNA polymerase α activity. Since no such activity has thus far been associated with viral reverse transcriptase, it is possible both that a cellular DNA polymerase α activity is necessary for normal viral replication and that this represents one of the sites of the *Fv-1* gene effect.

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