ANALYSIS OF DNA POLYMERASE ACTIVITIES INVOLVED IN BOVINE PARVOVIRAL DNA REPLICATION

by

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(ABSTRACT)

The polymerase activities involved in bovine parvoviral (BPV) DNA replication in vivo and in vitro have been described. In the in vitro system, purified and partially purified enzymes were used and the replication products were analyzed by gel electrophoresis and restriction enzyme digestion. DNA pol γ purified from fetal bovine liver, replicated BPV ssDNA to a unit-length covalently-linked dsDNA hairpin molecule but was unable to utilize purified BPV dsDNA as a template. Partially purified calf thymus DNA pol α replicated BPV DNA to a product 1 kbp smaller than unit-length dsDNA (11 kbp). Mapping of this product showed that the middle of the genome was under-represented. Purified pol α from bovine fetal lung (BFL) cells was capable of only end-labeling BPV ssDNA. If HeLa cell DNA pol α, which consisted of the core enzyme plus cofactors C_1C_2, was used, the products consisted of both noncovalently- and covalently-linked unit-length dsDNA hairpin molecules. Hence, purification of pol α removed factor(s) necessary for the activity of the enzyme on BPV DNA. The polymerase activities involved in vivo in BPV DNA

replication were analyzed using aphidicolin, a specific inhibitor of DNA pol α, and/or L-canavanine, an inhibitor of protein synthesis. DNA present in infected cells was visualized by autoradiography of Southern blots after probing with nick-translated BPV DNA. Aphidicolin, added at any time after-infection, reversibly inhibited each step of BPV DNA synthesis. Conversely, L-canavanine slowed the replication process, inhibited the synthesis of the viral-coded proteins NP-1 and VP3, and inhibited the production of replicative intermediates (RI) and progeny ssDNA. After removal of L-canavanine, both protein and DNA synthesis resumed. These results demonstrate that 1) pol α is involved in every stage of the replication process including the production of parental replicative form (RF), daughter RF, RI, and progeny genomes, 2) taken in conjunction with the in vitro data, that a pol α holoenzyme complex is required for BPV DNA replication, and 3) viral proteins are required for RI and progeny DNA synthesis,

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INTRODUCTION

Bovine parvovirus (BPV) is a member of the family Parvoviridae, the smallest of all animal viruses. The viral particle consists of an unenveloped icosahedron containing four capsid proteins (Lederman et al., 1983) which surround the ssDNA genome of 5500 bases (Snyder et al., 1982), 100-200 of which occur in hairpin structures at each terminus. Viral DNA replication occurs in the nucleus of host cells and requires an S phase specific function for initiation of replication (Parris and Bates, 1976; Rhode, 1973; Siegl and Gautschi, 1973; Tattersall, 1972; Tennant and Hand, 1970; Wolter et al., 1980). As a group, these viruses are important as tools to the molecular biologist studying cellular DNA replication. They offer a simple, naturally occurring, self-priming DNA template which normally utilizes the host cell polymerases and other cellular replication machinery (Pritchard et al., 1978b). Thus, they can be studied both in vivo and in vitro as indicators of polymerase activity.

In addition, these viruses are known to be pathogenic for various animal species. BPV and porcine parvovirus are both associated with reproductive failure due to fetal infection and will cause severe neonatal diarrhea (Kahrs, 1981; Molitor et al., 1983). Canine parvovirus and feline panleukopenia virus (FPV) cause a severe disease in neonates and FPV is also extremely pathogenic for adult cats (Tattersall and Ward, 1978). Aleutian disease of mink (ADV) causes a

persistent viral infection in mink and results in estimated losses to the North American mink industry of 10 million dollars annually (Porter and Cho, 1980). Moreover, there is a suggested link between parvoviruses and several human disorders including aplastic anemia in sickle-cell disease (Pattison et al., 1981), bone marrow aplasia (Mortimer et al., 1983), and aquired immune deficiency syndrome (AIDS) (Tattersall, personal communication). On the other hand, parvoviruses have been shown to decrease spontaneous, virus- and chemical- induced tumors in rodents (Blacklow and Cukor, 1978; Toolan, 1967; Toolan and Ledinko, 1968; Toolan et al., 1982), and inhibit cell transformation by SV40 (Mousset and Rommelaere, 1982). Hence, information concerning the replication of these viruses is of great interest.

The first model for parvoviral DNA replication (Tattersall and Ward, 1976) was based heavily on a model proposed by Cavalier-Smith for replication of linear chromosomal DNA (Cavalier-Smith, 1974). The most recent models (Astell et al., 1983a; Rhode and Klaassen, 1982) are a derivation of that first model which have evolved to incorporate all of the recent evidence concerning replication of the parvoviral genome. The model shown in Fig. 1 is one presented by Berns and Hauswirth (1982) which is a compilation of the models proposed by Astell et. al. (1983a) and Rhode and Klaassen (1982).

The first step in parvoviral DNA replication is postulated to be conversion of the ssDNA genome to a covalently closed hairpin duplex (parental RF) via initiation off of the 3' hairpin primer. This

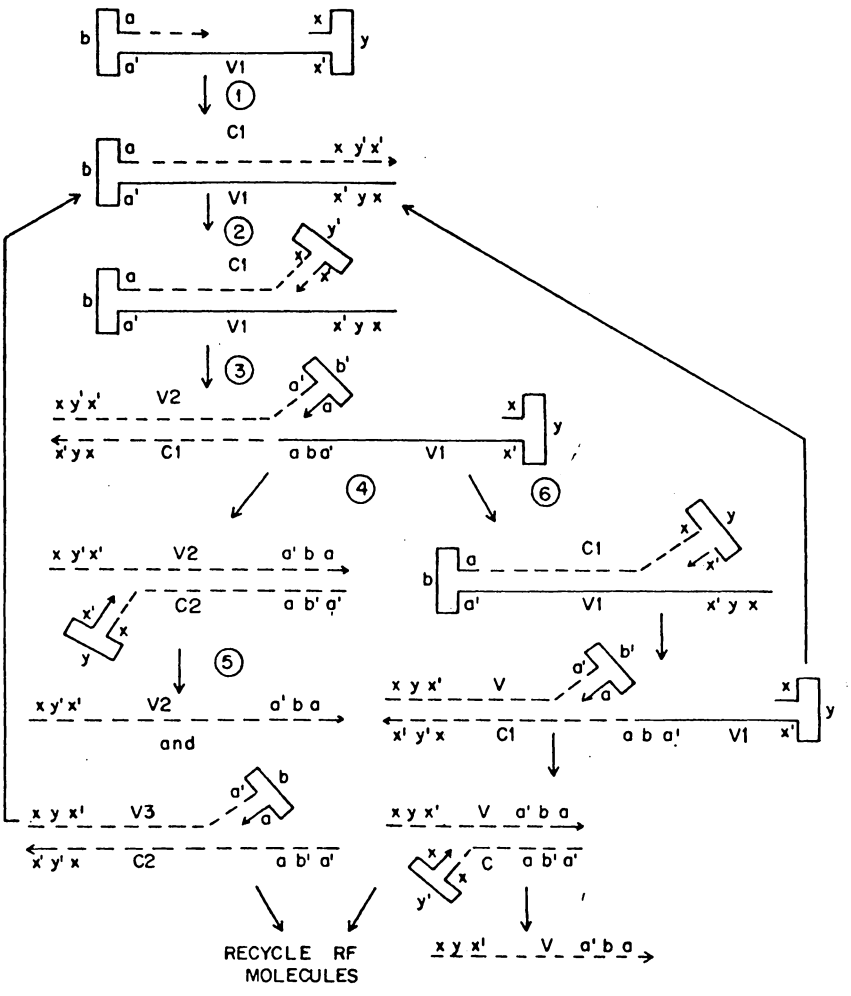

FIG. 1. Model for autonomous parvovirus DNA replication. This model is as presented by Berns and Hauswirth (1982). The terminal 3' sequences (a b a', or a b' a') and the terminal 5' sequences (x y x', or x' y' x) are displayed as is the complementarity of each strand. In this model, parental RF is produced from step 1, dimer RF from steps 2 and 3, and progeny strands from step 5.

was based on the observations (Hayward et al., 1978; Rhode, 1974; Siegl and Gautschi, 1978; Strauss et al., 1976; Ward and Dadachanji, 1978) made using alkaline agarose gel analysis and hydroxylapatite chromatography that covalently closed duplex forms were found in vivo. Interestingly enough, this hypothesis has not yet been proven in vivo even though more sensitive gel and blotting procedures are now available.

The second step in the process involves amplification of the parental RF to daughter RF molecules. The nature of the RF molecules has been studied extensively (Hayward et al., 1978; Li et al., 1978; Rhode, 1974; Siegl and Gautschi, 1976; Tattersall et al., 1973). They are of a discrete size (by definition) and are of either unit-length double-stranded or higher oliogomeric size (i.e., dimer, trimer, etc.). RF synthesis begins at the 5' end of the viral strand (Singer and Rhode, 1977b) and proceeds through a probable dimer intermediate although the exact requirement and/or function of the dimer is unknown. The dimer molecule itself consists of two double-stranded unit-lengths of viral and complementary strands arranged in tandem (Rhode, 1977; Strauss et al., 1976). The replicative intermediate (RI) molecules (Singer and Rhode, 1977a) are not of a discrete size, but, as determined by electron microscopy, are Y-shaped molecules from which ssDNA progeny are being produced.

Overall, the process begins during mid to late S phase (Rhode, 1973; Siegl and Gautschi, 1976; Wolter et al., 1980) in localized

euchromatic or nucleolar sites and spreads outward (Singer and Rhode, 1978). Involvement of the nuclear matrix in the replication process has also been postulated (Briggs, personal communication).

Viral proteins are known to be involved in various stages in the replication cycle. Although, the first step in the process, production of the parental RF does not require viral protein synthesis, amplification of RF DNA does (Rhode, 1978). According to the current models, part of this requirement is postulated to be two nickase activities. The first nickase may be associated with the 5' binding protein. This protein was first identified by Revie et al., (1979) associated with the 5' ends of RF DNA of the parvovirus H-1. As determined by gradient and restriction enzyme analysis, the protein is covalently bound to the 5' termini and has an approximate molecular weight of 60-70 kd. Astell et al. (1983a) now also have evidence to suggest the presence of a protein binding to the 5' end of minute virus of mice (MVM) DNA. Using polyacrylamide gel electrophoresis, they estimate the molecular weight to be 60 kd. The proposed function of the protein is as a site-specific nickase, creating a single strand nick within the dimer intermediate 75 nucleotides distal to the 5' end of a single viral genome. Both the protein and the extra nucleotides would be cleaved off during the final stages of virion maturation since neither are found in the mature virus particle. The second nickase has not been found, but it is postulated to function within the 5' hairpin termini of the dimer molecule. It would create a nick on the viral

strand allowing for a gap filling type of DNA replication which would, thereby, conserve the 5' hairpin. Replication of this sort would involve the formation of a flip-flop (ie., inverted complementary) sequence at this end and evidence to support this has been demonstrated (Rhode and Klaassen, 1982; Astell et al., 1983b).

Synthesis of progeny ssDNA is dependent upon capsid protein production. Based on studies using inhibitors of protein synthesis and the observation that pools of progeny ssDNA do not exist in infected cells, it has been postulated that the ssDNA genomes are packaged into preformed capsids (Muller and Siegl, 1983a and 1983b; Myers and Carter, 1980). In addition, these capsids are required for the displacement synthesis of progeny genomes off of RI molecules. The final stage of replication, maturation of the virions, occurs via a two step process. After encapsidation, the viral particle has a density of 1.46 g/cc. Following a final maturation process, the density of the particle is shifted to 1.41 g/cc (Muller and Siegl, 1983a and 1983b).

These viruses do not possess a virion-associated DNA polymerase (Pritchard et al., 1978b) and, given the limited coding capacity of the genome, they, most likely, do not code for an entire polymerase molecule. Hence, they must rely heavily, if not entirely, on cellular DNA polymerases. Identification of polymerase activity(ies) involved in each phase of the process has received a great deal of attention.

It has been well established that DNA polymerase alpha (pol α) is the major enzyme involved in cellular DNA replication (Weissbach, 1979). This enzyme has been purified from many different sources (reviewed in Fry, 1982; Scovassi et al., 1980; Weissbach, 1977) and the most generally accepted hypothesis at present is that the core enzyme consists of a single polypeptide of approximately 140 kd. However, in vivo, the enzyme is normally found complexed with several other enzymatic activities including a DNase and primase activity (Baril, personal communication) and the protein cofactors C_1 and C_2 (Lamothe et al., 1981). Together, with other as yet unidentified activities, they form a multienzyme complex which is proposed to be associated with the nuclear matrix of cells and upon which or through which DNA replication occurs (Pardoll et al., 1980; Vogelstein et al., 1980). Two of these factors, C_1 and C_2, have been studies in greater detail. They were first purified by Baril and colleagues (Lamothe et al., 1981; Novak and Baril, 1978) and in vitro investigations have led to the postulation that they are responsible for the increased ability of pol α to replicate single-stranded DNA molecules by serving as primer recognition proteins and inhibiting non-productive binding of the enzyme to ssDNA templates (Pritchard and DePamphilis, 1983; Pritchard et al., 1983). Pritchard et al., (1983) have shown that without the cofactors, core pol α is unable to replicate BPV DNA. However, if the cofactors are present on the pol α enzyme (holoenzyme) then the complex is capable of incorporating labeled dNTPs into the

BPV DNA template. In these studies, the size and/or quality of the final product was not investigated.

Other investigators have also analyzed the involvement of polymerase α in parvoviral replication. Faust and Rankin (1982) demonstrated the ability of partially purified pol α to completely replicate MVM DNA in vitro. The reaction required ATP and was suppressed by low enzyme/template ratios, apparently due to nonproductive binding of the enzyme. In vitro studies in isolated nuclei (Kollek et al., 1982) and in a nuclear lysate system (Pritchard et al., 1981) have also demonstrated the participation of DNA pol α in parvoviral replication.

Two other cellular DNA dependent DNA polymerases are known to exist (Fry, 1982; Scovassi et al., 1980; Weissbach, 1977). DNA polymerase beta (pol β) functions in repair synthesis and its exact involvement in replicative DNA synthesis is unknown. Polymerase gamma (pol γ) is the mitochondrial enzyme responsible for "D-loop" type synthesis of the mitochondrial genome. Although it has been shown to be present in the cell nucleus, its involvement, if any, in nuclear DNA replication is unclear (Weissbach, 1979). The ability of these enzymes to replicate parvoviral DNA has also been studied. Ikeda et al., (1980) showed that purified DNA pol β was capable of replicating MVM DNA in vitro. However, questions have been raised concerning the specific identify of the enzymatic activity involved. On the other hand, DNA pol γ has been shown capable of completely replicating H-1 DNA in vitro

(Kollek and Goulian, 1981). Furthermore, an activity that replicated H-1 DNA in isolated nuclei was identified as DNA pol γ (Kollek et al., 1982).

During the course of this research, my overall objective was to attempt to define the cellular polymerase and viral protein requirements for BPV DNA replication. The specific objectives were to:

1. Define the ability of partially purified and purified DNA pol α to replicate BPV DNA in vitro and to determine which protein factors were responsible for the activity of the enzyme on ssDNA.
2. Define the activity of purified DNA pol γ on purified BPV ssDNA, dsDNA, and virion DNA.
3. Determine the polymerase activity responsible in vivo for the first step in BPV DNA replication, and for RF DNA synthesis.
4. Identify the involvement of DNA pol α in BPV DNA replication in vivo and the requirement for the viral proteins NP-1 and VP3 in the later stages of the replication cycle.

Chapter I

REPLICATION OF BOVINE PARVOVIRAL DNA BY PURIFIED AND PARTIALLY PURIFIED DNA POLYMERASE ALPHA

INTRODUCTION

Bovine parvovirus (BPV) is a member of the family Parvoviridae, a group of small DNA viruses which replicate in the nucleus of host cells. For two major reasons, parvoviruses are considered to be excellent models for the molecular biologist studying eukaryotic DNA replication. First, they possess a small single-stranded DNA genome (4.7-5.5 kb) (Snyder et al., 1982) with 3' and 5' terminal hairpin structures. Thus, they serve as a naturally occuring self-priming template for in vitro analysis of DNA polymerase activity and give results which can be correlated directly to the in vivo situation. Secondly, these viruses are dependent on the host cell machinery for their replication since there is no viral-associated DNA polymerase (Pritchard et al., 1978b) and since, most likely, they do not code for a partial or complete DNA polymerase molecule. Furthermore, parvoviral replication, unlike that of other DNA viruses, is limited specifically to cellular S phase (Tennant and Hand, 1970) and recent evidence suggests that parvoviral DNA is associated with the nuclear matrix of infected cells (Briggs et al., manuscript in preparation). Hence, they can be used for both in vitro and in vivo studies

dissecting polymerase and other enzymatic activities which are present and/or functioning during cellular DNA replication.

The major enzyme involved in cellular DNA replication is DNA polymerase alpha (pol α) (Weissbach, 1979). In vivo (Hardt et al., 1983; Pritchard et al., 1978a) and in vitro (Faust et al., 1982; Kollek et al., 1982; Pritchard et al., 1981) results have indicated that pol α is involved in parvoviral DNA replication as well. However, identification of the specific protein(s) required for complete replication of the viral genome has not been obtained.

Preparations of DNA pol α enzymes obtained from 3 different sources and purified by 3 different procedures, have been tested for their ability to replicate BPV DNA. Purified viral DNA was used as a template and the products were analyzed by agarose gel electrophoresis. I found that partially purified pol α replicated BPV DNA to a dsDNA molecule slightly smaller than unit-length dsDNA, that purified pol α was only able to end label the DNA molecule, and that purified pol α with cofactors, C_1C_2, replicated the BPV template to unit-length dsDNA molecules, only part of which were covalently-linked hairpin forms.

MATERIALS AND METHODS

Materials

DEAE-cellulose and phosphocellulose were from Sigma. DEAE-Trisacryl was from LKB. CNBr-activated Sepharose was obtained from Pharmacia and ssDNA was coupled to it by the method suggested by the manufacturer. Hydroxylapatite was BioRad DNA grade. Radioactive nucleotides were from Amersham (^{32}P) or ICN (^{32}P and ^{3}H). Calf thymus DNA was activated by the method of Baril (1977) and ssDNA was prepared by boiling activated calf thymus DNA for 10 min and then quick chilling on ice. Restriction enzymes and calf thymus DNA pol α were purchased from BRL and P. L. Biochemicals, respectively.

Enzyme purification

Bovine fetal calf thymus (200 g) or SV40-transformed bovine fetal lung (BFL) cells (20-30 g) which had been stored at -80°C, were used. Unless noted otherwise, all steps were performed at 4°C and all buffers contained 1 mM DTT and 20% glycerol. For the calf thymus enzyme (CT-pol α), tissue was thawed in 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, homogenized in a Waring blendor, filtered through cheesecloth, and centrifuged at low speed (14,000 x g, 10 min). Twice more, the pellet was homogenized in a Brinkman Polytron Homogenizer and recentrifuged at low speed. All the supernatants were combined,

centrifuged at low speed and then at high speed (25,000 rpm, SW27 rotor, 1 hr). The final supernatant was adjusted to 0.4 M KCl and chromatographed on a DEAE-cellulose column equilibrated with 0.4 M KCl, 40 mM potassium phosphate (KPO_4) (pH 7.5), to remove contaminating nucleic acid. The flow through was concentrated versus polyethylene glycol 6000, then dialyzed versus 20:20 buffer (20 mM KPO_4, pH 7.5, 20 mM KCl). Protein precipitated with ammonium sulfate (35-55% saturation) was dialyzed versus 20:20 buffer, and chromatographed on a DEAE-Trisacryl column equilibrated with the same buffer. Enzyme was eluted with a 20-400 mM KCl gradient, dialyzed versus 20:20 buffer and chromatographed on a phosphocellulose column also in 20:20 buffer. A 20-500 mM KPO_4 (pH 7.5) gradient was used to elute the enzyme, active fractions were pooled and dialyzed versus Te buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Finally, this preparation was chromatographed over ssDNA-Sepharose equilibrated with Te buffer containing 50% glycerol and stored at -80°C.

Pol α was isolated from BFL cells (BFL-pol α) by the same procedure except for the following changes. After homogenization of the tissue, nuclei in the low speed pellet were lysed and extracted with 0.4 M KPO_4 (pH 7.5) for 30 min. Also, active fractions off of the phosphocellulose column were dialyzed versus 5 mM KPO_4 (pH 7.5), 400 mM KCl, chromatographed over hydroxylapatite equilibrated with the same buffer, and the enzyme was eluted with a 5-200 mM KPO_4 (pH 7.5) gradient. As before, active fractions were dialyzed versus Te

buffer, chromatographed over the ssDNA-Sepharose column, and stored in 20:20 buffer with 50% glycerol.

The purified HeLa cell DNA pol α (HL-pol α) consisted of the pol α core enzyme and the cofactors C_1C_2. HL-pol α was purified by the method of Baril (Lamothe et. al., 1981) and was supplied by him.

Enzyme Assays

To detect active fractions off of the columns, reactions of 100 µl total volume contained: 50 mM Tris-HCL (pH 8.0), 5.0 mM $MgCl_2$, 1 mM DTT, 300 µg/ml BSA, 100 µM dATP, dGTP, and dCTP, 10 µM dTTP, 10 µCi (^{3}H)dTTP (55-70 ci/mmole), 400 µg/ml activated calf thymus DNA and 5 µl of column fraction. After 1 hr at 37°C, acid precipitable radioactivity was measured as described (Stout and Arens, 1970).

For assays containing BPV DNA, reactions of 100 µl total volume contained: 50 mM Tris-HCl (pH 8.0), 5.0 mM $MgCl_2$, 1 mM DTT, 300 µg/ml BSA, 4 mM ATP, 100 µM dATP, dGTP, and dTTP, 10 µM dCTP, 5 µCi (^{32}P)dCTP (400 Ci/mmole), 0.25 - 1 µg of BPV DNA, and 0.25 - 1 U of enzyme (Table 1). In some cases, when HL-pol α was used, the total reaction volume was 10 µl. To determine polymerase activity, 5 µl of each sample was electrophoresed directly or the entire sample was treated with proteinase K (100 µg, 60°C, 8 hr), extracted with chloroform/isoamyl alcohol, and ethanol precipitated prior to electrophoresis.

For restriction enzyme analysis, precipitated DNA was resuspended in the appropiate enzyme assay buffer, and digested with Bgl II or Hinc II according to the manufacturers suggestions.

Gel Electrophoresis

Agarose gel electrophoresis was performed using either 1.4% horizontal neutral gels or 1.4% vertical alkaline gels as described previously (Robertson et al., 1984a and 1984b). Gels were dried onto DE81 (Whatman) paper, and exposed directly to Kodak X-Omat AR film.

Polyacrylamide gels were according to the method of Laemmli (1970) except that 5-15% gradient resolving gels with a 4% stacking gel were used.

RESULTS

Activity of Bovine Fetal Calf Thymus DNA Pol

The activity of CT-pol α is shown in Table 1. The enzyme was determined to be pol α by its sensitivity to aphidicolin, N-ethylmaleimide, and salt (100 mM) and by its insensitivity to ddTTP. The enzyme was inactive on poly rA:oligo dT, a homopolymer template which is a specific template for DNA pol γ. This enzyme was, however, active on single-stranded calf thymus DNA as well as activated calf thymus DNA. When analyzed by polyacrylamide gel electrophoresis, the numerous bands (’50) present on the gel indicated that CT-pol α was only partially pure.

TABLE 1. THE ACTIVITY OF FETAL CALF THYMUS DNA POLYMERASE ALPHA

	SPECIFIC ACTIVITY (pmoles/hr/mg protein)			
	rA:dT[a]	Act. CT DNA[b]	CT ssDNA[c]	BPV DNA[d]
Standard Reaction	27	11,000	10,200	2,610
+ Aphidicolin (1 mM)	nd	5,420	2,530	810
+ ddTTP (50 μM)	3	12,430	9,270	1,960
+ NEM (10 mM)	3	160	4	50

[a]50 μg/ml Poly rA:oligo dT (20:1).

[b]400 μg/ml DNase I activated calf thymus DNA.

[c]400 μg/ml calf thymus ssDNA.

[d]10 μg/ml BPV virion DNA.

The ability of the enzyme to replicate BPV DNA is shown in Fig 1. The major product was approximately 10 kb, which was 1 kb smaller than unit-length dsDNA. Species labeled during the reaction ranged in size from ssDNA (5.5 kb) to dsDNA (11 kb) (Fig. 1, lane 2). The same result was obtained when P. L. DNA pol α was used (Fig. 1, lane 1).

Restriction enzyme analysis of the products of CT-pol α replication are shown in Fig 2. When compared to the restriction patterns generated by digestion of pol I (Klenow) replicated BPV DNA, two differences were noted. First, when the CT-pol α replication products were digested, the middle fragments (i.e. Bgl II fragment B and Hinc II fragment A) were under represented (Fig. 2, lanes 4 and 6). Second, the 5' end fragments generated from the digestion of the CT-pol α replicated BPV DNA (i. e. Bgl II fragment A and Hinc II fragment E) were smaller than the pol I (Klenow) replicated and subsequently digested counterparts. Thus, CT-pol α had not completely replicated the BPV DNA genome and had preferentially replicated the ends of the ssDNA molecule.

Analysis and Activity of Purified BFL DNA Pol

To circumvent the problems of excess tissue and DNA encountered in purifying pol α from calf thymus, I switched to BFL cells. BFL-pol α eluted in three peaks off of the ssDNA-Sepharose column. The purity of each enzyme fraction was monitored by

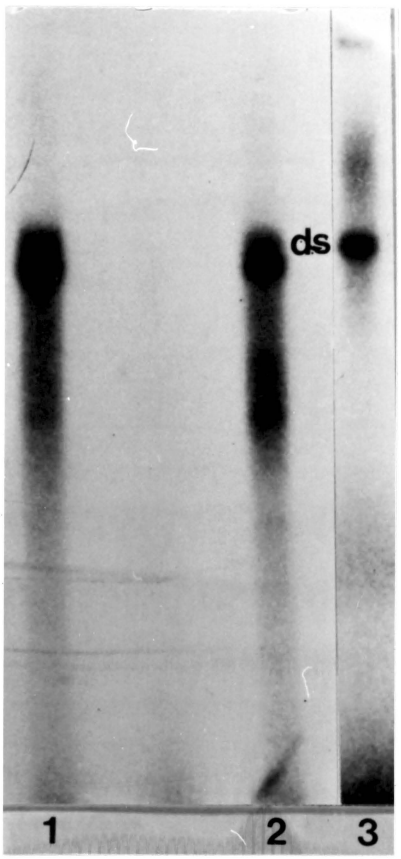

FIG. 1. CT-pol α replication of BPV DNA. BPV DNA was replicated by CT-pol α and electrophoresed directly on 1.4% neutral agarose gels (lane 2). P. L. Biochemical pol α (from calf thymus) replicated BPV DNA was used for comparison (lane 1). Pol I (Klenow) replicated BPV DNA (lane 3) was used to mark the position of dsDNA (ds).

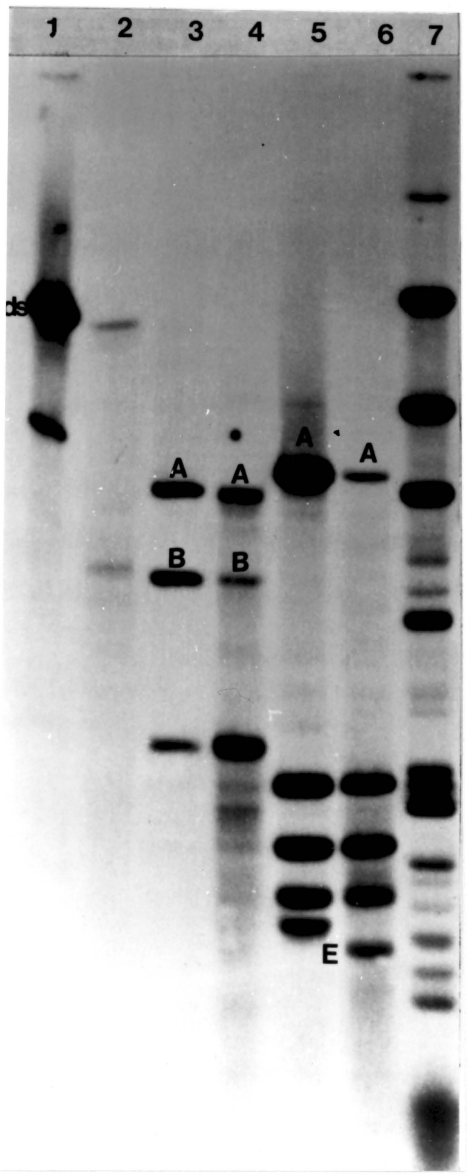

FIG. 2. Restriction enzyme analysis of BPV DNA replicated by CT-pol α. BPV DNA was replicated with CT-pol α (lanes 2, 4, 6) or pol I (Klenow) (lanes 1, 3, 5), proteinase K treated, ethanol precipitated, and digested with either Bgl II (lanes 3 and 4) or Hinc II (lanes 5 and 6). The position of the Bgl II fragments A and B and the Hinc II fragments A and E are noted. M13 size markers (lane 7) are 6.4, 3.55, 2.5, 1.6, .83, 0.82, and 0.65 kb.

polyacrylamide gel electrophoresis on 5-15% gradient gels (Fig. 3). The peak 2 fraction off of the ssDNA-Sepharose column did not contain enough protein to show up by silver staining on the acrylamide gel. In the peak 1 fraction, there was one major protein (64 kd) and 5 minor protein species (56 - 68 kd). In the flow through, the 6 proteins identical to those in peak 1 were visible as were a group of 4 proteins (28 - 35 kd) and 1 larger protein of 118-130 kd. These results are consistent with those of others who have also found more than one protein associated with "pure" preparations of pol α (Albert et al., 1982; Chen et al., 1979; Filpula et al., 1982; Holmes et al., 1976; Mechali et al., 1980; Yamaguchi et al., 1982).

The ability of BFL-pol α to replicate BPV DNA and its sensitivity to aphidicolin are shown in Fig. 4. BFL-pol α was able only to end label the BPV ssDNA molecule, resulting in radioactivity incorporated but no appreciable shift in the mobility of the product on agarose gels (Fig. 4, lanes 2, 4, and 6). In all three cases, the enzyme was sensitive to aphidicolin. This indicated that the purified enzyme had lost the protein factors necessary for replication of BPV DNA in vitro which were present in the CT-pol α preparation.

Activity of HeLa Cell DNA Pol

HL-pol α, as purified by Baril (Lamothe et al., 1981), consists of a complex of core enzyme plus cofactors C_1C_2. It replicated BPV DNA to a unit-length dsDNA molecule (Fig. 5A). When

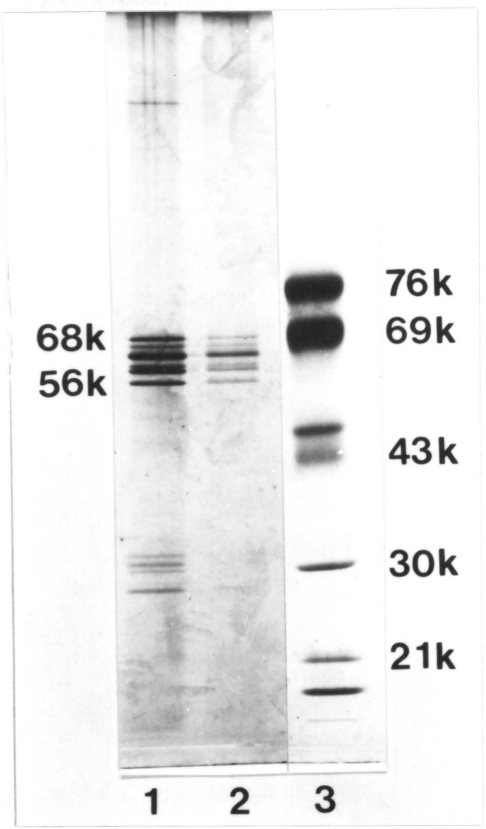

FIG. 3. Polyacrylamide gel analysis of BFL-pol α. BFL-pol α was electrophoresed on a 5-15% polyacrylamide gradient gel and visualized by silver staining. Lane 1, BFL-pol α that eluted in the flow through off of the ssDNA-Sepharose column. Lane 2, BFL-pol α that eluted in the peak 1 fraction off of the ssDNA-Sepharose column. Lane 3, marker proteins with sizes in kd as indicated.

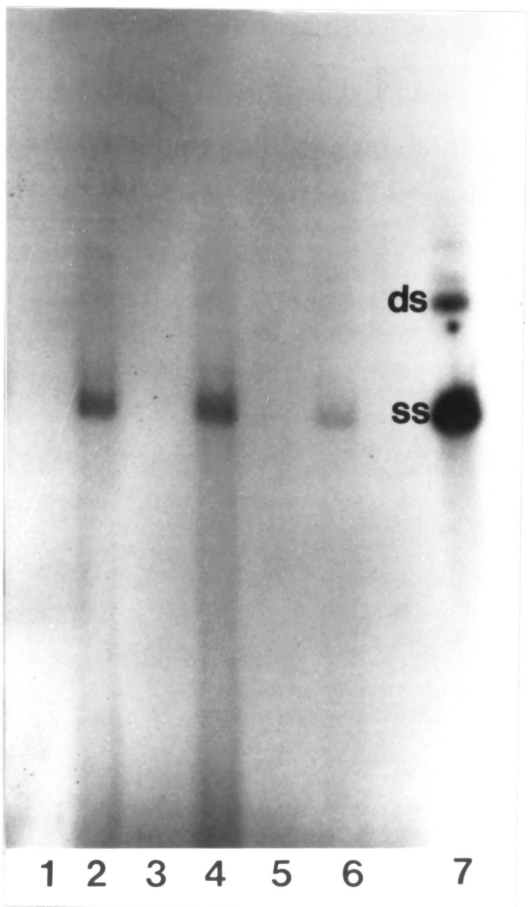

FIG. 4. BFL-pol α replication of BPV DNA in the presence or absence of aphidicolin. BPV DNA was replicated by the BFL-pol α in the peak 2 (lanes 1 and 2), peak 1 (lanes 3 and 4) or flow through (lanes 5 and 6) fraction and in the presence (lanes 1, 3, 5) or absence (lanes 2, 4, 6) of aphidicolin. The positions of dsDNA (ds) and ssDNA (ss) are noted in the marker lane (lane 7).

this product was run on an alkaline gel (Fig. 5B), a small quantity banded at the position of a covalently linked dsDNA hairpin, but most banded at a position slightly greater than unit-length monomer DNA (6.4 kb). In addition, numerous smaller bands were seen. This indicated that the cofactors C_1C_2 are required for activity of pol α on BPV DNA but they do not confer upon the enzyme the ability to replicate BPV ssDNA to a covalently closed dsDNA hairpin.

DISCUSSION

I have shown that partially purified CT-pol α replicated BPV DNA forming a major product which was approximately 1 kb less than unit-length dsDNA and many minor products. I have also shown, by restriction enzyme analysis, that the middle portions of the genome were under represented in the final product and the 5' terminal fragments were shorter than those produced by digestion of pol I (Klenow) replicated BPV DNA. Further purification of pol α resulted in loss of the ability of the enzyme to replicate BPV ssDNA. However, when pol α was complexed with cofactors C_1C_2 (i.e., HL-pol α), the enzyme was again able to utilize the parvoviral ssDNA template, although the major product was not the expected covalently closed dsDNA hairpin.

There are several problems with these data. First, a discrepancy is seen when comparing the products of CT-pol α replication of BPV DNA (Fig. 1) with the patterns generated from restriction enzyme digestion of the same (Fig. 2). Specifically, the 5'

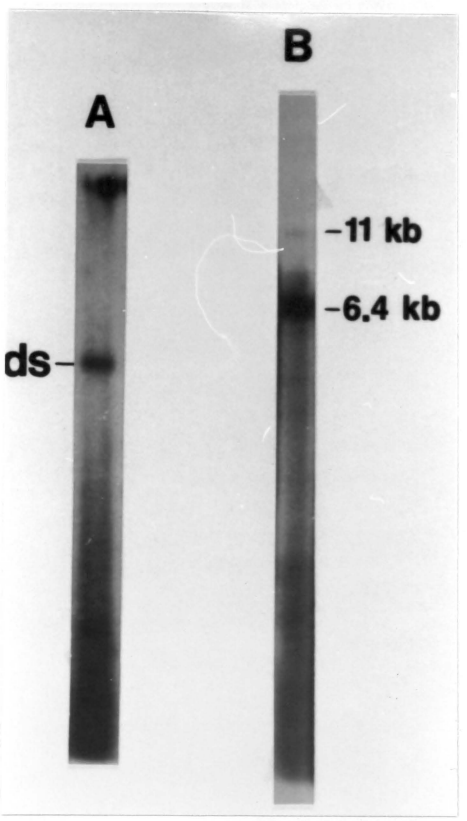

FIG. 5. Analysis of HL-pol α replication of BPV DNA. BPV DNA was replicated by HL-pol α and electrophoresed directly on either a neutral (A) or alkaline (B) agarose gel. The position of dsDNA (ds) is noted on the neutral gel. The positions of unit-length covalently linked hairpin molecules (11 kb) and the 6.4 kb band is indicated on the alkaline gel.

end fragment of the Bgl II and Hinc II digests is only 0.1 kbp smaller than expected whereas the major product seen in Fig. 1 is 1.0 kbp smaller than unit-length dsDNA. This discrepancy apparently results from differences in the age of the enzyme used. The fresher enzyme, used to prepare the template for restriction enzyme analysis, produced a dsDNA product which was only 100-200 bases shorter than full unit-length dsDNA (Fig. 2, lane 2). On the other hand, once the enzyme had been stored for longer periods of time at -80°C and had been frozen and thawed, it lost the ability to produce the longer product. That is, the major DNA specices seen after replication was 1.0 kb smaller than unit-length dsDNA. Age may also account for the differences seen between the products from CT-pol α relication and HL-pol α replication of BPV DNA. When HL-pol α was used, it was fresh. Overall, the "aging effect" most likely reflects susceptibility of a protein activity to inactivation as a result of freezing and thawing.

Another problem with the data in Fig. 2 concerns the under representation of the middle fragments generated from restriction enzyme digestion. This may result from a high concentration of defective BPV DNA species present in the sample used for the template. Such species have been shown to contain the ends of the parvoviral DNA genome but lack the middle segments (Faust and Ward, 1979). On the other hand, replication of unit-length BPV genomes by DNA CT-pol α may have generated DNA species which lacked the middle parts of the genome. For example, the BPV DNA genome may be in the form of a

"T" type structure in vitro where secondary structure in the middle regions of the genome produce the stem of the "T". Then, if CT-pol α initiated replication at the 3' end of the genome, it could "jump" to a region close to the 5' end of the genome, skipping the middle (stem of the "T") and replicating only the ends (top of the "T"). In fact, it has been shown that "hairpin" structures serve as arrest sites for pol α in vitro (Weaver and DePamphilis, 1982). One final explanation is possible. CT-pol α may, in most cases, only replicate a small portion of the genome before falling off, thus generating an abundance of 3' end fragments and considerably fewer middle fragments. The 5' end fragments would then be generated from partial replication of contaminating complementary strands. This would fit data (Faust and Rankin, 1982) which suggests that a strong stop signal exists at approximately 0.4 map units on the parvoviral genome.

The inability of the purified enzyme, BFL-pol α, to replicate BPV DNA whereas purified pol α plus cofactors (HL-pol α) could, indicates that the cofactors, C_1C_2, are responsible for the ability of pol α to replicate ssDNA. However, caution must be used when interpreting these results since the two pol α preparations were from different sources and were purified by different procedures. Furthermore, HL-pol α has not been analyzed by polyacrylamide gel electrophoresis and, therefore, the nature of the proteins present is unknown. In addition, the results obtained when using HL-pol α were not always consistent with each other. In some cases, the two major

products labeled in vitro were unit-length dsDNA and a molecule slightly larger than ssDNA. Restriction enzyme analysis was not performed on these molecules and, therefore, the extent of label incorporation is not known.

These data indicate that other protein factors, besides the pol α core enzyme, are required for activity of pol α on ssDNA templates. Detailed analysis of one system in which these factors are added and removed is required before definitive conclusions can be drawn concerning their activity on BPV DNA.

Chapter II

PURIFIED DNA POLYMERASE GAMMA REPLICATES BOVINE PARVOVIRUS DNA TO A UNIT-LENGTH PRODUCT

SUMMARY

DNA polymerase gamma, purified from fetal bovine liver, replicated virion single-stranded DNA from bovine parvovirus to a unit-length double-stranded DNA molecule. This product was not nicked and was covalently linked to the 3' hairpin primer. The reaction was inhibited by dideoxythymidine 5'-triphosphate, but was unaffected by ATP or aphidicolin. Double-stranded viral DNA was not a functional template for purified DNA polymerase gamma.

INTRODUCTION

Parvoviruses are small ssDNA viruses which rely on eukaryotic host cell DNA polymerase(s) for their replication (Pritchard, et. al., 1978b). Both ssDNA and dsDNA molecules function as templates during viral DNA synthesis (Berns, et. al., 1982). Although in vitro studies have implicated DNA pol α (Faust and Rankin, 1982; Kollek, et. al., 1982; Pritchard, et. al., 1978a; Pritchard, et. al., 1981) pol β (Ikeda, et. al. 1980) and pol γ (Kollek, et. al., 1982; Kollek, et. al., 1981; Robertson, et. al., 1982) in various phases of parvoviral DNA synthesis, the enzymatic requirements for specific steps have not been identified.

In this communication, I report the ability of purified bovine DNA pol γ to replicate BPV DNA in vitro. Using neutral and alkaline agarose gel electrophoresis and restriction enzyme analysis, I have shown that both virion DNA (which consists of a mixture of ssDNA and dsDNA) and purified ssDNA were replicated to a unit-length dsDNA product which was covalently linked to the 3' hairpin primer. Conversely, purified BPV dsDNA was not used as a template by pol γ.

MATERIALS AND METHODS

DNA Preparation

BPV virion DNA was isolated as previously described (Robertson et al., 1984b). These preparations contain up to 25-30% dsDNA molecules which have resulted from reannealing of separately encapsidated viral and complementary strands (Robertson, et. al., 1984b). To separate ssDNA and dsDNA, virion DNA was electrophoresed on 1.4% low melt agarose gels. DNA bands were excised, dissolved at 60°C in 0.5 M ammonium acetate and 2.0 mM EDTA, extracted by the method of Wieslander (1979), ethanol precipitated and dissloved in 50 mM Tris-HCl (pH 8.0). Marker dsDNA was synthesized from BPV DNA with pol I which has been shown to replicate parvoviral DNA to a unit-length dsDNA product (Bourguignon, et. al., 1976).

Enzyme Purification

DNA pol γ was purified from fetal bovine liver that had been stored at -80°C. Unless otherwise noted, all steps were performed at 4°C and all buffers contained 20% glycerol and 7 mM 2-mercaptoethanol (BME). The tissue (200 g) was thawed in 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 0.5 mM MgCl, 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride. It was homogenized in a Waring Blender, filtered through cheese cloth, centrifuged at 14,000 x g for 15 min then homogenized with a Polytron homogenizer and centrifuged twice. The pellets were resuspended in 50 mM KPO_4 (pH 7.5), 20 mM KCl and the nuclei were extracted for 1 hr after the addition of an equal volume of 0.8 M KPO_4 (pH 7.5). The supernatants were centrifuged first at low speed (14,000 x g, 10 min) then at high speed (25,000 rpm, SW27 rotor, 1 hr), adjusted to 0.4 M KCl, and chromatographed on a DEAE-cellulose column (4.5 x 13 cm) equilibrated with 0.4 M KCl, 50 mM KPO_4 (pH 7.5). Protein in the flow-through was precipitated with $(NH_4)_2SO_4$ (35%-55% saturation), and dialyzed against buffer A (20 mM KPO_4, pH 7.5, 20 mM KCl). The next two columns were equilibrated with buffer A and the enzyme was eluted as follows: DEAE-cellulose, 40-400 mM KCl gradient; phosphocellulose, 50-600 mM KPO_4 (pH 7.5) gradient. Active fractions were pooled, dialyzed against 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and chromatographed on a ssDNA-Sepharose column. The enzyme, eluted with a 20-400 mM KCl gradient in the above Tris buffer, was precipitated with

$(NH_4)_2SO_4$, dialyzed and stored in buffer A with 50% glycerol at -80°C.

Enzyme Assays

To determine pol γ activity during purification, reactions contained, in a final volume of 100 µl: 50 mM Tris-HCl (pH 7.5), 0.5 mM MnCl, 100 mM KCl, 0.5 mM DTT, 50 µM dATP, dGTP, and dCTP, 5.0 µM dTTP, 10 µCi [^{3}H]dTTP (55-77 Ci/mmole), 300 µg/ml BSA, 2.5 µg poly rA:oligo dT (freshly hybridized), and 5.0 µl of each column fraction. Reactions were incubated for 1 hr at 30°C and incorporation was measured as previously described (Pritchard, et. al., 1978a).

For assays with activated calf thymus DNA and BPV DNA, reactions contained, in a final volume of 100 µl, 50 mM Tris-HCl (pH 8.5), 7.5 mM MgCl, 1 mM DTT, 100 mM KCl, 20 mM KPO_4 (pH 8.5), 300 µg/ml BSA, 100 µM each dATP, dGTP, and dTTP, 10 µM dCTP, and 10 µCi/ml [^{32}P]dCTP (410 Ci/mmole), from 0.2 to 1.0 µg of BPV DNA or 40 µg of activated calf thymus DNA, and 0.25-1.0 unit of pol γ (Table 1). Reactions were incubated for 1 hr at 37°C and incorporation of radioactivity was measured (Pritchard, et. al., 1978a). Reactions containing BPV DNA were stopped by adding EDTA to 10 mM and treated with 500 µg/ml proteinase K for 1 hr at 60°C. The samples were then either electrophoresed directly on an agarose gel or extracted with chloroform/isoamyl alcohol (24:1) and ethanol precipitated. For restriction enzyme analysis, the precipitates were resuspended in H_2O

and digested with Eco RI, Hinc II, or Bgl II according to the instructions provided by the supplier (Bethesda Research Laboratories).

Gel Electrophoresis

Alkaline and neutral 1.4% agarose gels were as described (Robertson, et. al., 1984a, 1984b) except that vertical (14 x 20 x 0.25 cm) or horizontal mini-gels (2 mm thickness) were used. After drying the gels onto DE81 paper, autoradiograms were prepared.

RESULTS

The activity of the final enzyme preparation is shown in Table 1. It was identified as DNA pol γ by its insensitivity to aphidicolin, even at concentrations as high as 1 mM, and its sensitivity to N-ethylmaleimide (10 mM) and ddTTP (50 μM or a 5:1 ratio of ddTTP to dTTP). The enzyme was more active on BPV DNA than on activated calf thymus DNA, although it was most active on poly rA:oligo dT. The ability of the enzyme to replicate various BPV DNA species was monitored by autoradiography following electrophoresis on agarose gels. As shown in Fig. 1A (lane 1), pol γ replicated BPV virion DNA to a discrete dsDNA molecule. In addition, pol γ replicated purified BPV ssDNA (Fig. 1A, lane 2) but was unable to utilize isolated BPV dsDNA as a template (Fig. 1A, lane 3).

It has been shown (Faust and Rankin, 1982; Bates et. al., 1983) that ATP is required for in vitro activity of pol α on parvoviral

TABLE 1. THE ACTIVITY OF FETAL BOVINE LIVER POL γ

	SPECIFIC ACTIVITY (pmoles/hr/mg protein)		
	rA:dT[a]	Act CT DNA[b]	BPV DNA[c]
Standard reaction	256	45	114
+ Aphidicolin (1 mM)	nd	36	106
+ ddTTP (50 μM)	12	13	0
+ NEM (10 mM)	60	4	0

[a] 50 μg/ml Poly rA:oligo dT (20:1).

[b] 400 μg/ml DNase I activated calf thymus DNA (15).

[c] 10 μg/ml BPV virion DNA.

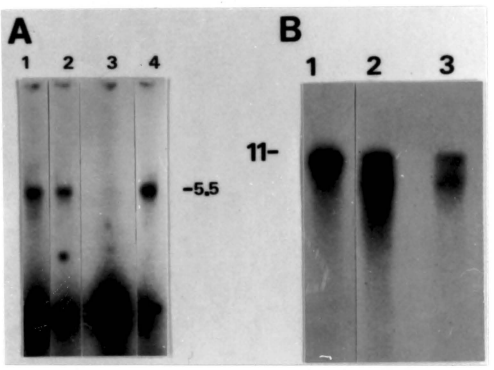

Fig. 1. Pol ɣ replication of BPV virion, ssDNA and dsDNA. BPV DNA was incubated in the standard reaction mixture. After incubation, samples were analyzed directly on 1.4% neutral and alkaline agarose gels. A: Neutral gel analysis of in vitro replication products. Lanes 1, 2 and 3 are pol ɣ replicated BPV virion, purified ssDNA, and purified dsDNA, respectively. Lane 4 is pol I replicated BPV virion DNA. The position of unit-length dsDNA (5.5 kbp) is indicated. B: Alkaline gel analysis of in vitro replication products. Lane 1, pol I replicated BPV virion DNA; lanes 2 and 3, pol ɣ replicated virion and purified ssDNA respectively. The position of the 11 kb molecule is noted.

DNA. To determine if pol γ activity on BPV DNA was influenced by ATP and to prove that the activity observed was pol γ activity and not a result of pol α contamination, I incubated BPV DNA with pol γ in the presence or absence of ATP and in the presence of aphidicolin or ddTTP. As shown in Fig. 2, synthesis of BPV dsDNA was not sensitive to aphidicolin (lanes 2 and 5) but was completely inhibited by ddTTP (lanes 3 and 6). In addition, the ability of DNA pol γ to replicate BPV DNA was not affected by ATP (Fig 2. compare lanes 1 and 4). In certain cases, both a unit-length dsDNA molecule (5.5 kbp) and a minor discrete dsDNA form of approximately 4.5 kbp were produced after replication by pol γ (Fig. 2). The presence of this minor band appeared to be related to the age of the enzyme as it was seen consistently after the enzyme preparation had been stored for extended periods at -80°C.

To verify that the dsDNA products were unit length, they were analyzed by restriction enzyme analysis. Pol γ replicated virion and ssDNA were digested with Eco RI as described above. The resulting patterns were compared to that obtained from Eco RI digestion of BPV DNA replicated with pol I (Fig. 3, lanes 2, 4, and 6). In addition, the products were digested with Hinc II and Bgl II (data not shown). For each enzyme, no differences were detected between the restriction patterns of the pol γ and the pol I replicated products.

To characterize further the DNA product, pol γ replicated BPV DNA was electrophoresed on alkaline gels (Fig. 1B). One major

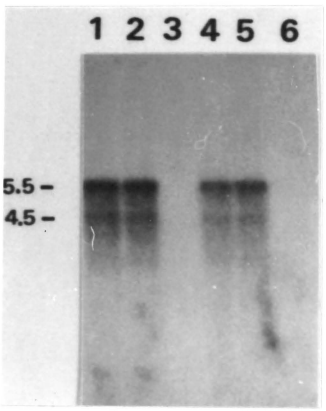

Fig. 2. Effect of inhibitors and ATP on BPV DNA replication. BPV DNA was incubated in the presence (lanes 1-3) or absence (lanes 4-6) of 4 mM ATP and without inhibitor (lanes 1 and 4) or in the presence of 20 μM aphidicolin (lanes 2 and 5) or 50 μM ddTTP (lanes 3 and 6). The positions of dsDNA (5.5 kbp) and the 4.5 kbp band are noted.

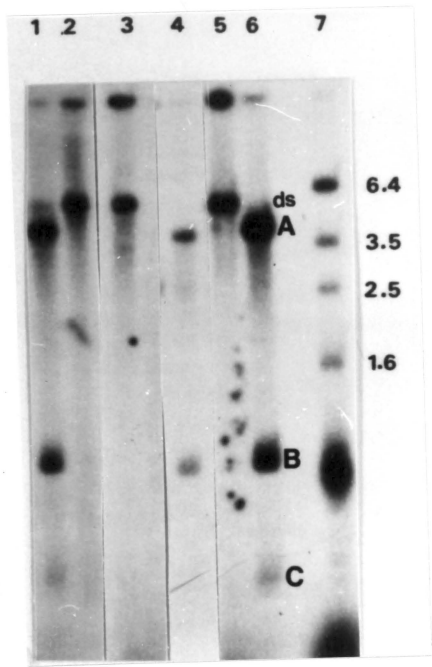

Fig. 3. Restriction enzyme analysis of pol ɣ replicated BPV virion and purified ssDNA. After BPV DNA was replicated in the standard reaction mixture, it was treated with proteinase K, extracted, ethanol precipitated, and digested with Eco RI. Samples were then electrophoresed directly on vertical agarose gels. Lane 1, pol I replicated BPV virion DNA digested with Eco RI; lane 2, pol I replicated BPV virion DNA; lane 3, pol ɣ replicated BPV virion DNA; lane 4, DNA from lane 3 digested with Eco RI; lane 5, pol ɣ replicated purified BPV ssDNA; lane 6, DNA from lane 5 digested with Eco RI; lane 7, M13 size markers (kbp). The position of dsDNA (ds) is noted. The location of the Eco RI restriction fragments, in map units, are as follows: A, 16-92.6; B, 0-16; C, 92.6-100 (Burd et al., 1983).

band of 11 kbp was produced (Fig. 1B, lanes 1 and 2) indicating that the product was not nicked and was covalently attached to the 3' hairpin primer. The minor band seen on neutral gels (4.5 kbp, Fig. 2) was also shown to be unnicked and covalently attached (9kb, Fig. 1B, lanes 1 and 2). Therefore, I conclude that pol γ replicated BPV DNA to fully extended dsDNA hairpin molecules which are identical to those seen in virus infected cells.

DISCUSSION

I have demonstrated in this report that isolated BPV virion and ssDNA can be used efficiently as templates by DNA pol γ. By restriction enzyme analysis, the product was shown to be a full unit-length dsDNA molecule. In contrast, pol γ cannot utilize isolated BPV dsDNA as a template (Fig. 1A, lane 3) nor can it use higher oligomeric double-stranded forms of BPV DNA (data not shown).

Unlike a previous report using pol γ from human placenta and H1 parvovirus DNA (Kollek and Goulian, 1981), we observed a unique dsDNA product from pol γ replication. Even when older enzyme preparations were used, only one additional band was produced. This smaller band did not appear in pol I replicated samples, was not altered by the inclusion of ATP (Fig. 2, lanes 4 and 5) by the addition of aphidicolin (Fig. 2, lanes 2 and 5), by changing the enzyme/template ratio, or by increasing the length of incubation (data not shown). However, it was identical in size to a DNA band observed during the later stages of BPV DNA replication in vivo (Robertson et. al., 1984b).

Based on these results, it seems most likely that, if involved, DNA pol γ would function in those steps of BPV DNA replication which utilize self-primed single-stranded viral DNA as templates.

Chapter III

APHIDICOLIN INHIBITION OF THE PRODUCTION OF REPLICATIVE FORM DNA DURING BOVINE PARVOVIRUS INFECTION

SUMMARY

Since parvoviruses apparently do not possess a DNA polymerase activity, one or more of the host cell DNA polymerases must be responsible for replicating the ssDNA genome. I have focused on determining which polymerase, alpha, beta, or gamma, is responsible for the first step in bovine parvoviral DNA replication: conversion of the ssDNA genome to a parental RF. In this study, I used aphidicolin, a specific inhibitor of DNA pol α to assay for the requirement of pol α activity in parental RF formation in vivo. Synchronized cell cultures were infected with BPV with or without aphidicolin and the products of viral replication were separated on agarose gels and identified by Southern blot analysis. I found complete inhibition of viral DNA synthesis resulted when 20 μM aphidicolin was present throughout the infection. In addition, viral DNA synthesis was inhibited by as little as 1 μM aphidicolin while lower concentrations (0.1 and 0.01 μM) resulted in partial inhibition of the replication process. Using ^{32}P-labeled BPV input virus, I differentiated parental RF from daughter RF and progeny DNA synthesis. I conclude that DNA pol α is required for the production of RF during BPV replication in vivo and that this

requirement is most likely for the conversion of BPV input ssDNA to parental RF. These results do not rule out a possible role for DNA pol γ in the first step nor do they rule out a role for pol α or pol γ in later stages of the replication cycle.

INTRODUCTION

Parvoviruses are small, unenveloped DNA viruses composed of three or four major capsid proteins and a linear single-stranded DNA (ssDNA) genome with hairpins at both termini (Tattersall and Ward, 1976; Tattersall and Ward, 1978). Replication of these viruses occurs exclusively in the nucleus of host cells and is cell cycle dependent, requiring an S phase function for initiation of DNA synthesis (Parris and Bates, 1976; Rhode, 1973; Siegl and Gautschi, 1973; Tattersall, 1972; Tennant and Hand, 1970; Wolter et al., 1980). Several models have been proposed for parvoviral DNA replication (Astell et al., 1983a; Berns and Hauswirth, 1982; Rhode and Klaassen, 1982; Tattersall and Ward, 1976). The first step in each model involves self-priming of the ssDNA genome at the 3' terminus (Strauss et al., 1976) and formation of a double-stranded DNA (dsDNA) replicative form which is covalently closed at the original 3' end (parental RF). The second phase involves replication of the parental RF through a possible dimer intermediate to daughter RF molecules which may or may not be covalently closed. These serve as the template for the production of progeny genomes. Although the exact requirement(s) for viral proteins in the replication

cycle is unknown, there is no virion-associated DNA polymerase (Pritchard et al., 1978), nor is there any evidence to suggest that a virally coded DNA polymerase exists. Therefore, one or more of the cellular DNA polymerases, alpha (pol α), beta (pol β), or gamma (pol γ), must be responsible for viral DNA synthesis.

The activity of both DNA pol α and pol γ, on parvoviral DNA has been previously studied in vitro. Purified DNA pol γ was able to utilize the 3' hairpin as a primer and replicate the viral DNA to a unit length RF (Kollek and Goulian, 1981; Robertson et al., 1982). Studies using polymerase inhibitors in isolated nuclei led Goulian and coworkers (Kollek et al., 1982) to conclude that pol γ was responsible for the first step of the replication process in vivo. In addition, partially purified DNA pol α (Faust and Rankin, 1982;), and purified pol α reconstituted with specific protein cofactors (but not purified pol α alone) (Pritchard et al., 1983) utilized the parvoviral DNA template. Studies using specific inhibitors, indicated that in vitro, pol α functioned in parvoviral DNA replication in both isolated nuclei (Kollek et al., 1982) and a nuclear lysate system (Pritchard et al., 1981). Moreover, in vivo levels of DNA pol α increased during the parvoviral infection cycle (Pritchard et al., 1978).

I have undertaken a series of in vivo experiments aimed at identifying the DNA polymerase requirements for the synthesis of viral RF DNA. I used bovine parvovirus (BPV), which possesses the largest genome of the autonomous parvoviruses (5500 bases) (Snyder et al.,

1982) and aphidicolin, a tetracyclic diterpenoid from Cephalosporium aphidicola which specifically inhibits DNA pol α (Huberman, 1981; Spadari et al., 1982). In this report, I used a synchronized cell system and Southern blot analysis to examine the ability of aphidicolin to block the production of viral RF DNA. I found that aphidicolin inhibited viral RF DNA synthesis which indicated that cellular DNA pol α is required for BPV DNA replication in vivo.

MATERIALS AND METHODS

Virus Growth and Purification

BPV stocks were prepared as described (Parris and Bates, 1976). For preparation of ^{32}P-labeled virus, bovine fetal lung (BFL) cells were infected with 1.0 PFU/cell of BPV. When 20% cytopathic effect (CPE) was observed, the medium was changed to phosphate-free minimal essential medium (MEM) containing 10% dialyzed fetal bovine serum (FBS) and 50 μCi/ml of $^{32}P_i$. At 100% CPE, the cells were harvested, frozen and thawed, and BPV was isolated by CsCl centrifugation.

Cell Culture, Synchronization and Infection

To obtain synchronized cell cultures, BFL cells were seeded into 60 mm petri plates (500,000 cells/plate) in the presence of 2 mM hydroxyurea (Parris et al., 1975). After 26-32 hr, the cells were washed twice with Dulbecco's phosphate buffer to remove hydroxyurea and infected with 1.0 ml of BPV (20-50 PFU/cell). After 1 hr

adsorption, the virus was removed and 5.0 ml of MEM with 10% FBS was added. Aphidicolin, dissolved in dimethylsulfoxide to a final concentration of 0.1 M, was added to the cultures at this point (time 1 hr) or as indicated in the individual experiment. Since 20 μM (7 μg/ml) aphidicolin gave maximal inhibition of cellular DNA synthesis (Fig. 5), this concentration was routinely used. When total DNA synthesis was to be measured, 2 μCi/ml of [^{3}H]thymidine (TdR) (60-70 Ci/mmol) was added in the media after adsorption. For pulse labeling, 2 μCi/ml of [^{3}H]TdR was added 30 min prior to harvesting the cells.

DNA Preparation

To measure [^{3}H]TdR incorporation, the cells were washed twice with cold Dulbecco's phosphate buffer, scraped into 1.0 ml of buffer, and stored at -80°C. Just prior to assay, the samples were thawed and sonicated for 20 sec at 60 W. From each sample, 100 μl was applied to a Whatman 3 mm filter disk, and trichloroacetic acid (TCA) precipitable radioactivity was measured as previously described (Stout and Arens, 1970).

To prepare DNA for gel analysis, the media was removed from each plate, and the cells were lysed by adding 0.2 ml of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.6% sodium dodecyl sulfate (Hirt, 1967). The lysate was scraped into a 1.5 ml centrifuge tube and proteinase K was added to a final concentration of 100 μg/ml. After 4 hr at 60°C, 200 μl of H_2O was added, and the DNA was extracted with

chloroform/isoamyl alcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in 100 µl of H_2O, heated to 60°C for 10 min, and stored at 4°C.

Agarose Gel Electrophoresis

DNA samples were adjusted to 10% formamide, 0.1% bromophenol blue and electrophoresed on submerged horizontal 1.4% agarose gels (8 mm thick) with 40 mM Tris-acetate (pH 8.3), 20 mM Na-acetate, and 2 mM EDTA as running buffer. Electrophoresis was for 15 hr at 50 V or until the dye front had migrated 10 cm. The gel was then stained with ethidium bromide (5 µg/ml) and exposed to short wave UV for 5 min. Alkaline gel electrophoresis was performed according to the method of Favaloro et. al. (1980).

Southern Blot Analysis

For blotting analysis, Gene Screen membranes (New England Nuclear, Boston, MA) were used and all procedures were according to the modification of the Southern method (Southern, 1975) published in the Gene Screen instruction manual. After denaturation and neutralization, gels were passively transferred for at least 24 hr.

Prehybridization and hybridization were performed for 24 hr each at 42°C in the presence of 50% formamide according to Method I in the Gene Screen instruction manual. All gels were probed with 3×10^6 dpm of nick-translated BPV DNA (sp. act. $2.5\text{-}3.7 \times 10^7$ dpm/µg).

After hybridization, the membranes were washed, dried at room temperature and exposed to Kodak SB-5 X-ray film with a Cronex Lightning-Plus intensifying screen at -80°C. Calculations based on the specific activity of the probe and the number of cells electrophoresed per gel lane indicate that, with this technique, I should be able to detect, at the minimum, 1 genome equivalent/cell.

Dot Blot Hybridization

Dot blot assays were performed using a Schleicher and Schuell 96-place microsample filtration manifold by a modification of the method suggested by the manufacturer. DNA samples were denatured in 50 mM Tris-HCl (pH 7.4), 0.2 N NaOH, and 0.6 M NaCl for 10 min at 80°C. The samples were neutralized by adding 0.1 volume of 2 M Tris-HCl (pH 7.4) and quick chilling on ice. Serial ten-fold dilutions of each sample were made in 25 mM $NaPO_4$ (pH 6.5). The samples were applied onto a sheet of Gene Screen which had been presoaked in 25 mM $NaPO_4$ (pH 6.5). After air drying, the membrane was heated for 2 hr at 80°C and hybridized and exposed as above. Purified BPV DNA was used as a standard.

RESULTS

Aphidicolin Inhibition of DNA Synthesis

DNA synthesis in BPV-infected and uninfected cells was measured by [^{3}H]TdR incorporation into acid precipitable material after release from synchronization and in the presence or absence of 20 μM aphidicolin (Fig. 1). Without aphidicolin, the peak of [^{3}H]TdR incorporation was at 4 hr post release for both infected and uninfected cells and, in both cases, S phase was over by 10 hr post-release. When aphidicolin was added after adsorption of the virus at the time of release from synchronization, incorporation was less than 3% of that in uninhibited cells.

The effect of aphidicolin on viral specific DNA synthesis was demonstrated using Southern blot analysis. As shown in Fig. 2, during a normal infection cycle, viral input DNA (Fig. 2A, lane 1) was converted to parental RF by 8-10 hr pi (Fig. 2A, lane 4). Dimer RF molecules (Fig. 2A) could be visualized at the time of conversion to parental RF, but were not seen consistently at multiplicities of infection lower than used here (data not shown). In addition, a band smaller than viral ssDNA of approximately 4.5 kb appeared at this time (Fig. 2A).

The second step in the replication process is postulated to be conversion of parental to daughter RF molecules. This was monitored as an increase in intensity of the monomer dsDNA band up to 26 hr pi when progeny ssDNA was first detected (Fig. 2A and 2B, lanes 4-10).

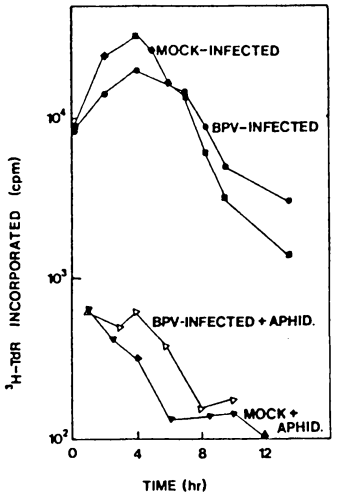

FIG. 1. DNA synthesis in mock-infected or BPV-infected BFL cells, with or without aphidicolin treatment. Synchronized BFL cells were released, infected and incubated in the presence or absence of 20 µM (7 µg/ml) aphidicolin. [^{3}H]TdR was added 30 min prior to harvesting of the cells and TCA precipitable radioactivity was measured. Symbols: No aphidicolin: , mock-infected; , BPV-infected: 20 µM aphidicolin: , mock-infected; , BPV-infected.

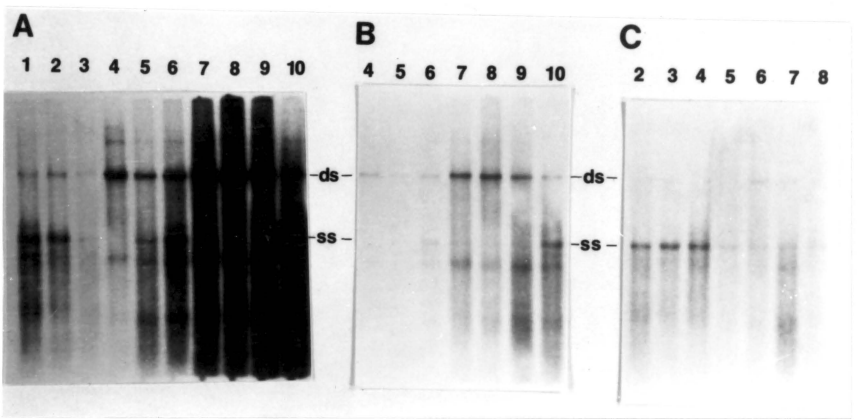

FIG. 2. Autoradiograms of blot hybridizations of a normal or aphidicolin inhibited infection. Synchronized BFL cells were released, and infected in the presence or absence of 20 μM aphidicolin. Samples were collected at various times pi by the modified Hirt procedure and electrophoresed on 1.4% agarose gels. Samples were then transferred to Gene Screen and probed with 3 x 10^6 dpm of nick-translated ^{32}P-labeled BPV DNA (sp. act. 2.5-3.7 x 10^7 dpm/μg). (A). Autoradiogram of the normal infection, 24 hr exposure. (B). Same as (A) except a 2 hr exposure. (C). Same as (A) except 20 μM aphidicolin was present throughout the infection cycle. Lane 1 was harvested at 1 hr pi and represents input BPV DNA. Lanes 2-10 represent samples which were harvested at the times indicated: lane 2, 4 hr pi; lane 3, 7 hr pi; lane 4, 10 hr pi; lane 5, 12 hr pi; lane 6, 14 hr pi; lane 7, 16 hr pi, lane 8, 18 hr pi; lane 9, 21 hr pi; lane 10, 26 hr pi. The positions of BPV ssDNA (ss) and dsDNA (ds) are labeled as well as the position of dimer DNA ( ) and the 4.5 kb DNA band (>).

Although the dimer DNA band never increased in intensity over that seen early in infection, other viral specific DNA of all sizes was greatly increased, as indicated by the dark smear in Fig. 2A, lanes 7-10. Since some of these sizes were greater than dsDNA, it is unlikely that they are products of nucleolytic degradation. In addition, similar DNA species have been observed for other parvoviruses.

When 20 μM aphidicolin was included, the entire replication process was greatly inhibited. Single-stranded DNA was detected through 18 hr pi (Fig. 2C, lanes 2-8), and detectable dsDNA did not increase over input (compare Fig. 2A, lane 1 with 2C, lanes 2-8). In other experiments, minor amounts of dsDNA could be detected late in infection (22 hr pi or later) (data not shown). However, the total amount of inhibition by aphidicolin was 97% or more as determined by [^{3}H]TdR incorporation (Fig. 1).

It is well established that parvoviruses require a late S phase function for DNA synthesis (Parris and Bates, 1976; Rhode, 1973; Siegl and Gautschi, 1973; Tattersall, 1972; Tennant and Hand, 1970; Wolter et al., 1980). Therefore, in order to insure that I were observing inhibition of DNA pol α activity and not indirect inhibition of other factor(s), aphidicolin was added at 1, 3.5, 6, and 8 hr pi. This enabled us to specifically inhibit viral DNA synthesis, which began at 8 hr pi (Fig. 2A), while allowing cell DNA synthesis to occur normally. As shown in Fig. 3, viral DNA synthesis was completely inhibited for up to 37 hr pi when aphidicolin was added as late as 6 hr pi (Fig. 3,

lanes 1-11). In all cases, ssDNA was visible until 26 hr pi but could not be detected by 37 hr pi. When aphidicolin was added 8 hr pi, only minor amounts of dsDNA were detectable (Fig. 3, lanes 12-15). Comparison of autoradiograms of different exposure times suggested that this amount was less than 2% of that seen during the normal infection.

Analysis of the Conversion of Input DNA

Once viral DNA synthesis begins, daughter RF molecules and other replicative forms accumulate rapidly masking the fate of input DNA when observed on a probed blot (Fig. 2A). To circumvent this problem, cells can be infected with ^{32}P-labeled virus. However, the low specific activity of the ^{32}P-labeled input DNA and the high particle/infectivity ratio of the parvoviruses (300-400:1) make it difficult to follow, conclusively, the fate of the infecting genome (Ward and Dadachanji, 1978). To overcome both problems, cells were infected with ^{32}P-labeled virus (10^5 cpm/µg: 300,000 cpm/plate) and the DNA was isolated at various times pi, electrophoresed on 1.4% agarose gels, blotted, and exposed for autoradiography before and after probing. For a direct comparison between aphidicolin treated and normal infections, corresponding samples were analyzed on the same gel. On the unprobed blot, a dsDNA species was detectable throughout the normal viral infection cycle (Fig. 4A). By 12 hr pi, ssDNA was no longer seen (Fig. 4A, lanes 3-6). By observing the corresponding lanes on the probed blot (Fig. 4C, lanes 2-6), it was obvious that the

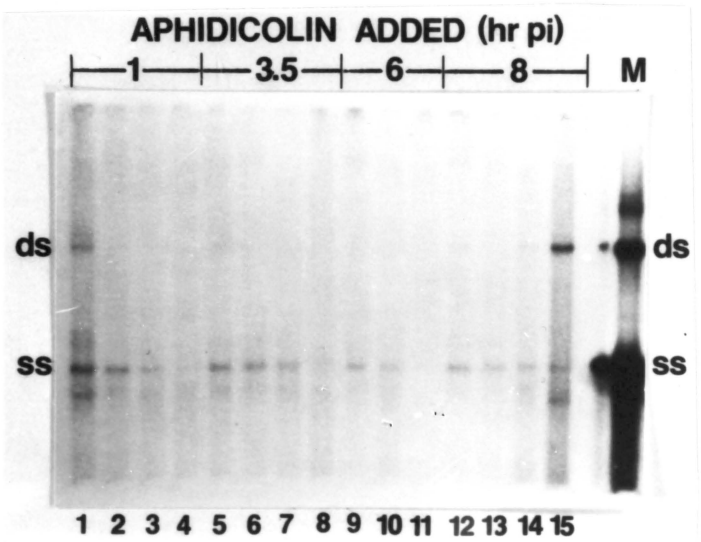

FIG. 3. Autoradiogram demonstrating the effect of adding aphidicolin at various times post release. BFL cells were infected, the DNA samples prepared and the gel blotted and probed as in Fig. 2. Aphidicolin (20 μM) was added at 1 hr pi (lanes 1-4), 3.5 hr pi (lanes 5-8), 6 hr pi (lanes 9-11), or 8 hr pi (lanes 12-15). At each time of addition of aphidicolin, a sample was harvested: lane 1, 1 hr pi; lane 5, 3.5 hr pi; lane 12, 8 hr pi. Other samples were harvested at 14 hr pi (lanes 2, 6, 8, and 13), 26 hr pi (lanes 3, 7, 10 and 14) or 37 hr pi (lanes 4, 8, 11, and 15). Lane M is marker BPV single-stranded (ss) and double-stranded (ds) DNA.

conversion of ssDNA to RF had occurred. When aphidicolin was added, ssDNA was visible throughout the infection cycle on the unprobed blot (Fig. 4B). When the blot was probed (Fig. 4D), ssDNA was still visible, and the intensities of the DNA bands were not increased (compare 4B and 4D).

Effect of Varying Concentrations of Aphidicolin on DNA Synthesis

To determine the effect of varying concentrations of aphidicolin on DNA synthesis, cells were released from synchronization, infected with BPV or mock-infected and incubated in the presence of [^{3}H]TdR and either 0, 0.1, 1.0, 20, 100, or 1000 µM aphidicolin. The cells were harvested at various times pi (depending on the experiment) and TCA precipitable radioactivity was determined. Continuous exposure to 20 µM aphidicolin and [^{3}H]TdR resulted in an inhibition of [^{3}H]TdR incorporation of at least 80% with less than 2% of the normal activity remaining when 100-1000 µM aphidicolin was added (Fig. 5).

The total amount of viral-specific DNA synthesized in the experiment shown in Fig. 5 was measured by a dot-blot assay (Fig. 6A). Compared to a normal infection, there was a 10 fold decrease in viral specific DNA when 0.1 µM aphidicolin was present and a 100 fold decrease when 1.0 µM was used. This highest level of inhibition (i.e., 100 fold) was also seen in samples where 20, 100, or 1000 µM aphidicolin had been added.

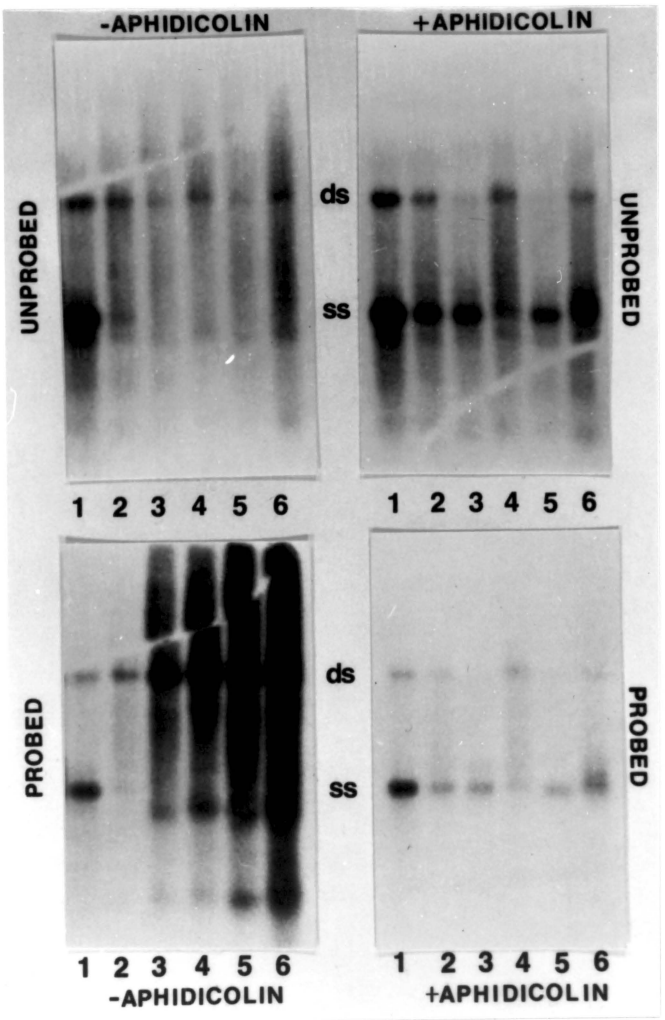

FIG. 4. Autoradiograms of a normal or aphidicolin inhibited infection of ^{32}P-labeled BPV before and after probing. ^{32}P-labeled BPV (300,000 cpm/plate) was used to infect synchronized BFL cells. Samples from the normal and aphidicolin treated infection were electrophoresed on the same gel, blotted onto Gene Screen and exposed before and after probing with nick-translated ^{32}P-labeled BPV DNA as in Fig. 2. (A) and (B). Autoradiograms of the unprobed blot showing samples from the normal (A) and aphidicolin treated (B) infections, 2 day exposure. (C) and (D). Probed autoradiograms of (A) and (B), respectively, 8 hr exposure. Samples were harvested as follows: lane 1, 4 hr pi; lane 2, 8 hr pi; lane 3, 12 hr pi; lane 4, 15 hr pi; lane 5, 18 hr pi; lane 6, 21 hr pi.

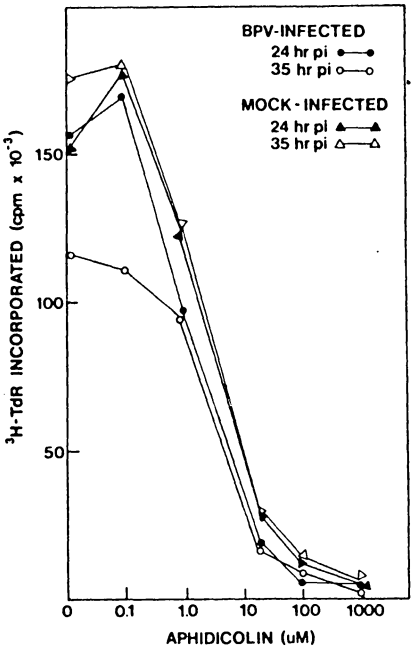

FIG. 5. Effect of varying concentrations of aphidicolin on total DNA synthesis. At release from synchronization, BFL cells were BPV- or mock- infected, incubated with [^{3}H]TdR (2 µCi/ml), and aphidicolin at final concentrations of 0, 0.1, 1.0, 20, 100, or 1000 µM. Cells were harvested at either 24 or 35 hr pi, and TCA precipitable radioactivity was measured. Symbols: , BPV-infected, 24 hr pi; BPV-infected, 35 hr pi; , mock-infected, 24 hr pi; mock-infected, 35 hr pi.

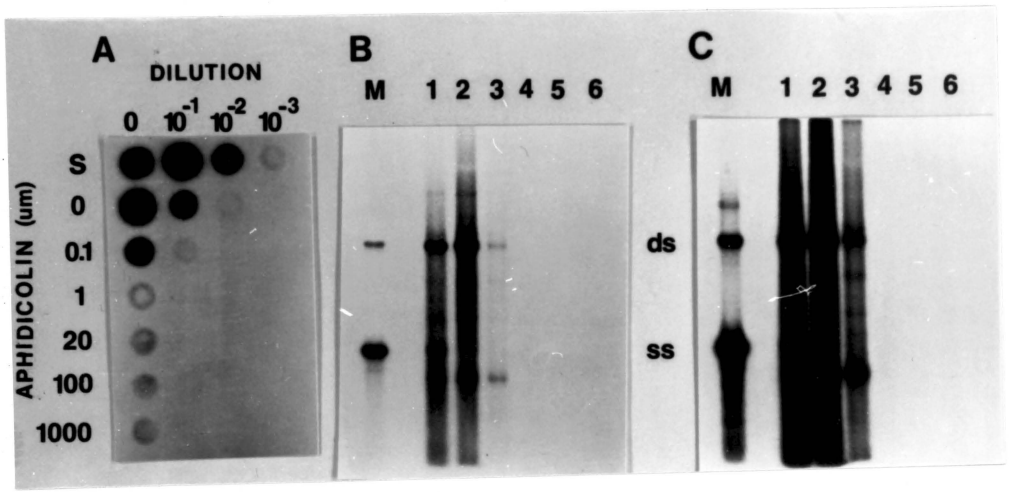

FIG. 6. Analysis of the effect of varying concentrations of aphidicolin on BPV DNA synthesis. BPV-infected BFL cells, which had been incubated in the presence of varying concentrations of aphidicolin were harvested at 35 hr pi and DNA samples were processed as described in Fig. 2. (A). Autoradiogram of the dot-blot hybridization of serial ten-fold dilutions of samples which had been exposed to the concentrations of aphidicolin indicated. Undiluted samples consisted of the DNA from approximately 250,000 infected cells. BPV DNA (0.5 µg) was used as a standard. (B) and (C). Autoradiograms of the blots of 1.4% agarose gels of samples which had been incubated in the presence of aphidicolin at a final concentration of 0 µM (lane 1), 0.01 µM (lane 2), 0.1 µM (lane 3), 1.0 µM (lane 4), 20 µM (lane 5) or 100 µM (lane 6). (B). 3 hr exposure. (C). 5 day exposure. The position of dimer DNA (d) is noted and the arrow (→) indicates the 4.5 kb DNA band.

I assumed that the residual viral-specific DNA seen at high concentrations of aphidicolin (Fig. 6A) represented input BPV DNA. To verify this, samples were electrophoresed on 1.4% agarose gels, blotted and probed (Fig. 6B and 6C). With 1.0 μM or greater aphidicolin, there was no evidence that viral DNA replication had occurred (Fig. 6B and 6C, lanes 4-6). Even when the autoradiograms were exposed for 2 weeks, no BPV specific DNA other than input DNA was seen (data not shown). However, when 0.01 μM or 0.1 μM aphidicolin was added, viral DNA replication did occur (Fig. 6B and 6C, lanes 2 and 3). In this case, the major viral DNA bands seen were dsDNA and the 4.5 kb band, while BPV ssDNA was not visible at 35 hr pi.

DISCUSSION

In this report, I have focused on the ability of aphidicolin to inhibit BPV DNA replication. At concentrations of 20 μM or greater, aphidicolin inhibited the production of viral RF DNA indicating that DNA pol α activity is required for BPV DNA replication in vivo.

The conversion of parvoviral DNA to RF has been reported to require a cellular function which is not available (Spalholz and Tattersall, 1983) and/or expressed until mid to late S phase (Wolter et al., 1980). In our experiments, BPV ssDNA was not converted to RF until 8-10 hr pi, or 4-6 hr after the peak of cellular S phase. During a normal infection, ssDNA remaining after this point was, apparently,

degraded. This is consistent with the results of others (Ward and Dadachanji, 1978; Wolter et al., 1980) who showed that input MVM ssDNA was not converted to parental RF until mid-S phase. However, when aphidicolin was present, ssDNA was detectable until as late as 26 hr pi and there was no visible increase in the amount of dsDNA. Even when cellular S phase was allowed to proceed, by delaying the addition of aphidicolin until 6 hr pi, synthesis of RF still was not seen. If the addition of aphidicolin was delayed to 8 hr pi, only in some experiments were small amounts of dsDNA observed. These probably are ssDNA genomes that were converted to RF just before the addition of aphidicolin. These results indicate that the ability of aphidicolin to inhibit RF production is due to a direct effect on DNA pol α and not an indirect inhibition of another S phase function(s).

Aphidicolin completely inhibited BPV DNA replication at concentrations down to 1.0 μM. Thus, BPV RF synthesis is at least as sensitive to aphidicolin in vivo as is cellular DNA synthesis. This is unlike results obtained with adenovirus (Kwant and van der Vliet, 1980) and previous results obtained, in vitro, with H1 parvovirus (Kollek et al., 1982) where viral DNA synthesis was less sensitive to aphidicolin than was cellular DNA replication. The disparity between our results and those obtained with H1 probably reflect differences in both the stages of replication being examined and the assay systems being used (in vitro vs. in vivo). When I used H1 or Lu III parvovirus in our standard in vivo experiment (ie. 20 μM aphidicolin), complete inhibition of viral DNA synthesis was observed (data not shown).

At concentrations of aphidicolin less than 1.0 μM (0.01 and 0.1 μM) BPV replication was reduced. By gel analysis, the major BPV specific DNA forms were unit length RF and a band of approximately 4.5 kb. This 4.5 kb band consistently appeared at the time of conversion of the ssDNA genome to RF. Although it may or may not represent defective genomes, it seems to be a normal byproduct of the replication cycle.

To monitor the fate of ^{32}P-labeled BPV input DNA, and to control for the high particle/infectivity ratio of parvoviruses, autoradiograms were made from blots both before and after probing. Before probing the blot containing DNA from the normal infection, I were unable to detect the increase in RF due to the high background levels of dsDNA which had resulted from reannealing of genomic plus and minus strands. However, after probing, the visible increase in BPV specific DNA indicated that replication had occurred. On the blot of the aphidicolin treated samples, the presence of ssDNA throughout the infection cycle and the inability of the probe to identify any new BPV specific bands, or intensify bands already present, demonstrated that replication had not occurred.

By these methods, I could detect as few as 1 genome equivalent/cell and therefore should have been able to detect conversion of the ssDNA genome to the parental RF. However, it is possible that in the presence of aphidicolin, low levels of RF synthesis could have been masked by input dsDNA. I used alkaline gel electrophoresis in an attempt to circumvent this problem. But, since parental RF was nicked

as soon as it was produced (unpublished observation), alkaline gels could not be used to distinguish between parental RF and fully linear dsDNA until late in infection when there is an accumulation of hinged (hairpin) species. However, as opposed to a normal infection, alkaline gels of aphidicolin inhibited cells never displayed viral specific forms greater than unit-length. In addition, even when araC (an inhibitor of both DNA pol α and pol ɣ) was used in place of aphidicolin, I observed no difference in viral DNA synthesis over that seen when pol α activity alone was blocked. This data suggests that pol α activity is required for the first step of BPV DNA replication. However, these results do not rule out the possibility that both pol α and pol ɣ activities are necessary for the production of parental RF.

Previous studies using subcellular systems to study parvoviral DNA replication have focused on the in vitro polymerase requirements for later stages in the replication cycle (Kollek et al., 1982; Pritchard et al., 1981). Data obtained using partially purified (Faust and Rankin, 1982; Robertson et al., 1982) or purified enzyme preparations (Ikeda et al., 1980; Kollek and Goulian, 1981; Pritchard et al., 1983) have led to conflicting results concerning the polymerase requirements for the first step in parvoviral DNA replication. The data presented in this report clearly demonstrates a requirement for pol α in the production of BPV RF DNA in vivo and strongly suggests that this requirement for pol α occurs at the first step in the replication process. These data do not rule out a role for DNA pol ɣ in this step

nor do they exclude a role for pol α or pol γ in other steps of the replication cycle. I are currently undertaking experiments aimed at identifying the polymerase requirements for later stages in BPV DNA replication.

Chapter IV

ANALYSIS OF BOVINE PARVOVIRUS DNA REPLICATION IN VIVO USING APHIDICOLIN AND L-CANAVANINE

SUMMARY

The replication of the autonomous parvovirus, bovine parvovirus (BPV) has been studied in vivo. Neutral and alkaline gel electrophoresis were used to determine the effect of aphidicolin, a specific inhibitor of DNA polymerase alpha, and/or L-canavanine, an inhibitor of protein synthesis, on specific stages of viral DNA replication. Synchronized cell cultures were infected with ^{32}P-labeled or unlabeled BPV in the presence or absence of aphidicolin and/or L-canavanine. Cells were harvested at various times post-infection, and DNA was electrophoresed and blotted. When aphidicolin was added to cells at the time of infection, then removed 8 hr later, BPV RF synthesis began within 2 hr after the removal of aphidicolin. This preceeded the peak of cellular DNA synthesis by 2 hr, unlike an uninhibited infection, when viral RF synthesis follows the peak of S phase by 2-4 hr. Furthermore, if aphidicolin was added at any point during the replication cycle, BPV DNA synthesis stopped. This effect was shown to be completely reversible and indicated that aphidicolin did not disrupt the replication apparatus required for viral DNA synthesis. On the other hand, L-canavanine slowed the entire BPV replication cycle by inhibiting synthesis of the viral specific proteins NP-1 and

VP3 and thus permitting synchronization of the infection cycle prior to RI and progeny DNA synthesis. Upon removal of L-canavanine, RI DNA synthesis began unless aphidicolin was added. Thus aphidicolin specifically inhibited synthesis of daughter RF, RI, and progeny ssDNA. These results indicate that a specific S phase function other than cellular DNA synthesis, per se, is required for BPV RF replication, that DNA polymerase alpha plays a major role in BPV DNA replication in vivo, and that these inhibitors can be used to reversibly inhibit various stages of BPV replication.

INTRODUCTION

Parvoviruses possess a single-stranded DNA (ssDNA) genome with 3' and 5' terminal hairpin structures, replicate in the nucleus of host cells, and are divided into two groups (defective and nondefective) depending upon their requirement for a helper virus during viral DNA synthesis (reviewed in Berns and Hauswirth, 1982). For the autonomous parvoviruses, several models of DNA replication have been proposed (Astell et al., 1983a; Berns and Hauswirth, 1982; Rhode and Klaassen, 1982). The first step in the replication cycle, conversion of the ssDNA genome to a covalently closed (hairpin) replicative form (parental RF) has been studied (Rhode, 1974; Strauss et al., 1976; Ward and Dadachanji, 1978). The final two steps of the process, encapsidation of progeny genomes and virus particle maturation, have also been characterized (Muller and Siegl, 1983a; Muller and Siegl,

1983b; Myers and Carter, 1980). In addition, studies focused on the intermediate steps in the replication cycle have led to: 1) a definition of the viral DNA species present in infected cells (Hayward et al., 1978; Li et al., 1978; Rhode, 1974; Siegl and Gautschi, 1976; Tattersall et al., 1973), 2) localization of the initiation of RF → RF DNA synthesis to the right end of the RF molecule (Singer and Rhode, 1977a; Singer and Rhode, 1977b), 3) identification of a flip-flop sequence structure at the 5' termini of genomic DNA (Astell et al., 1983b; Rhode and Klaassen, 1982) and, 4) demonstration of a protein covalently bound to the 5' termini of RF DNA (Revie et al., 1979). However, definitive answers concerning the exact mechanism by which the intermediate stages of the replication cycle proceed have not been obtained due, in part, to the lack of suitable experimental systems.

I have developed a system by which each stage in the parvoviral DNA replication cycle can be specifically examined in vivo. I used bovine parvovirus (BPV) which possesses the largest genome of the autonomous parvoviruses, 5500 bases (Snyder et al., 1982). Using aphidicolin, a specific inhibitor of DNA polymerase alpha (pol α) (Huberman, 1981; Spadari et al., 1982) and the amino acid analog, L-canavanine, I can reversibly inhibit each step during BPV DNA replication, including the production of daughter RF, RI (replicative intermediate), and progeny genomes. I discuss the potential of this system in defining various aspects of the parvoviral replication cycle.

METHODS

Cell Culture, Synchronization, and Infection

Preparation of ^{32}P-labeled and unlabeled BPV virus stocks and growth and synchronization of bovine fetal lung (BFL) cell cultures were as previously described (Robertson et al., 1984b). Briefly, BFL cells were seeded into 60 mm petri plates in the presence of 2 mM hydroxyurea (HU). After 26-32 hr, cells were released from synchronization and infected with BPV at a multiplicity of 20 PFU/cell. Aphidicolin (20 µM) or L-canavanine (5 mM) were added to the cultures with the media after adsorption of the virus or at the times indicated in the individual experiment. For labeling of protein samples, [^{35}S]methionine (5 µCi/ml) was used as described (Lederman et al., 1983). To remove the inhibitors, cultures were washed twice with Dulbecco phosphate buffer and fresh media was added.

Sample Preparation

Infected cells were harvested at various times post-infection (pi) by washing the cultures twice with Dulbecco phosphate buffer. For DNA isolation, a modified Hirt procedure was used (Robertson et al., 1984b). Cells were lysed in 200 µl of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.6% SDS. The lysates were digested with proteinase K (100 µg/ml) for 8 hr at 60°C. The DNA was ethanol precipitated, resuspended in H_2O, heated to 60°C for 10 min, and stored at 4°C. For analysis of protein, cells were lysed with 200 µl Laemmli sample

buffer (Laemmli, 1970) containing 1 mM phenylmethylsulfonyl fluoride and 2.3 U/ml aprotinin. The samples were sonicated briefly to decrease their viscosity and stored at 4°C until ready for use.

Gel Electrophoresis

Polyacrylamide gel electrophoresis of the protein samples was carried out by the method of Laemmli (Laemmli, 1970). In some cases, 5-15% gradient gels were used with a 4% stacking gel. Gels were stained with Coomassie brilliant blue R (Lederman et al., 1983) and fluorographed using Enhance (New England Nuclear, Boston, MA) according to the manufacturers instructions.

DNA samples were analyzed on neutral or alkaline 1.4% agarose gels. For neutral gel electrophoresis, samples were adjusted to 10% formamide, 0.1% bromophenol blue and electrophoresed on submerged horizontal gels (8 mm thickness) with 40 mM Tris-acetate (pH 8.3), 20 mM Na-acetate, and 2 mM EDTA as running buffer. Electrophoresis was for 15 hr at 50 V or until the dye front had migrated 10 cm. Gels were stained with ethidium bromide (5 μg/ml) and exposed to UV. Alkaline gel electrophoresis was performed according to a modification of the method of Favaloro (1980). Vertical 1.4% agarose gels (40 x 20 x 0.25 cm) containing 50 mM NaCl and 2 mM EDTA (pH 8.0) were run in 50 mM NaOH and 2 mM EDTA electrophoresis buffer. The gels were prerun for 30 min at 80 V before loading the samples which had been adjusted to 50 mM NaOH, 2 mM EDTA, 2.5% Ficoll, and 0.1% bromocresol

green. Electrophoresis was for 18 hr at 80 V or until the dye front had traveled 18 cm. The buffer was recirculated throughout the run and changed after 12 hr. After electrophoresis, the gel was renatured in 3 changes of 25 mM $NaPO_4$ (pH 6.5) for 1 hr and stained with ethidium bromide as above.

Southern Blot Analysis

Southern blots of the agarose gels were made using Gene Screen membrane according to the modification of the Southern method (Southern, 1975) published in the Gene Screen instruction manual and described previously (Robertson et al., 1984b). Neutral gels were denatured, neutralized, and blotted for 24 hr. Ethidium bromide stained alkaline gels were passively transferred directly to the Gene Screen membrane for 12 hr. Prehybridization and hybridization were performed in the presence of 50% formamide as described (Robertson et al., 1984b). All gels were probed with 3 x 10^6 dpm of nick-translated BPV DNA (spec. act. 2.5-3.7 x 10^7 dpm/µg). All blots were exposed for autoradiography and those containing ^{32}P-labeled BPV input were exposed before and after probing. By using probe of this specific activity, and by electrophoresing approximately 250,000 infected cells per gel lane, I should detect, at the minimum, 1 genome equivalent/cell.

RESULTS

Aphidicolin synchronization of RF DNA synthesis

It has been shown that in synchronized cell cultures, parvoviral DNA replication does not begin until mid to late S phase after the peak of cellular DNA replication (Parris and Bates, 1976; Robertson et al., 1984b; Wolter et al., 1980). I have also shown that this stage of BPV DNA replication is sensitive to aphidicolin (Robertson et al., 1984b). To determine the effect of delaying cellular S phase on BPV DNA synthesis, synchronized BFL cells were infected with ^{32}P-labeled or unlabeled BPV in the presence of aphidicolin. After 8 hr, the cells were released from the aphidicolin block, and viral and cellular DNA synthesis were monitored. By analysis of [^{3}H]thymidine incorporation (data not shown), it was determined that the cells passed through a normal S phase with the peak of DNA synthesis occurring 4 hr after removal of aphidicolin (Robertson et al., 1984b).

BPV RF DNA synthesis was analyzed during this period. To specifically follow the fate of infecting genomes, ^{32}P-labeled input virus was used. On the unprobed blot of the neutral gel, BPV input ssDNA was converted to RF within 2 hr after the removal of aphidicolin or 2 hr before the peak of cellular DNA synthesis (Fig. 1A). This conversion was begun by 0.5 hr post-release and was completed between 1 and 2 hr post-release (data not shown). Analysis of the probed blot indicated that amplification of RF DNA had begun between 2 and 4 hr post-release (Fig. 1B).

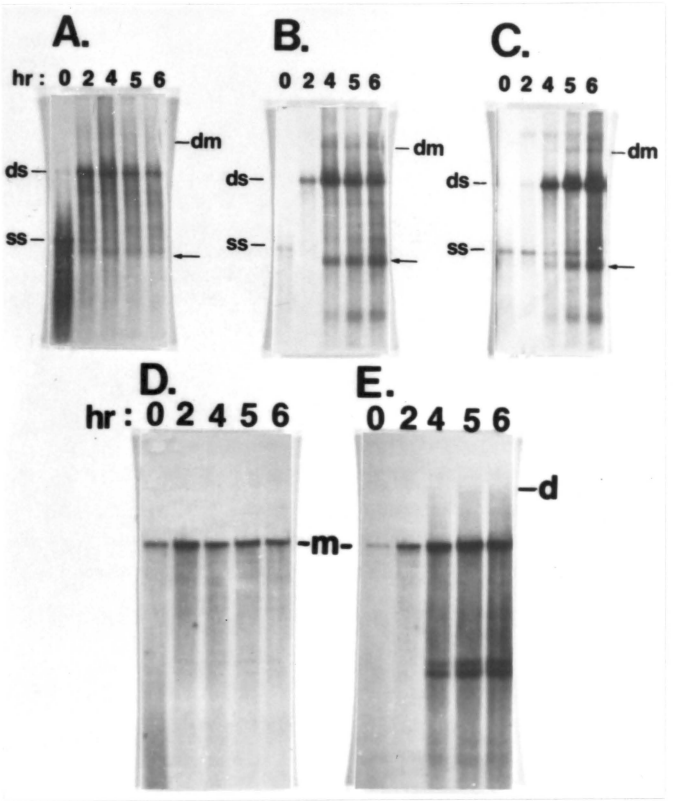

Fig. 1. Aphidicolin synchronization of RF production. Cells were released from HU and infected with ^{32}P-labeled BPV (A, B, D, E) or unlabeled virus (C) in the presence of 20 μM aphidicolin. After 8 hr, aphidicolin was removed and cells were harvested at the times indicated (post removal of aphidicolin). BPV DNA synthesis was monitored by neutral (A, B, C) and alkaline (D, E) gel electrophoresis. (A) Unprobed blot of the neutral gel using ^{32}P-labeled input virus, 48 hr exposure. (B) Panel A after probing with nick-translated BPV DNA, 4 hr exposure. (C) Probed blot of a BPV infection using unlabeled virus at 1-5 PFU/cell, 48 hr exposure. (D) Unprobed blot of the alkaline gel using ^{32}P-labeled input virus, 48 hr exposure. (E) Panel D after probing, 4 hr exposure. The positions of dsDNA (ds), ssDNA (ss), dimer-length RF (dm), the 4.5 kb band (→), monomer unit-length DNA (m), and the covalently closed hairpin duplex (d) are noted.

Aklaline gel analysis of this experiment revealed that, as seen on the unprobed blot (Fig. 1D), the major BPV DNA species present was of monomer unit-length. Thus, ^{32}P-labeled input virus was not immediately converted to a detectable, fully linear, covalently closed hairpin molecule (Fig. 1D). Furthermore, on the probed blot (Fig. 1E), species greater than monomer unit-length were not seen until 4 hr post-release or concomitantly with the appearance of daughter RF synthesis on neutral gels (Fig. 1B). This indicated that conversion to parental RF was closely linked to a nicking event (assuming, of course, that conversion to a covalently closed hairpin is the first step in the replication cycle).

Further analysis demonstrated that the time of conversion from ssDNA to RF was dependent upon the multiplicity of infection (Fig. 1C). If cells were infected with 1-5 PFU/cell, the time of conversion to RF was delayed to 4 hr post-release. This effect was not due to an inability to detect earlier conversion since, on a 48 hr exposure of the autoradiogram, viral ssDNA could be seen. Lower multiplicities of infection resulted in even later conversion times (data not shown). If aphidicolin was removed prior to 8 hr pi, the time of conversion (ie, 10 hr pi) was not altered. That is, RF synthesis never occurred earlier than 7-8 hr pi regardless of the presence or absence of aphidicolin during 0-8 hr pi. This data indicates that the completion of cellular DNA synthesis is not required for viral RF synthesis but some other S phase specific function is.

Inhibition of BPV DNA Replication by Aphidicolin

Cells were released from synchrony, infected with BPV, and aphidicolin was added at 1 and 4 hr pi, hourly from 6 to 14 hr pi, and at 16 and 18 hr pi. Cells were harvested at various times after the addition of aphidicolin and at 28 hr pi. After electrophoresis, gels were blotted, and the membranes were probed with nick-translated BPV DNA. A comparison was made between DNA species present at different times during the normal infection (Fig. 2A and 2B) and between DNA species present in aphidicolin treated samples (Fig. 2C). Early in the normal infection (Fig. 2A), background levels of dsDNA were detected. An increase in dsDNA was first seen at 10 hr pi (Fig. 2A). After aphidicolin was added, the amount of viral specific DNA did not increase (Fig. 2C) as it did during a normal infection (Fig. 2A and 2B). If aphidicolin was added at 8-10 hr pi, a slight decrease in the amount of dsDNA was seen over a period of time. This appeared to correspond to an increase in ssDNA and a 4.5 kb band. I believe this results from a nicking event rather than DNA synthesis. If aphidicolin was added before progeny DNA was seen (ie, up to 18 hr pi in this experiment), the production of progeny genomes was inhibited. These results indicate that if aphidicolin was added any time during the normal infection cycle, BPV DNA synthesis was blocked.

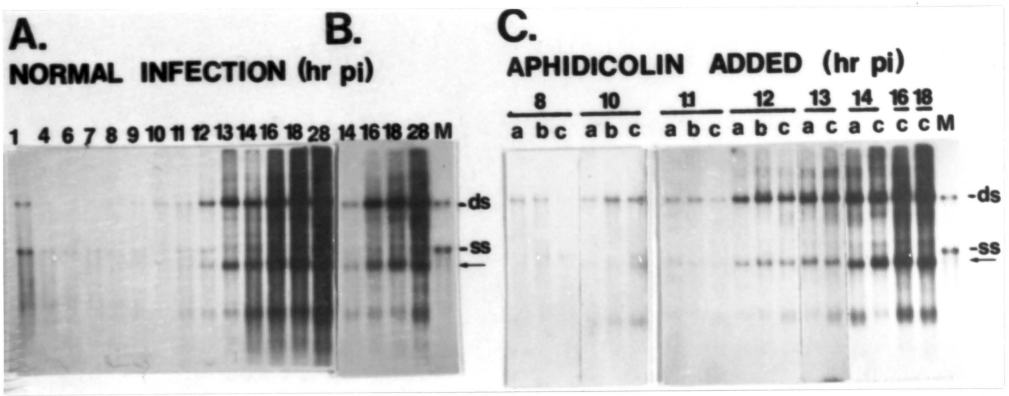

Fig. 2. Aphidicolin inhibition of BPV DNA synthesis. Synchronized BFL cells were released from HU, infected with BPV, and harvested at various times pi (A and B). (C) Aphidicolin was added at the hours pi indicated and samples were harvested at 4 hr post-addition of aphidicolin (a), 8 hr post-addition (b), and 28 hr pi (c). Each gel was exposed for autoradiography as follows: A, 12 hr; B, 6 hr; C, lanes 8a-10c, 15 hr and lanes 11a-M, 12 hr. The positions of dsDNA (ds) and ssDNA (ss) are indicated in the marker lane (M) and the position of the 4.5 kb band (→) is noted.

Reversibility of Aphidicolin Inhibition

To determine whether aphidicolin inhibition of BPV DNA replication was reversible, cells were released 4 hr after treatment with aphidicolin, harvested, and the DNA was electrophoresed, and blotted. If aphidicolin was added at 8, 10, or 12 hr pi, BPV DNA replication was inhibited (Fig. 3, compare lanes 8a with 8b and c, 10a with 10b and c, and 12a with 12b and c). If aphidicolin was removed after a 4 hr incubation, BPV DNA synthesis resumed (Fig. 3, lanes 8d, 10d, and 12d). Furthermore, in each lane where BPV DNA synthesis had been interrupted by aphidicolin, the only bands observed were those which were normally seen during an infection cycle. This indicated that, as far as could be determined by gel analysis, DNA replication was proceeding normally. It also demonstrated that addition and removal of aphidicolin could be used to stop and start BPV DNA synthesis throughout replication.

L-canavanine Inhibition of BPV DNA Synthesis

It has been shown for the defective parvovirus, adeno-associated virus (AAV), that L-canavanine inhibited progeny DNA synthesis and assembly of virus particles by inhibiting viral protein synthesis (Buller and Rose, 1978; Myers and Carter, 1980; Myers and Carter, 1981). I wanted to define the effect of L-canavanine on BPV DNA replication and the reversibility of its action to determine if L-canavanine could be used to synchronize the final stages of RF (RI) replication and progeny DNA synthesis.

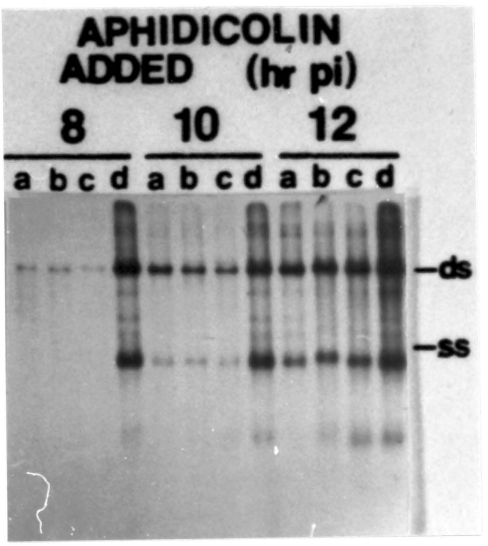

Fig. 3. Reversibility of aphidicolin inhibition. BFL cells were infected as described. At the times indicated pi, aphidicolin was added and a sample was harvested (a). Four hr after the addition of aphidicolin, one sample was released from the aphidicolin block (d) and another was harvested (b). Four hr later, both the released sample (d) and an unreleased sample (c) were harvested. The positions of BPV ssDNA (ss) and dsDNA (ds) are marked.

BFL cells were infected with BPV in the presence or absence of 5 mM L-canavanine. Comparison between a normal infection (Fig. 4, lanes 2, 4, 6, 8) and one in which L-canavanine was present (Fig. 4, lanes 1, 3, 5, 7) showed that L-canavanine slowed the entire replication process. At 16 hr pi (Fig. 4, lane 7), the major viral DNA species present in the inhibited sample was unit-length dsDNA (5.5 kbp). In most cases, a dimer-length RF band and a band (4.5 kb) smaller than unit-length ssDNA were visible at this point (Fig. 6A, lane 1). RI molecules, which are a heterogeneous class of molecules the size of ssDNA and larger (Singer and Rhode, 1977a; Singer and Rhode, 1977b), were not seen (Fig. 4, lane 7).

To determine if this effect was reversible, cells were infected and incubated with 5 mM L-canavanine for 15 hr. The cells were then released and samples were collected for protein and DNA analysis at 0, 0.25, 0.5, 1, and 2 hr post-release.

Analysis of the proteins present on Coomassie stained polyacrylamide gels (Fig. 5A) demonstrated that synthesis of the most abundant capsid protein, the 62 kd protein, VP3, was blocked by L-canavanine, as was synthesis of the smaller nonstructural 28 kd protein, NP-1 (Lederman et al., 1984). When [^{35}S]methionine was added at release from L-canavanine, label incorporated into NP-1 and VP3 was detectable by 30 min post-release and had increased by 1 hr post-release (Fig. 5B). These data indicate that L-canavanine inhibited synthesis of specific viral proteins (NP-1 and VP3) and that this inhibition was reversible.

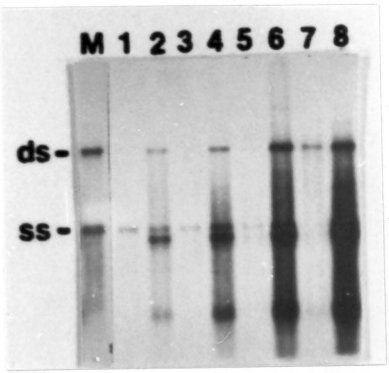

Fig. 4. L-canavanine inhibition of BPV DNA synthesis. Cells were released from HU and infected with BPV in the presence (lanes 1, 3, 5, 7) or absence (lanes 2, 4, 6, 8) of L-canavanine. Cells were harvested at 10 hr pi (lanes 1 and 2), 12 hr pi (lanes 3 and 4), 14 hr pi (lanes 5 and 6), and 16 hr pi (lanes 7 and 8). The marker (M) is BPV dsDNA (ds) and ssDNA (ss).

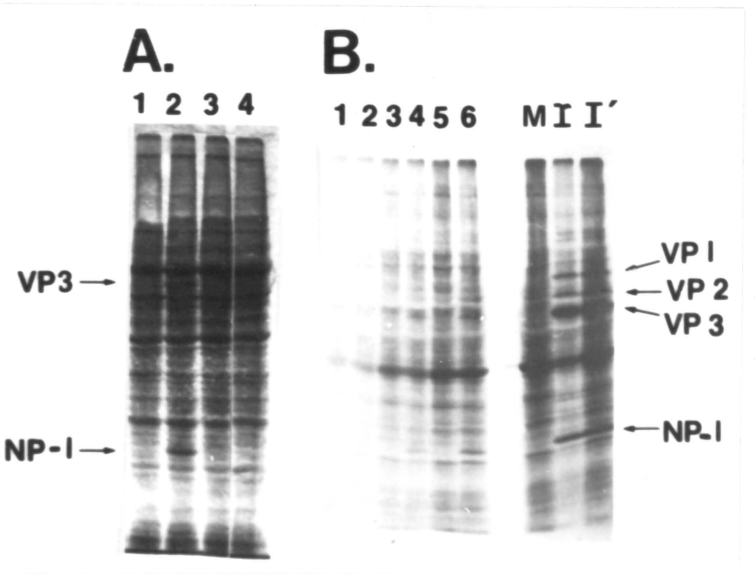

Fig. 5. Polyacrylamide gel analysis of viral proteins. (A) Cells, incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of L-canavanine, were harvested at 15 hr pi and the proteins were electrophoresed on a 10% polyacrylamide gel and stained with Coomassie brilliant blue R. Lanes 1 and 4, mock-infected; lanes 2 and 3, BPV-infected. (B) BPV- or mock- infected cells were released from the L-canavanine block at 15 hr pi and incubated in the presence of [^{35}S]methionine. Both mock-infected (lanes 1, 3, 5) and BPV-infected (lanes 2, 4, 6) samples were harvested at 15 min (lanes 1 and 2), 30 min (lanes 3 and 4), and 1 hr (lanes 5 and 6) post-release. Also shown are mock-infected (M) and BPV-infected (I and I') samples that had not been treated with L-canavanine but were pulsed with [^{35}S]methionine at 15 hr pi for 1 hr (lanes M and I) or 2 hr (lane I'). The positions of NP-1 (28 kd), VP3 (62 kd), VP2 (72 kd), and VP1 (80 kd) are noted.

To determine the effect of L-canavanine on DNA synthesis, neutral and alkaline gel analysis was used. Prior to release, the major DNA species present on the blot of the neutral agarose gel, were unit-length RF, dimer-length RF, and the 4.5 kb band (Fig. 6A, lane 1). None of these forms consisted of covalently linked viral and complementary strands since only monomer unit-length DNA was seen on the alkaline gel (Fig 6B, lane 1). After removal of L-canavanine, synthesis of RI DNA increased (Fig. 6A, lanes 2-5). On the alkaline gel (Fig. 6B), this was monitored as an increase in unit-length DNA molecules and an increase in BPV specific DNA ranging up to the size of, and including, the parental RF duplex (ie., covalently closed hairpin molecules) (Fig. 6B, lane 5). Hence, inhibition of BPV DNA synthesis by L-canavanine was reversible.

Aphidicolin inhibition of the final stages in BPV DNA replication

I have shown that I can interrupt BPV DNA replication in vivo at any step by adding aphidicolin and that L-canavanine will inhibit progeny DNA synthesis and the final stages of RF (RI) DNA replication. To observe the effects of aphidicolin on RI and progeny DNA synthesis, specifically, I synchronized the infection by incubating the cells in the presence of L-canavanine for 15 hr, then released the cells, and incubated them in the presence or absence of aphidicolin. As shown (Fig. 6C, lane 4) when the cells were released from L-canavanine, BPV DNA synthesis resumed. However, if aphidicolin

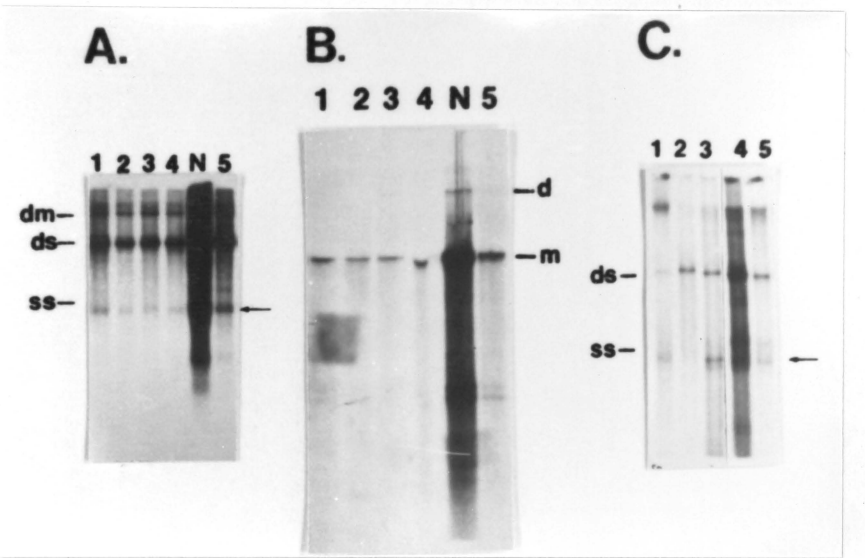

Fig. 6. Sensitivity of RI synthesis to L-canavanine and aphidicolin. BFL cells were infected with BPV in the presence of L-canavanine. (A) and (B) At 15 hr pi, samples were released from L-canavanine. Neutral (A) and alkaline (B) gel analysis of samples harvested at 0 (lane 1), 15 min (lane 2), 30 min (lane 3), 1 hr (lane 4) and 2 hr (lane 5) post-release. One sample (N) from a normal infection harvested at 17 hr pi is included for comparison. (C) To test for sensitivity of RI synthesis to aphidicolin, BPV-infected cells were incubated in the presence of L-canavanine. Samples were harvested at 16 hr pi (lane 1), 20 hr pi (lane 2), and 24 hr pi (lane 3). At 24 hr pi, cells were released from L-canavanine (lanes 4 and 5) and incubated for 4 hr in the presence (lane 5) or absence (lane 4) of aphidicolin. The positions of dimer-length RF (dm), dsDNA (ds), ssDNA (ss), the 4.5 kb band (→), covalently closed hairpin duplex (d), and monomer unit-length DNA (m) are noted.

was added, RI and progeny DNA synthesis were blocked (Fig. 6C, lane 5). This indicates that aphidicolin inhibited RI as well as progeny DNA synthesis.

DISCUSSION

I have defined the effect of aphidicolin and L-canavanine on BPV DNA replication in vivo. I have shown that daughter RF synthesis was interrupted by aphidicolin, that RI and progeny DNA synthesis were blocked by both L-canavanine and aphidicolin, and that both of these reactions were reversible. In addition, I demonstrated that rapid conversion of ssDNA to RF DNA occurs after removal of aphidicolin at 8 hr pi.

It has been shown previously that viral proteins were required for parvoviral progeny DNA synthesis (Myers and Carter, 1981; Rhode, 1976) and that L-canavanine inhibited ssDNA production during replication of the defective parvovirus, AAV (Myers and Carter, 1980; Myers and Carter, 1981). Like AAV, the nondefective parvovirus BPV possesses a nonstructural protein of comparable molecular weight (24,000 for AAV, 28,000 for BPV) which is found in high concentration in vivo and its synthesis is inhibited by L-canavanine. Also like AAV, synthesis of the major capsid protein was inhibited by L-canavanine as was progeny ssDNA production and encapsidation while cellular protein synthesis remains largely unaffected (Fig. 5; Fig. 6; Buller and Rose, 1978). For BPV, I demonstrated by gel analysis that L-canavanine

slowed the entire replication cycle, inhibited RF → RI and RI → ssDNA synthesis, and produced effects which were completely reversible. Others had shown that production of parvovirus progeny genomes was dependent on viral protein synthesis (Myers and Carter, 1981; Rhode, 1976; Tattersall et al., 1973). I conclude that viral proteins are required for BPV RI as well as for progeny DNA synthesis.

The effects of aphidicolin, like those of L-canavanine, are reversible (Fig. 3; Pedrali-Noy et al., 1981). Hence, aphidicolin does not disrupt the replication machinery responsible for viral DNA replication. In addition, if aphidicolin was removed at 8 hr pi, BPV RF synthesis occured prior to the peak of cellular DNA synthesis. This indicates that an S phase specific event, other than cellular DNA synthesis per se, is required for RF synthesis. Since this phenomena is multiplicity dependent and since BPV is known to associate with the nuclear matrix of infected cells (Briggs et al., manuscript in preparation), part of this S phase requirement may involve association with the matrix.

I also found that aphidicolin, when added to infected cells at various times pi, stopped BPV DNA replication at every stage. This included RF, RI, and progeny DNA synthesis. Since aphidicolin is a specific inhibitor of DNA pol α, these results suggest that pol α plays a major role in all aspects of BPV DNA replication in vivo. However, more detailed analysis is required before a conclusion can be drawn concerning the exact polymerase requirements for each specific step in the replication cycle.

Recently, Hardt and coworkers (1983) also presented evidence to support this conclusion. They used radiolabeling and gradient analysis to show that MVM DNA synthesis was sensitive to aphidicolin. In addition, in their system, MVM DNA synthesis occurred concomitantly with cellular DNA replication.

Use of both L-canavanine and aphidicolin has allowed us to specifically observe RI DNA synthesis. By synchronizing BPV replication with L-canavanine, then releasing the cells and adding aphidicolin, I demonstrated that pol α was involved in RI production. It has been shown (Pritchard et al., 1981; Kollek et al., 1982) that, in vitro, pol α was involved in the displacement synthesis of parvoviral progeny DNA (ie, RF → RI → ssDNA). In addition, Goulian and coworkers (1982) had demonstrated that, in isolated nuclei, pol γ was involved in conversion of ssDNA back to an RF molecule. Our in vivo results corroborate the first conclusion and do not address the latter. That is, in these experiments, I did not focus on the conversion of ssDNA to RF during the late stages of replication.

This system has allowed us to examine specific stages in BPV DNA replication in vivo. Further uses of this system include identification of the polymerase and viral protein requirements for each stage in the replication cycle. In addition, incubation in the presence of aphidicolin should allow for separation of pol α activity from other enzymatic functions and would, thus, allow for identification of these functions and the role they play in parvoviral DNA replication.

DISCUSSION

In this dissertation, I have focused on identifying the cellular DNA polymerase(s) and viral proteins required for the replication of BPV DNA. A summary of these finding is presented in Table 1. It was demonstrated that partially purified DNA pol α replicated BPV DNA to a dsDNA molecule 1.0 kb less than unit-length, that purified pol α was incapable of replicating the ssDNA template and that purified pol α with associated cofactors, C_1C_2, replicated BPV DNA to a unit-length dsDNA molecule. It was also demonstrated that purified pol γ was able to replicate BPV DNA from virions or separated ssDNA to a covalently linked dsDNA hairpin molecule which was identical to the product of pol I (Klenow) replicated BPV DNA. Pol γ was not capable of utilizing dsDNA as a template. Furthermore, it was shown that, in vivo, DNA pol α was required for RF DNA synthesis and that this requirement, most likely, occured at the first step in the replication cycle. In vivo results obtained using aphidicolin and L-canavanine indicated that pol α was involved in most stages of BPV DNA replication, including RF → RF synthesis and the production of RI and progeny genomes. It was shown that the initial steps in the replication process did not require viral protein synthesis or cellular DNA replication, per se, but did require some S phase function. Finally, the late stages in DNA replication, RI and progeny ssDNA production, required viral protein synthesis and were inhibited by aphidicolin. Thus they, too, required pol α activity.

TABLE 1. ROLE OF DNA POLYMERASES IN PARVOVIRAL DNA REPLICATION

Stage	Description	DNA Polymerase
I.	Conversion of virion single-stranded DNA to double-stranded replicative form	alpha (gamma ?)
II.	Conversion of parental double-stranded replicative form to replicative intermediates and daughter replicative form.	alpha
III.	Synthesis of progeny single-stranded DNA from replicative intermediates	alpha (*)

* denotes requirement for viral proteins

Throughout the course of this work, other, basic facts concerning BPV DNA replication have been identified. In essence, I have verified that earlier claims made for the other parvoviruses are true for BPV by using the more sensitive gel analysis and blotting procudures. First, as is consistent with the data for MVM (Wolter et al., 1980) BPV replication did not begin until 8 hr after the beginning of S phase and the first step was the cell cycle dependent conversion to a dsDNA molecule. Using alkaline gel electrophoresis, a covalently linked hairpin form was not present early in the replication cycle. Later during replication (i.e., 14 hr pi) these were seen. Using other, less sensitive methods, others (Hayward et al., 1978; Rhode, 1974; Siegl and Gautschi, 1978; Strauss et al., 1976; Ward and Dadachanji, 1978) had also found covalently linked dsDNA hairpin molecules in vivo. Ward and Dadachanji, using parasynchronous cell cultures and hydroxylapatite chromatography, demonstrated that by 8 hr pi, 5% of the total input DNA was double-stranded and, of this, 80% was in a "snap-back" configuration. That is, 4% of the input ^{32}P-labeled DNA was in a covalently closed hairpin form. Using these methods (ie., probing blots of alkaline gels of ^{32}P-labeled input virus) I should have been able to detect this percentage. Therefore, BPV appears to be different from MVM and this difference may be due to increased nucleolytic activity in our system.

Dimer DNA molecules were visualized shortly after/or concomitantly with the appearance of parental RF. At the time of dimer

DNA synthesis, a band of approximately 4.5 kb always appeared. This 4.5 kb band increased in concentration throughout the infection cycle. It is not known what portions of the genome this represents, whether or not it is encapsidated and whether or not it represents defective genomes. It is larger than the genomes of defective interfering (DI) particles previously described for MVM (Faust et al., 1979) which averaged 2.0 kb in size. However, these molecules are comparable in size to the DI particles described for H1 (Rhode, 1978) which were the same size as the H1 genome or 10% smaller. DI particles from both viruses were generated by propagation at high multiplicities of infection, conditions comparable to those used in these experiments with BPV. Therefore, it is very possible that the 4.5 kb band represents defective genomes, however, these molecules most definitely are a byproduct of the normal replication cycle.

By 12-14 hr pi (during a "good" infection) RI synthesis had begun. This synthesis, and the production of progeny ssDNA were dependent on viral protein synthesis. Specifically, if NP-1 and VP3 were blocked, RI synthesis did not occur although RF synthesis did. This particular point has not been shown for other parvoviruses since this is the first comprehensive study using these more sensitive techniques and previous studies were unable to visualize RI DNA synthesis specifically. Furthermore, these studies indicate that NP-1 is involved in the later stages of the replication cycle. As had been demonstrated for the other parvoviruses (Muller and Siegl, 1983a and

1983b; Myers and Carter, 1981; Rhode, 1976) capsid proteins are required for ssDNA synthesis.

Concerning the polymerase requirements for each step in the process, it should be noted that none of the in vivo data presented here indicates that pol γ is not involved, but only that pol α is required. Since pol γ readily replicates BPV DNA in vitro (Kollek and Goulian, 1981; Robertson et al., 1983), and since it does so preferentially in the presence of pol α (Faust et al., 1983; unpublished data), a role for pol γ in replication cannot be ruled out. It may function during the late stages of the cycle by converting newly synthesized ssDNA molecules back to RF, as was shown to occur in isolated nuclei by Goulian and coworkers (Kollek et al., 1982). Furthermore, it may function at the first step in BPV DNA replication, and this activity was masked in our system by the presnce of contaminating complementary strands (see Chapter 3). Whatever the case, I have demonstrated that aphidicolin inhibited each phase in BPV DNA replication but I recognize that a more thorough investigation is needed before it can be concluded that pol α is responsible for each step. This question can be answered by using the methods developed in the last chapter.

The methods described in chapter 4 can also be used to separate polymerizing activity from other enzymatic functions in vivo and/or in vitro. Thus, the identificaion of these activities (ie., nicking activities, etc.), the steps at which they function, and the identity of the genes from which they are produced could be described.

Finally, by comparing the in vitro data in chapter 1 and the in vivo data in chapters 3 and 4, it can be concluded that, although pol α may be involved in every stage of BPV DNA replication in vivo, it is not solely the activity of the core enzyme which is required. Instead, cofactors and other enzymatic functions are needed to form the functional pol α holoenzyme complex. In vitro data from other laboratories corroborates this conclusion. Hubscher et al (1982) have purified separately a core α enzyme and a holoenzyme. The holoenzyme preparation, but not the core enzyme, was able to replicate MVM DNA. At this point, their holoenzyme preparation contained numerous protein species and, therefore, they could not identify the exact factors which were responsible for the ability of this enzyme to replicate the MVM DNA molecule. Evidence from chapter 1 and other laboratories (Pritchard and DePamphilis, 1983; Pritchard et al., 1983) indicates that these factors may be the cofactors, C_1 and C_2. In the case of parvoviral DNA replication in vivo, the possibility also exists that there is a viral-coded protein which functions at the polymerase level, either as a cofactor or other enzymatic activity. Purification of pol α complexes from infected cells and identification of the associated proteins would help to answer this question.

Overall, I can conclude that cellular factors alone appear to be responsible for the first stages in BPV DNA replication, that pol α is involved, in one form or another, in every stage of BPV DNA replication in vivo, and that the steps occurring after daughter RF

synthesis are the most likely candidates for steps in which viral proteins (especially noncapsid viral proteins) are involved.

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