Bovine Parvovirus DNA-binding Proteins: Identification by a Combined DNA Hybridization and Immunodetection Assay

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SUMMARY

We have investigated the interaction between bovine parvovirus (BPV) capsid and non-capsid proteins and restriction fragments of the BPV genome by a combined DNA hybridization and immunodetection assay. $^{32}$P-labelleled DNA was bound to nitrocellulose membranes bearing lysates of mock-infected and virus-infected cells whose proteins had been separated by SDS–polyacrylamide gel electrophoresis. The position of bound DNA was determined by autoradiography. The proteins on the membrane were still accessible to specific antibodies, allowing confirmation of the DNA-binding species by an immunodetection reaction. In 0-2 M-NaCl, BPV capsid proteins VP2 (72000 daltons) and VP3 (62000 daltons) bound the 0 to 16 map unit EcoRI fragment of BPV DNA which contained label in either the minus or plus strand. At higher salt concentration (0·5 M), only VP2 still bound DNA. Within this fragment, the capsid protein binding was restricted to those nucleotides between map units 0 and 4. No binding to capsid proteins was seen with the fragment spanning the middle of the genome and minor binding to VP3 was seen with the 5' end. Binding to the BPV non-capsid protein NP-1 was observed with the 0 to 16 map unit fragment when label was in the virion strand and to other possibly BPV-coded proteins when label was in the plus strand. The NP-1 binding was localized to map units 4 to 16. We did not detect binding to the BPV homologue(s) of the autonomous parvovirus non-capsid protein NS1, due in part to its low concentration in the cell lysates used. Points of the parvovirus replication cycle at which DNA-binding proteins may serve controlling functions are discussed.

INTRODUCTION

The characterization of the life cycles of both autonomous and defective parvoviruses has shown that there are points at which DNA–protein interaction(s) may serve a regulatory function. Both structural and non-structural proteins may serve in this role. The structural and non-structural proteins of both classes of parvoviruses are coded by the same areas of their respective genomes. Capsid proteins are translated from overlapping mRNAs spanning the right half of the genome, generating proteins sharing major amino acid homology, while non-capsid proteins are encoded by the left half of the genome (Carter et al., 1984). Sequence analysis of the bovine parvovirus (BPV) genome shows that this virus conforms to the above scheme (Chen et al., 1986).

A requirement for capsid protein(s) in the synthesis of progeny single-stranded DNA has been demonstrated for adeno-associated virus (AAV) (Buller & Rose, 1978) and for BPV (Robertson et al., 1984) by inhibition of protein synthesis with L-canavanine. Deletion and insertion mutagenesis of the region of the AAV genome coding for capsid proteins (cap mutants) restricts synthesis of single-stranded DNA (Hermonat et al., 1984; Tratschin et al., 1984). Another level of control is the effect of a trans-activator on the promoter for parvovirus capsid proteins (Rhode, 1985). This activator maps to the left half of the genome and in the rodent parvovirus H-1 may be the 76000 dalton non-structural protein NS1 (Rhode & Paradiso, 1983;
Paradiso, 1984). A region of the genome coding for a trans-acting element has also been identified in AAV (Hermonat et al., 1984). Mutagenesis of the left half of the AAV genome results in a replication-deficient phenotype, showing that at least one AAV protein is required for viral DNA replication. These rep mutants complement cap mutants, showing a parallel with H-1 in the interaction of the genes coded by the left and right halves of autonomous and defective parvovirus genomes.

The data described above have been obtained either by biochemical or by genetic means. To complement these analyses, we investigated directly the binding of parvovirus DNA to parvovirus proteins by hybridization of 32P-labelled fragments of the BPV genome to the proteins of mock-infected and BPV-infected cells which had been separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Although this method has been used by others (Bowen et al., 1980; Hoch, 1982; Blair & Honess, 1983; Russell & Precious, 1982; White et al., 1985; Miskimins et al., 1985), it has not been widely applied in viral systems and never to parvovirus proteins. We have further extended the utility of DNA binding to proteins on membranes by the finding that the binding of DNA did not saturate the antigenic sites on the transferred proteins: their reaction with specific antibodies whose position could be visualized via an enzyme-coupled second antibody allowed confirmation of the BPV protein species binding DNA.

**METHODS**

**Cell culture and virus infection.** Bovine foetal lung cells were grown and infected with plaque-purified BPV as previously described (Parris & Bates, 1976).

**Labelling and purification of viral DNA.** Viral DNA was extracted from virions purified by one cycle of CsCl centrifugation (Lederman et al., 1983). After dialysis against 50 mM-Tris-HCl pH 8.0, the virus was treated with proteinase K (EM Reagents) (50 μg/ml) overnight at 55°C. The DNA was extracted with chloroform–phenol (1:1), ethanol-precipitated and centrifuged on a neutral 5 to 30% sucrose gradient in 0.7 M NaCl (Pritchard et al., 1981). The DNA fractions were analysed on a 1% agarose gel in TAE buffer (Maniatis et al., 1982) which contained 0.01% ethidium bromide. The fractions containing ssDNA were pooled and precipitated with ethanol. To prepare viral DNA in radioactive form, the medium was replaced 24 h after infection with phosphate-free medium containing 10% dialysed foetal calf serum and 5 μCi/ml 32P (ICN Pharmaceuticals).

In vitro synthesis of double-stranded DNA, restriction enzyme digestion and recovery of fragments. Viral DNA was replicated to the double-stranded form taking advantage of the priming activity of the 3’ terminal hairpin of the genome (Bourguignon et al., 1976). Virion DNA 32P-labelled in vitro was incubated in 50 mM-Tris-HCl pH 8.0, 50 mM-NaCl, 10 mM-MgCl2 with all four deoxyribonucleoside triphosphates (0.1 mM) and 0.5 units/ml of the exonuclease-free (Klenow) fragment of Escherichia coli DNA polymerase I (Boehringer) at 42°C for 3 h, deproteinized with proteinase K (50 μg/ml) at 60°C in the presence of 10 mM-EDTA. The specific activity of the virion DNA labelled in vivo was 105 to 2 × 106 c.p.m./μg. To label the complementary strand, non-radioactive virion DNA was replicated as described above except that the dCTP concentration in the reaction mixture was reduced to 0.02 mM and 5 μCi/ml [α-32P]dCTP (650 Ci/mmol, New England Nuclear) was added. Unincorporated nucleotides were removed using a spin-column of Sephadex G50-150 prior to proteinase K treatment. The final specific activity of material labelled in vitro was 107 to 2 × 107 c.p.m./μg.

Restriction endonuclease digestion of equal masses of both types of dsDNA was carried out following the directions specified by the suppliers (Bethesda Research Laboratories, IBI). The fragments were separated on an 11 × 14 cm 1% low melting point agarose gel (Sea-Plaque; FMC, Rockland, Me., U.S.A.) in sterilized TAE buffer containing ethidium bromide by electrophoresis in the cold either overnight at 30 V or for 4 h at 60 V depending on the size of the fragment to be recovered. The positions of the fragments were determined by u.v. transillumination, and those portions of the gel were excised and melted in 0.5 ml water either at 65°C for 5 min or in a boiling water-bath for 2 min. Hybridization buffer (see below) was added and the solution was used immediately for probing.

**DNA hybridization.** Nitrocellulose membrane (Millipore) containing electrophoretically transferred proteins were prehybridized overnight in buffer containing 10 mM-Tris–HCl pH 7.4, 1 mM-EDTA, 50 mM-NaCl, 1 mM-2-mercaptoethanol, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 50 μg/ml native calf thymus DNA and 50 μg/ml heat-denatured calf thymus DNA by shaking at room temperature in sealed bags. They were then hybridized with 32P-labelled fragments of the BPV genome in this buffer without calf thymus DNA for 1 h at room temperature. Duplicate strips which had been exposed to the same DNA fragment were washed either with the hybridization buffer brought to 0.2 M-NaCl or to 0.5 M-NaCl for 1 h with shaking at room temperature. After drying in air, the membranes were autoradiographed at ~80°C using Kodak XAR-5 film and...
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(a) 3' 5'

(b) ........ 5'

(c) PstI EcoRI EcoRI

Fig. 1. Diagram of the structure of various forms of BPV DNA. (a) Structure of virion DNA. (b) Structure of BPV DNA after replication with the Klenow fragment of E. coli DNA polymerase I. (c) Location of restriction endonuclease sites on the structure shown in (b).

PAGE and immunoblotting. Lysates of mock-infected and BPV-infected cells were prepared 18 to 24 h post-infection after two washes in a phosphate-buffered saline (Tamm, 1977). After centrifugation at 5000 g, the cell pellets were resuspended in gel application buffer (Laemmli, 1970) containing pyronin Y, sonicated and after boiling for 3 min protein from 2 x 10^4 cells was loaded per lane of 10% or 12% SDS-polyacrylamide gels. The separated proteins were transferred electrophoretically (Towbin et al., 1979) to nitrocellulose membranes (Millipore) which were stored if needed, in sterile water containing 0.01% thimerosal (Sigma) at 4 °C. After DNA hybridization and autoradiography, immunodetection of BPV proteins was done by the procedure of Binder et al. (1985). First antibody was either a 1/100 dilution of polyclonal antibody prepared in a rabbit against CsCl-purified BPV capsids or a 1/25 dilution of polyclonal antibody raised in a rabbit against the BPV non-capsid protein, NP-1, eluted from an SDS-polyacrylamide gel. Second antibody was Protein A coupled to horseradish peroxidase (Sigma), visualized with a 4-chloro-1-naphthol reagent obtained from and used according to the directions of Bio-Rad, in the presence of 0.0015% hydrogen peroxide. The position of the positive bands obtained with either specific antibody correlated with the position of the respective viral proteins in stained SDS-polyacrylamide gels.

RESULTS

Experimental design

The secondary structure of bovine parvovirus DNA contains non-identical, 75 and 60 base pair, double-stranded regions at the 3' and 5' ends, respectively, of a single-stranded genome (Fig. 1 a) (Chen et al., 1986). The first step in parvovirus replication is thought to be the synthesis of a complementary strand, covalently linked to the viral strand, using the foldback 3' end as a primer (Fig. 1 b). This step can be mimicked in vitro using purified virion DNA and the Klenow fragment of E. coli DNA polymerase I. If single-stranded DNA is isolated from virions labelled during infection with 32P, and DNA is replicated with unlabelled deoxyribonucleoside triphosphates, a double-stranded hairpin monomer with label in the viral strand is generated. Alternatively, non-radioactive single-stranded DNA can be replicated using an α-32P-labelled deoxyribonucleoside triphosphate as one of the substrates, yielding double-stranded DNA labelled in the complementary strand.

Both these DNAs can be cut with restriction endonucleases. The location of the EcoRI sites (at map units 16 and 92 on the 5500 base pair genome) (Fig. 1 c) (Burd et al., 1983) permits the isolation of two small fragments, each containing an end of the genome, and a large piece containing the middle 75% of the genome. The piece corresponding to the 3' end can be further cut with PstI to yield pieces covering map units 0 to 4 and 4 to 16. Depending on whether the DNA was labelled in vivo or in vitro, the fragments have label in either the viral or complementary strand.
Fig. 2. Binding of the 0 to 16 map unit EcoRI fragment of BPV DNA labelled with $^{32}$P in the virion strand to lysates of mock-infected and BPV-infected cells. (a) Autoradiogram of a strip probed with DNA and washed in 0.2 M-NaCl. (b) Immunoblot of this strip probed with anti-NP-1 antibody. (c) Autoradiogram of a strip probed with DNA and washed in 0.5 M-NaCl. (d) Immunoblot of this strip probed with anti-capsid antibody. Within each strip, proteins of BPV-infected cells are on the left and proteins of mock-infected cells are on the right. The positions of VP1, VP2 and VP3 are indicated and the position of NP-1 is indicated by arrows. The bands were aligned by superimposition of the film on the immunoblot.

It was also found that after binding of DNA to the nitrocellulose strips, the proteins on the membrane were still accessible to antibody. The position of bound antibody on the strip could be visualized by horseradish peroxidase-coupled Protein A in combination with 4-chloro-1-naphthol and hydrogen peroxide. Based on the known specificity of the antibodies used, a correlation can be made between the position of binding of radioactive DNA fragments and the location of BPV-specific proteins, leading to an identification of viral DNA-binding proteins.

**Preliminary studies**

On the basis of the protocols of Bowen *et al.* (1980) and Russell & Precious (1982), we optimized the conditions for binding of BPV DNA to lysates of mock-infected and BPV-infected cells on nitrocellulose membranes. The criteria were sharpness of the bands on the autoradiogram, specificity of binding and absence of binding to lysates of mock-infected cells. For washing of the blots, salt conditions were chosen to retain some bound DNA at the higher level and, at the lower level, to demonstrate binding at positions characteristic of BPV proteins without indiscriminate binding to many sites on the membrane. Conditions tested included comparison of binding of viral DNA labelled by nick translation rather than by replication, addition of different concentrations of heterologous DNA, both native and denatured, and of heparin, to the prehybridization buffer, and washing the blots in increasing concentrations of NaCl up to 1 M. As little as $10^4$ c.p.m. of DNA gave specific binding. The conditions selected are given in Methods.

A combination of native and heat-denatured calf thymus DNA was chosen as the blocking agent in the prehybridization step. The choice of calf thymus DNA raised the possibility that, since it is from the host organism of the virus under study, it might bind to proteins and prevent access of labelled BPV DNA. To control for this, membranes were prehybridized with salmon sperm DNA and then exposed to $^{32}$P-labelled nick-translated calf thymus DNA. No binding of calf thymus DNA was observed, indicating that it does not compete with binding of BPV DNA (data not shown).

**Binding of EcoRI fragments of the BPV genome**

When the 0 to 16 map unit fragment of BPV DNA labelled in the virion strand was used for binding and the strip was washed in 0.2 M-NaCl, bound radioactivity was observed at positions corresponding to the capsid proteins VP2 (72000 daltons), VP3 (62000 daltons) and the non-capsid protein NP-1 (28000 daltons) (Lederman *et al.*, 1983, 1984) (Fig. 2). For each experiment
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Fig. 3. Binding of the 16 to 92 map unit (a to d) and the 92 to 100 map unit (e to h) EcoRI fragments of the BPV genome labelled with 32P in the virion strand to lysates of mock-infected and BPV-infected cells. (a) Autoradiogram of a strip probed with the 16 to 92 map unit fragment and washed with 0.2 M-NaCl. (b) Immunoblot of this strip probed with anti-NP-1 antibody. (c) Autoradiogram of a strip probed with the 16 to 92 map unit fragment and washed with 0.5 M-NaCl. (d) Immunoblot of this strip probed with anti-capsid antibody. (e) Autoradiogram of a strip probed with the 92 to 100 map unit fragment and washed with 0.2 M-NaCl. (f) Immunoblot of this strip probed with anti-NP-1 antibody. (g) Autoradiogram of a strip probed with the 92 to 100 map unit fragment and washed with 0.5 M-NaCl. (h) Immunoblot of this strip probed with anti-capsid antibody. Binding to VP3 is indicated by an arrow.

shown in Fig. 2 to 6, the strips washed in 0.2 M-NaCl were probed with antibody to NP-1, whose location is marked with an arrow in Fig. 2. A duplicate strip washed in 0.5 M-NaCl showed bound radioactivity at the positions of VP2 and NP-1, although the intensity of the bands was decreased. Strips washed in 0.5 M-NaCl were probed with antibody to BPV capsids and the positions of VP1 (80000 daltons), VP2 and VP3 are indicated. These binding results were obtained when the DNA was released from the gel either by boiling and quick chilling or by heating the gel only sufficiently to melt the agarose.

When the two other EcoRI fragments of the BPV genome (map units 16 to 92 and 92 to 100), labelled in the virion strand, were used for binding after heating and quick chilling, no virus-specific binding was observed with the 16 to 92 map unit fragment, whereas a small amount of binding to VP3 was seen with the fragment covering the 5' terminus (Fig. 3). This binding was retained at 0.5 M-NaCl. No binding was observed with the undenatured form of the DNA (data not shown).

When the experiment was carried out with the 0 to 16 map unit EcoRI fragment of the BPV genome labelled in the complementary strand by replication in vitro and released from the gel by boiling, the same pattern of binding to the capsid proteins was observed as with DNA labelled in the virion strand: binding at the positions of VP2 and VP3 in 0.2 M-NaCl and binding at the position of VP2 in 0.5 M-NaCl (Fig. 4). However, no binding at the position of NP-1 was seen. Instead, binding at positions corresponding to approximately 30000, 35000 and 46000 daltons was seen after a 0.2 M-NaCl wash. This binding was abolished at the higher salt concentration. The same results were observed with DNA released from the gel by melting the agarose. No binding was observed with the fragments covering the rest of the genome.

Binding of an EcoRI–PstI fragment of the BPV genome

Since binding to BPV proteins was shown to be restricted to the 3' end of the BPV genome, we hoped to localize further the regions responsible for binding to capsid and non-capsid proteins. By double digestion with PstI and EcoRI (Fig. 1 c), two fragments from the 3' end of the BPV genome were generated, one covering map units 0 to 4 and the other covering map units 4 to 16. When these fragments were bound, a clear difference was seen between the regions of the genome with binding specificity for the capsid and non-capsid proteins. Binding at a location characteristic of capsid proteins was observed with the 0 to 4 map unit PstI fragment (Fig. 5) but not with the PstI–EcoRI 4 to 16 map unit fragment (Fig. 6). Conversely, weak binding at the position of NP-1 was observed with the 4 to 16 map unit fragment only with the undenatured DNA and no binding at a location characteristic of capsid proteins was seen (Fig. 6).
Fig. 4. Binding of the 0 to 16 map unit EcoRI fragment of the BPV genome $^{32}$P-labelled in the complementary strand to lysates of mock-infected and BPV-infected cells. The organization of this figure is the same as Fig. 2. The position of binding of novel proteins in BPV-infected cells is indicated by arrows.

Fig. 5. Binding of the 0 to 4 map unit EcoRI-PstI fragment of the BPV genome $^{32}$P-labelled in the complementary strand to lysates of mock-infected and BPV-infected cells. The organization of this figure is the same as Fig. 2. The positions of VP2 and VP3 are indicated.

**Binding to capsids**

Other autonomous parvoviruses have been shown to produce a non-capsid protein in the general size range of the capsid proteins. BPV, on the other hand, produces large amounts of the much smaller non-capsid protein NP-1. Analysis of the nucleotide sequence of BPV shows that there is an open reading frame in the left half of the genome which could code for a protein with a maximum size of 81000 daltons which would be analogous to proteins such as the NS1 of minute virus of mice (MVM) (Cotmore et al., 1983) and H-1 (Paradiso, 1984), the 71000 dalton non-capsid protein of Aleutian disease virus (Bloom et al., 1982) and the 84000 to 86000 dalton non-capsid protein of porcine parvovirus (Molitor et al., 1985).

We have preliminary evidence which suggests the existence of two BPV-specific non-capsid proteins in the size range of those of other autonomous parvoviruses. They were detected by immunoprecipitation of a lysate of BPV-infected cells (Bloom et al., 1982) labelled to a higher specific activity than we had used previously. Consequently, it was necessary to show that the DNA bound at a position defined as that of VP2 by immunoblotting was actually binding to
VP2 and not to a non-capsid protein with similar mobility. We have bound the 0 to 4 map unit fragment of the BPV genome, labelled in vitro in the complementary strand, to a nitrocellulose membrane bearing proteins of CsCl gradient-purified BPV. The same pattern of binding was observed with the capsid proteins as with the lysate of BPV-infected cells: binding to VP2 and VP3 in 0.2 M-NaCl and binding to VP3 in 0.5 M-NaCl (Fig. 7).

DISCUSSION

Methods have been published for the binding of DNA to nitrocellulose membranes which bear electrophoretically transferred proteins. The specificity of these methods has been validated by comparing DNA binding to proteins on membranes with the same interaction studied by other means such as filter binding assays and affinity chromatography. These confirmatory studies have been carried out for human plasma fibronectin (Hoch, 1982), the lac repressor (Bowen et al., 1980) and several structural and non-structural proteins of herpesvirus saimiri (Blair & Honess, 1983). However, binding of adenovirus type 5 DNA to the terminal protein on a membrane was not detected (Russell & Precious, 1982). The investigators suggest
this might reflect inappropriate washing conditions or blockage of the protein site by DNA which had been bound \textit{in vivo} and not removed during preparation. We have adapted these protocols for the analysis of the binding of restriction endonuclease fragments of the BPV genome to membranes bearing lysates of mock-infected and BPV-infected bovine foetal lung cells. We have shown the specificity of binding of the 3' end of the genome to two of the four capsid proteins and to the non-capsid protein NP-1. These proteins have been identified by probing the membrane with specific antisera subsequent to the binding of DNA.

The modifications we introduced in the prehybridization buffer, i.e. the inclusion of single-stranded as well as double-stranded DNA, was directly related to the desire to study the binding of both of these forms of BPV DNA to protein. BPV contains single-stranded DNA in the virion which replicates through a double-stranded form which is the template for the synthesis of progeny single strands. Our experimental conditions were designed, before the fact, to test whether differential binding occurred to either of these forms which might reflect interaction at specific points in the life cycle of the virus. However, the region of the genome found to bind to capsid proteins (0 to 4 map units, whether as the \textit{EcoRI–PstI} fragment or within the \textit{EcoRI} fragment) is a palindrome and cannot be irreversibly denatured. The salt conditions employed in the washing step were empirically selected to eliminate most non-specific binding. The use of two different salt concentrations allowed an assessment of the strength of the interaction of DNA and protein.

The novel aspect of the procedure we have developed is the ability to process the membrane through an immunodetection step after the DNA-binding reaction. Reaction of the membrane with an antibody of known specificity and correlation of the position of the immune reaction product with the exposed areas on the autoradiogram allowed the identification of the protein responsible for the binding. The source of the protein used here was a lysate of virus-infected cells. The demonstration of specific binding without any purification should allow, in other systems, a preliminary identification of DNA-binding proteins which can then be further characterized. It is likely that this method is generally applicable for any DNA and any protein for which an antibody is available and may prove useful for identifying proteins which have controlling functions through interaction with small regions of a genome (White \textit{et al.}, 1985). We have shown that fragments as small as 150 base pairs (the 0 to 4 map unit \textit{PstI} fragment) and fragments containing as few as 10^4 c.p.m. bind efficiently under the conditions used here. While this communication was in preparation, Miskimins \textit{et al.} (1985) reported the use of non-fat dry milk as the blocking agent during the DNA binding. Since milk is a common blocking agent for immunoblotting, its use in the DNA-binding step may mean that a filter can be exposed directly to antibody after autoradiographic exposure.

For this procedure or for any of the procedures which utilize SDS–polyacrylamide gels for protein separation, it is not known how 'native' or 'denatured' the proteins are when they interact with DNA after transfer to nitrocellulose. Shapiro & Maizel (1969) have shown that SDS is removed from protein in an electric field, such as is present during electrophoretic transfer; therefore, the proteins may be approaching the native configuration under conditions of DNA hybridization. The data presented here show that only two of the four BPV capsid proteins, VP2 (72000 daltons) and VP3 (62000 daltons) bind BPV DNA. The binding is stronger to VP2 since it remains in 0.5 M NaCl while that of VP3 is abolished at this ionic strength. Examination of the BPV nucleotide sequence suggests that the capsid proteins are coded as a nested set, co-terminal at the carboxyl end. VP1 would then contain most of the amino acids in VP2 and VP3, while VP2 would contain most of the amino acids in VP3. Therefore, binding of DNA to VP2 in the absence of binding to VP1 and stronger binding of DNA to VP2 than to VP3 is evidence for reversal of denaturation prior to binding and for binding which shows specificity for the native conformation. This is further supported by the ability of an antibody raised to native capsid to cross-react with the capsid proteins on the membrane.

Binding of DNA to VP2 and VP3 may play a physiologically important role in the replication cycle. Studies both \textit{in vivo} (Richards \textit{et al.}, 1977; Myers & Carter, 1980) and \textit{in vitro} (Muller & Siegl, 1983a, b) suggest that viral DNA interacts with structural proteins which are already assembled into a 'capsid' structure. The studies presented here, by virtue of the experimental
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design, cannot address the state of the capsid proteins when they interact with DNA. Analysis of the sequences coding for the capsid proteins of paroviruses suggest that a possible nuclear transport signal is absent from the largest capsid proteins (Lederman et al., 1986). Therefore, assembly of empty capsids prior to nuclear transport may be necessary to ensure that all structural proteins reach the nucleus. If so, then the encapsidation event is likely to involve interaction of single-stranded DNA with an empty capsid through a terminal palindrome.

By the nature of the restriction fragment used as a probe, the sequences that bind to protein must be located between nucleotides 1 and 291 on the minus strand and/or within the nucleotides complementary to nucleotides 151 and 291. This region contains the terminal hairpin and its internal palindromes, whose conformation in AAV has been shown to be required for DNA replication (Lefebvre et al., 1984), although the nature of the interaction between the DNA and cell or viral component(s) is not known. Faust & Ward (1979) have studied spontaneous deletion mutants of MVM and found encapsidated pieces of DNA as small as 10% of the genome, composed of the leftmost and rightmost ends of the genome. This suggests that the encapsidation signals reside in these areas. For BPV, the data presented here favor the location of this signal at the 3' end. This is a small region which, in an infectious clone, could be subjected to mutagenesis to determine both the absolute sequences involved in DNA-protein interaction and the phenotype of the mutant.

Involvement of capsid proteins in the regulation of parovirus DNA synthesis has been demonstrated for AAV (Buller & Rose, 1978; Hermonat et al., 1984), H-1 (Rhode, 1976) and BPV (Robertson et al., 1984), and it has been suggested that synthesis of progeny single strands from a double-stranded intermediate is driven by a packaging event (Tattersall & Ward, 1976). Paroviruses encapsidate varying proportions of plus and minus strands, ranging from equal amounts in LuIII (Bates et al., 1984) to 99% minus strand for paroviruses such as MVM (Bourguignon et al., 1976). BPV encapsidates about four minus strands for each plus strand (Bates et al., 1984). The mechanism by which strands are selected for inclusion in the virion is not known. The interaction of two capsid proteins with viral DNA may be relevant in this context. There may be specificity in the binding of an individual capsid protein for a strand of given polarity, with the strength of the binding determining the proportion of plus and minus strands encapsidated.

Binding to the abundant BPV non-capsid protein NP-1 has been detected and is also limited to the left half of the genome. When the 0 to 16 map unit fragment is further subdivided, binding to NP-1 is not detected with the 0 to 4 map unit fragment and binding to the 4 to 16 map unit fragment is greatly reduced. This decrease in binding suggests that there is some sequence in the proximity of the PstI site which is required for efficient binding. When the label is in the plus strand, binding to NP-1 is not observed, although binding is observed to several other proteins present only in lysates of BPV-infected cells whose sizes are similar to products of in vitro translation of BPV-specific RNA (Lederman et al., 1983). This result is not easily explained but the observation supports the virus-specificity of these proteins and suggests that they may function through interaction with DNA.

We have not been able to demonstrate DNA binding to the BPV homologue(s) of the non-capsid protein NS1 of other autonomous paroviruses. However, the amount of these proteins in the bovine cell lysates used is very low and detection of binding may be difficult. Of the BPV non-capsid proteins, it is NP-1 which interacts with a region of the genome which may contain two promoters, one at map unit 4 and another at map unit 12. We believe that one of these is the promoter at least for the non-structural proteins. This raises the possibility that the levels of non-capsid protein in the infected cell regulate either the rate of their own messenger RNA synthesis or that of other non-capsid proteins. As discussed above, deletion mutagenesis and expression assays will be required to determine the sequences in the BPV genome responsible for interaction with non-capsid proteins and the mechanism by which these interactions regulate the replication cycle.

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REFERENCES


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