JOURNAL OF VIROLOGY, June 1979, p. 917-922
0022-538X/79/06-0917/06$02.00/0

Vol. 30, No. 3

Transcription of the Bovine Parvovirus Genome in Isolated Nuclei

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Received for publication 24 October 1978

Transcription of the genome of the nondefective parvovirus BPV was examined in nuclei isolated from synchronized bovine fetal spleen cells. The relative levels of total RNA polymerase and RNA polymerase I, II, and III activities in nuclei isolated from BPV-infected and mock-infected cells were found to be similar throughout the course of infection. Hybridization of RNA synthesized in isolated nuclei indicated that BPV-specific RNA synthesis began during the period of 8 to 12 h postinfection and proceeded linearly until at least 20 h postinfection. By 20 h postinfection, 5% of the total RNA synthesized in nuclei from infected cells was virus specific. BPV-specific RNA synthesis was inhibited by 95% in the presence of 0.1 μg of α-amanitin per ml, suggesting that the viral genome is transcribed by cellular RNA polymerase II.

Although transcription of the defective parvovirus known as adeno-associated virus (AAV) has been extensively studied during the past several years (3), similar information is not available for this process in the replication cycle of the nondefective parvoviruses such as bovine parvovirus (BPV). Nuclear in vitro systems provide several distinct advantages for the analysis of RNA synthesis, since isolated nuclei are freely permeable to both nucleoside triphosphates and various inhibitors such as α-amanitin (19). Further, the fidelity of these in vitro systems is apparent in that those RNA species synthesized in isolated nuclei resemble those found in vivo (22, 23). Previous studies using isolated nuclei have shown that adenovirus (Ad) infection of KB cells results in a 10-fold increase of RNA polymerase II and III activities by 12 to 14 h postinfection (p.i.), whereas the activity of RNA polymerase I remains similar to that of mock-infected cells (21). Recent investigations revealed that total RNA polymerase activities in nuclei recovered from KB cells infected with both AAV and Ad were comparable to the activities found in nuclei infected with Ad alone through 12 h p.i. (1). However, after this time total RNA polymerase activity decreased dramatically in nuclei infected with both AAV and Ad as compared to those nuclei infected with Ad alone. In these studies, effects of the AAV infection on the RNA polymerase activities in the nuclei may have been masked to some extent by the helper Ad virus.

To avoid the influence of a helper virus on the system, we have utilized the nondefective BPV to examine the effects of infection on RNA polymerase activities in nuclei isolated from synchronized cells. We report here the levels of RNA polymerase activities in nuclei isolated from BPV-infected and mock-infected cells throughout the course of infection. We have also determined by hybridization the levels of BPV-specific RNA synthesized in vitro by nuclei isolated from infected cells. Finally, we have examined the effects of low concentrations of α-amanitin on the synthesis of virus-specific RNA in nuclei recovered from BPV-infected cells.

Primary bovine fetal spleen cells (BFS) were prepared and maintained as previously reported (7). BFS cells used in these studies were synchronized with hydroxyurea (7). Plaque-purified BPV, used as a source of inoculum, was freed of cellular debris by differential centrifugation. Cells synchronized with hydroxyurea were infected with BPV (multiplicity of infection, 15 to 20 PFU per cell) or mock-infected with minimal essential medium immediately upon removal of hydroxyurea at the beginning of the S phase. At specified times after infection, the nuclei were recovered from BPV-infected or mock-infected cells as described by Tamm (16). The nuclei were monitored by phase-contrast microscopy for the presence of contaminating cellular debris and undisrupted cells. Nuclei were stored in liquid nitrogen until assayed.

The levels of RNA polymerase activities in the isolated nuclei were assayed as described in Fig. 1. To differentiate between the three classes of endogenous RNA polymerases present in the nuclei, the fungal toxin α-amanitin was included

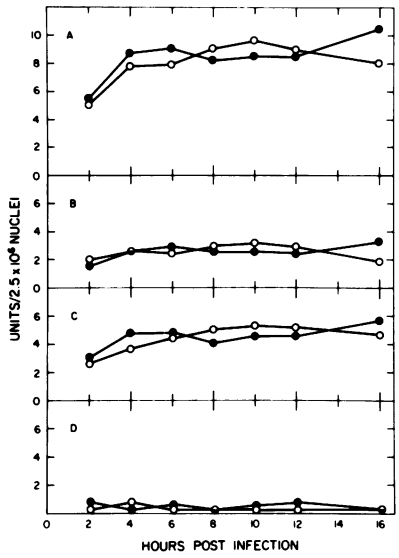

FIG. 1. *Levels of endogenous RNA polymerase activities during the course of infection. Nuclei from BPV-infected cells* (○) *and from mock-infected cells* (●) *were assayed for total RNA polymerase activity (A) and RNA polymerase I (B), II (C), and III (D) activities. RNA polymerase activity was measured as the incorporation of radioactively labeled substrate into acid-precipitable product. In a final volume of 100 μl, 2.5 × 10^6 nuclei were assayed in a reaction mixture containing 36 mM NaCl, 18 mM KCl, 0.72 mM each of ATP, GTP, and CTP, 0.8 mM dithiothreitol, 5.5 mM NaF, 1.8 mM $MnCl_2$, 0.27 mM $MgCl_2$, 45 mM Tris-hydrochloride (pH 8.2 at 37°C), 25% glycerol, and 1 μCi of [^{3}H]UTP (ICN; 27 Ci/mmol). For total RNA polymerase activity and RNA polymerase II activity, the reaction mixture contained 90 mM ammonium sulfate; RNA polymerases I and III were assayed in the presence of 0.27 mM $(NH_4)_2SO_4$. Reaction mixtures were incubated for 1 h at 37°C. The reaction was stopped by the addition of 100 μl of cold 5% trichloroacetic acid containing 2 mM $Na_4P_2O_7$ to each reaction tube. After incubation on ice for 10 min, each tube was treated by sonication for 6 s, using a microprobe at 60 W output. The amount of radioactivity incorporated was determined as described by Stout and Mans (15). The individual levels of RNA polymerase I, II, and III activity were distinguished with α-amanitin as described in the text. The data are averages for duplicate determinations for 2.5 × 10^6 nuclei. One unit of RNA polymerase activity is defined as the incorporation of 1 pmol of [^{3}H]UMP into acid-precipitable product during 1 h of incubation at 37°C.*

in some reaction mixtures. Each of these polymerases exhibits a characteristic sensitivity to this agent (4). RNA polymerase II activity was obtained by determining the difference in activity between nuclei in the presence and absence of 1 μg of α-amanitin (Calbiochem) per ml. RNA polymerase III activity was calculated as the difference in activity in the presence of 1 μg of α-amanitin per ml versus the presence of 150 μg of α-amanitin per ml. RNA polymerase I activity was determined by assaying the nuclei in the presence of 150 μg of α-amanitin per ml.

In some cases, RNA labeled in vitro was extracted from the nuclei as described by Bloom and Rose (1) for hybridization against BPV-specific DNA to detect the levels of virus-specific RNA synthesized. Virus, used as a source of BPV-specific DNA, was prepared by infecting asynchronous BFS cell cultures in roller bottles with 0.1 to 0.5 PFU of plaque-purified BPV per cell in the presence of 1 μCi of [^{14}C]thymidine (ICN; 58.2 mCi/mmol) per roller bottle. When approximately 95% of the cells showed cytopathic characteristics, they were scraped into the media and collected by centrifugation (850 × *g* for 15 min). The pellet was suspended in a small volume of 10 mM Tris-hydrochloride (pH 8.0) and stored at −20°C. Upon thawing, the cells were adjusted to 5 mM Na_2EDTA and pH 8.0. Afterwards, the cells were homogenized (6 × 15 s) with a Brinkmann polytron homogenizer at a setting of 5 and sonicated (6 × 15 s) using a large probe at 150 W output. After the cell suspension was made 0.5 mM each $CaCl_2$ and $MgCl_2$, the sample was incubated in the presence of 100 μg of DNase I, 5 units of micrococcal nuclease, and 1 mg of RNase per ml for 1 h at 37°C. The cell suspension was then adjusted to 1 mg of papain, 30 μg of trypsin, and 30 μg of chymotrypsin per ml and incubated at 37°C for 1 h. The resulting sample was brought to 1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol and layered over a CsCl-sucrose step gradient as described by Tattersall et al. (17). After centrifugation at 26,000 rpm for 20 h (17°C) in a SW27.1 rotor, the virus band was collected and dialyzed against 50 mM Tris-hydrochloride–0.5 mM Na_2EDTA (pH 8.7). The virus suspension was then adjusted to 0.5 mM each $CaCl_2$ and $MgCl_2$ and treated with 100 μg of DNAase 1, 5 units of micrococcal nuclease, and 1 mg of RNase per ml for 1 h at 37°C. Afterwards, the sample was incubated for 1 h at 37°C in the presence of 100 μg of proteinase K per ml and then adjusted to 0.3 M NaOH to release the virion DNA. The pH of the DNA sample was neutralized with Tris-hydrochloride after 30 min. After an additional incubation with 100 μg of proteinase K per ml, the DNA was passed through a hydroxyapatite column to remove nucleotides, sodium dodecyl sulfate, and proteins. Viral DNA used for hybridization eluted from the column at a sodium phosphate concentration of 0.17 to 0.22 M.

For hybridization, BPV-specific DNA was immobilized onto nitrocellulose filters as described by Gillespie and Spiegelman (5). Each 25-mm filter contained 2 μg of viral DNA. Exhaustive hybridization was carried out according to the procedure described by Shih and Khoury (13). Hybridization of RNA to the immobilized DNA was carried out in 1 ml of 50% formamide (vol/vol), 0.6 M NaCl, 2 mM Na_2EDTA, 0.2% sodium dodecyl sulfate, 0.06 M sodium phosphate (pH 7.0). Formamide was purified as described by Tibbetts et al. (18). Three consecutive DNA filters were incubated with each RNA sample at 37°C for 24-h intervals. After incubation, each filter was washed overnight in 2 ml of 2× SSC at 4°C with gentle shaking and then counted. Under these conditions, acid-precipitable RNA counts did not decrease after incubation of the third filter. The amount of viral RNA hybridized was calculated from the difference in radioactivity obtained from incubating RNA extracted from BPV-infected nuclei versus RNA recovered from mock-infected nuclei.

Nuclei isolated from uninfected, nonsynchronized BFS cells were used to determine optimal endogenous RNA polymerase conditions. Maximal incorporation of [^{3}H]UMP into acid-precipitable product was exhibited in the presence of both Mn^{2+} and Mg^{2+} at 37°C and at pH 8. The inclusion of 25% glycerol in the reaction mixture containing the isolated nuclei was found to increase [^{3}H]UMP incorporation by at least 25%. When constant numbers of nuclei were assayed for total RNA polymerase activity, incorporation of [^{3}H]UMP into acid-precipitable product was linear for at least the first 10 min but plateaued approximately 5 min later, indicating that extensive reinitiation by the polymerases was not occurring (not shown). To further test if reinitiation was occurring, heparin, which prevents the reassociation of RNA polymerase to DNA (16), was included in the reaction mixture. In the presence of 1 mg of heparin per ml, we did not observe any decrease in the amount of [^{3}H]UMP incorporation, indicating that reinitiation of transcription was not occurring in the isolated nuclei.

Viral infection may result in modifications of cellular RNA polymerases or the production of virus-coded RNA polymerases which can often be detected by changes of salt optima of the RNA polymerase activities in isolated nuclei (21). We examined the ammonium sulfate optima of the RNA polymerases in nuclei recovered from synchronized mock-infected and BPV-infected cells to determine if BPV infection results in any change of salt optimum. Experiments were conducted to determine the optimal ammonium sulfate concentration for total RNA polymerase activity, RNA polymerase II activity, and combined RNA polymerase I and III activities. These activities were differentiated on the basis of α-amanitin sensitivity. Under our assay conditions, as described in Fig. 1, we were unable to detect any differences in the ammonium sulfate optima between the mock-infected and BPV-infected nuclei at all times examined after infection (not shown). Total RNA polymerase activity and RNA polymerase II activity had an optimum of 90 mM ammonium sulfate, whereas the combined RNA polymerases I and III had an optimum between 27 mM and 40 mM ammonium sulfate.

By examining nuclei isolated from mock-infected and BPV-infected cells, we have determined the effect BPV infection has on levels of endogenous enzyme activities attributable to RNA polymerases I, II, and III (Fig. 1A to D). The nuclei were assayed under optimal conditions as described in Fig. 1. Total RNA polymerase activity (Fig. 1A) was found to be essentially the same in both BPV-infected and mock-infected nuclei, although in some experiments a slight increase in activity was found in the infected nuclei between 8 and 12 h p.i. as compared to mock-infected nuclei. After 12 h p.i. RNA polymerase activity decreased in BPV-infected nuclei versus mock-infected nuclei. This may be the result of mitosis occurring only in the mock-infected cells, which increases the number of uninfected nuclei being assayed as compared to the number of infected nuclei. Levels of RNA polymerase I, II, and III activities (Fig. 1B to D, respectively) were essentially the same in mock-infected and BPV-infected nuclei. RNA polymerase I represented 25 to 40% of the total endogenous activity in the nuclei at 4 to 12 h p.i. The level of RNA polymerase II activity represented at least 50% of the total endogenous RNA polymerase activity, whereas RNA polymerase III represented less than 5% of the total polymerase activity. However, in some cases a slight increase of RNA polymerase II activity in BPV-infected over that of the mock-infected nuclei occurred during 6 to 12 h p.i.

Exhaustive hybridization in 50% formamide was used to detect the synthesis of BPV-specific RNA in infected nuclei. Nuclei were assayed as described previously for total RNA polymerase activity except that the incubation time was only 35 min. Total in vitro-labeled RNA was recovered from these nuclei and was hybridized successively against three nitrocellulose filters containing BPV virion DNA. By this hybridization procedure, BPV-specific RNA synthesis was first detected in nuclei recovered from infected

BFS cells at 12 h p.i. (Fig. 2). The hybridization profile indicates that a large increase in the rate of viral RNA synthesis occurs between 8 and 12 h p.i., and suggests that the onset of BPV-specific transcription also occurs during this time period. However, viral RNA synthesis may have begun prior to this at levels below the detectability of our hybridization system. From the period of 8 to 20 h p.i., we found an approximately linear increase in the synthesis of viral RNA in infected nuclei. By 20 h p.i., 5.3% of the total RNA labeled in vitro in BPV-infected nuclei was virus specific.

Infected nuclei were also assayed for the presence of hemagglutinating antigens whose production must have been preceded by BPV-specific RNA synthesis (Fig. 2). In agreement with the initial detection of viral RNA synthesis, the production of hemagglutinating antigens was found in our system beginning at 10 to 12 h p.i.

The α-amanitin sensitivity of the RNA polymerase responsible for the production of BPV-specific RNA was determined by assaying BPV-infected nuclei in the presence and absence of the fungal toxin, followed by extraction and exhaustive hybridization of the in vitro-labeled RNA (Table 1). The presence of 1 μg of α-amanitin per ml in the assay mixture containing BPV-infected nuclei isolated at 16 h p.i. reduced the amount of BPV-specific RNA synthesized in vitro by 95% as determined by hybridization. In the presence of low levels of α-amanitin, the RNA synthesized in vitro in BPV-infected nuclei was only 0.17% virus specific whereas infected nuclei assayed without α-amanitin synthesized RNA that was 3.1% virus specific. The extreme sensitivity of BPV-specific RNA synthesis to low concentrations of α-amanitin indicates that viral transcription is performed by cellular RNA polymerase II. This is supported by recent findings in our laboratory that RNA polymerase II is associated with BPV transcription complexes (unpublished data).

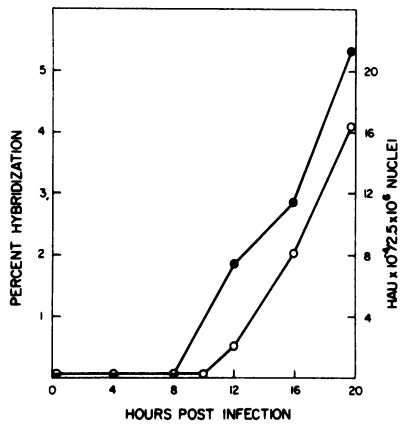

FIG. 2. *Synthesis of viral RNA (●) and levels of hemagglutinating antigens (○) in isolated nuclei during the course of infection. Nuclei were prepared from cells harvested at the indicated times after infection. At each time point, nuclei form BPV-infected and mock-infected cells were incubated with [³H]UTP as described in the text. The total RNA in the nuclei was purified and hybridized in 50% formamide (5,000 cpm) against three successive filters containing BPV DNA. Percent hybridization of mock-infected RNA to the filters (<0.1%) was subtracted from the percent of viral RNA hybridized. Hemagglutinating antigen levels were determined by sonic disruption of 10^6 nuclei from infected cells in 10 mM Tris-hydrochloride (pH 8.0), followed by assay of a sample for hemagglutinins as described previously (7). The levels of hemagglutinating antigens were normalized to 2.5 × 10^6 nuclei.*

In this study we have analyzed the transcription process of the BPV genome in nuclei isolated from BFS cells. We have found that BPV infection does not result in any substantial changes in total RNA polymerase activity or in RNA polymerase I, II, or III activities as compared to nuclei from mock-infected cells (Fig. 1A to D). This is in contrast to studies with nuclei isolated from Ad-infected KB cells, which showed a dramatic increase in total RNA polymerase activity and RNA polymerase II and III activities by 12 h p.i. as compared to nuclei isolated from uninfected cells (21). Bloom and Rose (1) have shown that total RNA polymerase activity in nuclei isolated from KB cells infected

TABLE 1. *Effects of low concentrations of α-amanitin on the synthesis of BPV-specific RNA in isolated nuclei*[a]

Assay conditions (α-amanitin concn)	Hybridization[b]	
	cpm bound[c]	Percent[d]
BPV-infected nuclei (none)	464	3.10
BPV-infected nuclei (1 μg/ml)	26	0.17
Mock-infected nuclei (1 μg/ml)	0	0

[a] Nuclei were prepared from BPV-infected cells or mock-infected cells harvested at 16 h p.i. The isolated nuclei were incubated under conditions described in Fig. 1 for total RNA polymerase activity. α-Amanitin was added to some reaction mixtures, as shown.

[b] To detect viral RNA, the total RNA in the nuclei was purified and hybridized in 50% formamide against three successive filters containing BPV DNA as described in the text.

[c] Represents the difference between labeled RNA binding to nitrocellulose filters containing BPV DNA and to blank nitrocellulose filters.

[d] Percent of total [³H]RNA hybridizing to nitrocellulose filters containing BPV DNA.

with both AAV and Ad increased through 12 h p.i., similar to those activities found in nuclei recovered from KB cells infected with only Ad. On the basis of our studies with a nondefective parvovirus, which showed no difference in RNA polymerase activities between nuclei recovered from BPV-infected and mock-infected cells, the increase of total RNA polymerase activity seen in nuclei from Ad- and AAV-infected cells may have been the result of the helper virus, Ad, and not the defective parvovirus, AAV.

It seems unlikely that the BPV genome (1.7×10^6 daltons; A. K. Saemundsen and R. C. Bates, manuscript in preparation) is transcribed by a virus-specific RNA polymerase, due to the limited coding capacity of the viral DNA. Since mammalian RNA polymerases have molecular weights of at least 500,000 (4), the synthesis of these enzymes would apparently require DNA templates with coding capacities in excess of those possible with the BPV genome. We have found essentially no changes in the endogenous RNA polymerase activities and ammonium sulfate optima in nuclei isolated from both BPV-infected and mock-infected cells, suggesting that the cellular RNA polymerases are not modified during BPV infection.

We have found that a low concentration of α-amanitin inhibits the production of viral RNA in isolated nuclei, indicating that BPV transcription is mediated by a polymerase with characteristics of cellular RNA polymerase II (Table 1). Since we have not detected modification of the endogenous cellular RNA polymerases in the isolated nuclei or the presence of any virus-coded RNA polymerase, we propose that the BPV genome is transcribed by cellular RNA polymerase II. This polymerase, which has been shown to be responsible for the production of pre-messenger RNA in eucaryotic systems (2, 4, 8), has also been implicated in the transcription of the AAV genome (1, 6).

Hybridization of RNA extracted from nuclei isolated from BPV-infected cells indicated that viral RNA is initially synthesized in vitro at detectable levels during the period from 8 to 12 h p.i. The actual onset of viral transcription may occur earlier than this time but at levels below the sensitivity of our hybridization system. In our synchronized cell system, the time period of 8 to 12 h p.i. corresponds to late S or early G_2 phase in the cell cycle (7). Previous investigations with the nondefective parvoviruses BPV, H-1, LuIII, and minute virus of mice have shown that the onset of viral DNA synthesis also begins during the period from late S to early G_2 phase (7, 9, 10, 12, 14, 20). Our data indicating that parvoviral transcription begins at this time in the cell cycle support the model for parvovirus replication as proposed by Rhode. His model proposes that viral transcription is required for the subsequent production of replicative form and progeny viral DNA (10–12).

To further study the components and process of BPV-specific RNA synthesis, we are currently analyzing virus-specific transcription complexes produced in BPV-infected cells.

This research was supported by American Cancer Society Grant no. IN-117.

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