THE ASSOCIATION OF BOVINE PARVOVIRUS DNA AND PROTEINS WITH THE NUCLEAR MATRIX OF INFECTED CELLS

by

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(ABSTRACT)

Bovine parvovirus DNA is associated with the nuclear matrix of infected bovine fetal lung cells as shown by Southern blot analysis of matrix DNA isolated by two procedures differing in the order of exposure of detergent-treated nuclei to high salt conditions and DNase I. Protein analysis of the two matrix types showed the polypeptide composition to be similar. Both procedures showed enrichment for BPV DNA with progressive DNase I digestion. Over the course of infection the amount of BPV DNA associated with the matrix increased, yet the amount of BPV DNA associated with matrix DNA as opposed to total DNA decreased from 21% at two hours to 7% at eight hours with a subsequent rise to 13% at sixteen hours. Restriction enzyme analysis of the matrix DNA indicated that no specific portion of the BPV genome was responsible for its attachment to the matrix at the selected times. In addition both the nonstructural BPV protein ,NP-1, and the capsid proteins VP1, VP2, and VP3 were associated with the matrix at sixteen hours. The association of BPV DNA and proteins with the nuclear matrix implies structural if not functional significance for the matrix in BPV replication.

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Finally I thank my parents and my brother for their forebearance and unflagging encouragement that sustained me and thus allowed the completion of this degree.

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I. Introduction

Studies have shown that the nuclei of eucaryotic cells contain a substructure, the nuclear matrix, to which the DNA of the nucleus is attached. The nuclear matrix is comprised of the peripheral lamina, nuclear pore complexes, residual nucleoli, and an internal fibrogranular network. Matrices are isolated via a series of steps using a non-ionic detergent, low and high salt buffers, and nuclease digestion (Reviewed in Berezney, 1979 and Shaper et al., 1979). It was shown in previous studies that the nuclear matrix is involved in DNA replication (Berezney and Coffy, 1975; Dijkwel et al., 1979; Pardoll et al., 1980; Hunt and Vogelstein, 1981), the synthesis, processing, and transport of RNA (Miller et al., 1978; Herman et al., 1978; van Eekelen and van Venrooij, 1981), and in addition may play a role in hormone binding (Barrack and Coffy, 1980; Sevaljevie et al., 1982).

Viral components such as the DNA, RNA, and protein of several viruses have been shown to be associated with the matrix during infection of host cells. These viruses include the following; SV40 (Nelkin et al., 1980; Staufenbiel and Deppert, 1983), adenovirus (Younghusband and Maundrell, 1982), polyomavirus (Buckler-White et al., 1980), and herpesvirus (Bibor-Hardy et al., 1982).

Like the above viruses, parvoviruses are DNA viruses that replicate in the nucleus of infected cells. Parvoviruses are small, unenveloped, and composed of three or four major capsid proteins (Lederman et al., 1983). The genome is a linear, single-stranded DNA molecule that

replicates through a double-stranded DNA intermediate. Cells in the S phase of the cell cycle are required for parvovirus DNA replication (Challberg and Kelly, 1982).

In vitro studies using viral replication complexes, or nuclear lysates, have failed to produce viral products resembling those synthesized in vivo. This aberrant viral replication could be a result of the disruption of the functional or structural characteristics of the nuclear matrix during the preparation of the nuclear lysate. The nature of this function(s) has yet to be identified. It may reflect structural and/or functional requirements of an intact nuclear matrix in order for virus replication to proceed. Therefore the objectives of this research were: (1) to prepare nuclear matrices from parvovirus-infected cells and to determine if parvoviral DNA sequences and/or viral proteins were structurally associated with the nuclear matrix of infected cells, and (2) to determine if any portion of the viral genome was specifically responsible for DNA attachment to the nuclear matrix.

II. LITERATURE REVIEW

A. The Nuclear Matrix

1. Structure

A ubiquitous feature of the nuclei of eucaryotic organisms is the presence of the nuclear matrix or nuclear skeleton. It has been identified in a variety of eucaryotic cells including the dinoflagellates (Kubai and Ris, 1969; Wolfe, 1972), the protozoan Tetrahymena pyriformis (Herlan and Wunderlich, 1976), Physarum polycephalum (Hunt and Vogelstein, 1981), plant cells (Franke and Falk, 1970), chicken oviduct cells (Robinson, et al., 1982), and in a variety of mammalian cells including six different rat tissues (Shaper et al., 1979) and human HeLa cells (Hodge et al., 1977). Nuclear structures were first isolated by Mayer and Gulick in 1942 when using 1 M NaCl in the extraction of nuclei found that insoluble proteins remained. Upon microscopic examination this material was observed to compose a residual nuclear structure (Zarbsky and Georgiev, 1959). Interest in these structures waned until the advent of non-ionic detergents and procedures to obtain highly purified nuclei rekindled interest in the characterization of this nuclear structure (Shaper et al., 1979).

2. Isolation

Highly purified nuclei, free of cytoplasmic remnants, are required to ensure isolation of a structure derived from the nucleus (Shaper et al., 1979). The source of nuclei will determine whether the isolated nuclei should be preincubated with low levels of DNase I before extraction

with low and high salt buffers. Rat liver nuclei contain an endogenous DNase that allows for the solubilization or removal of bulk DNA during the low magnesium buffer step. Other nuclei lack this endogenous DNase activity and without preincubation with exogenous DNase I, the high salt buffer step will result in the formation of a gelatinous pellet that will interfere with making reproducible preparations of nuclear matrices (Berezney and Coffy, 1977; Barrack and Coffy, 1980; Shaper et al., 1979).

Nuclear matrices are isolated by sequentially exposing highly purified nuclei to hypotonic low magnesium buffer, hypertonic 2 M NaCl buffer, a non-ionic detergent, and then digestion with the nucleases DNase and RNase (Berezney and Coffy, 1976; Berezney, 1979; Shaper et al., 1979).

The bulk (approximately 99%) of the nucleic acids and phospholipids are removed by the nucleases and non-ionic detergents respectively. As the majority of the nuclear proteins (approximately 90%) are removed by the hypo- and hper- tonic buffers. The resultant matrix is thus composed of 98% protein, 0.1% DNA, 1.1% RNA, and 0.5% phospholipid. It is comprised of the peripheral lamina, nuclear pore structures, a residual nucleolus, and an intranuclear network formed of nucleic acid and protein. The presence of the latter three components will vary depending on the conditions employed during the extraction procedure. These include 1) the nature of the starting material (nuclei versus a nuclear envelope); 2) the temperature and the order in which the nuclei are exposed to the high salt, detergent, and nuclease

treatments; 3) the types and concentrations of nucleases used; 4) the strength of the ionic agent used; and 5) the presence or absence of protease inhibitors. A tabulation of the characteristics of various matrix types and their isolation procedures are presented in the review by Shaper et al. (1979). Thus, the gross polypeptide composition of matrices from one batch of nuclei (isolated by different procedures) may be similar qualitatively yet can differ quantitatively. Slight changes in the conditions employed can result in matrices with a wide range of morphologies and compositions. Therefore it should be possible to isolate matrices of various composition and determine if a particular function is associated with the peripheral lamina or if is associated with the fibrillar network (Shaper et al., 1979).

3. Functions

The matrix as described thus far is that of a rigid skeletal structure (Berezney et al., 1977). The term matrix was adopted, according to Shaper, because it is defined by Friel (1974) as "the groundwork on which anything is cast, or the basic material on which a thing develops." The idea of a supporting framework for the replication of DNA was first proposed by Jacob, Brenner, and Cuzin (1963), and it has since been shown in a number of bacterial species that the replication complex of procaryotes is attached to the cell membrane (see Shaper et al., 1979).

Yet the nuclear matrix is far from being rigid, it has the ability to expand and contract (Herlan and Wunderlich, 1976; Berezney and Coffy, 1976; Wunderlich and Harlen, 1977; Wunderlich et al., 1978).

This ability concurs with the nuclear swelling phenomenon that is a prerequisite for RNA and/or DNA synthesis (Berezney and Coffy, 1977). Both newly-replicated DNA and the DNA polymerase alpha, responsible for DNA replication, are found to be preferentially associated with the nuclear matrix (Berezney and Coffy, 1975; Dijkwel et al., 1979; Pardoll et al., 1980; Hunt and Vogelstein, 1981; Berezney and Buchholtz, 1981; Smith and Berezney, 1980; Jones and Su, 1982). Furthermore matrix proteins have been shown to be phosphorylated to the maximal level immediately prior to the onset of DNA replication (Berezney et al., 1976; Allen et al., 1977). Nuclear matrix protein phosphorylation can be amplified by the specific binding of the cortisol [1, 2, 4(n)-3H] -triamcinolone acetonide to the nuclear matrix. This binding also results in the migration and binding of cytosol proteins to the nuclear matrix (Sevaljevie et al., 1982). Others have reported the presence of estrogen and androgen receptors within the nuclear matrix of sex hormone responsive tissues (Barrack et al., 1977; Barrack and Coffy, 1980). The sequences of transcribed genes of rRNA rat liver cells (Pardoll and Vogelstein, 1980), Alu family sequences in human tissue cells (Small et al., 1982), and the ovalbumin gene in chicken oviduct cells (Robinson et al., 1982) are all enriched in the nuclear matrix. Hence it is not surprising to find that RNA synthesis, processing, and transport all occur on the nuclear matrix. (Miller et al., 1978; Herman et al., 1978; van Eekelen and van Venrooij, 1981). In addition to normal nuclear processes occurring on the nuclear matrix, viral infection of host cells results in a composition change of

the nuclear matrix. Replicating DNA of the polyomavirus (Buckler-White et al., 1980) and of the adenovirus (Younghusband and Maundrell, 1982), as well as the transcribed sequences of SV40 are all associated with the nuclear matrix of virus-infected cells (Nelkin et al., 1980). Processing of HnRNA of the adenoviruses and of the SV40 virus also occurs on the nuclear matrix (Ben-Ze'ev and Aloni, 1983; Mariman et al., 1982). The polypeptide composition of the nuclear matrix also changes with virus infection, proteins of adenovirus (Chin and Maizel, 1977; Hodge et al., 1977; Feldman and Nevins, 1983; Sarnow et al., 1982), of SV40 (Deppert, 1978; Staufenbiel and Deppert, 1983; Verderame et al., 1983), of polyomavirus (Buckler-White et al., 1979), and of herpesvirus (Bibor-Hardy, et al., 1982; Quinlan and Knipe, 1983) have all been found associated with the nuclear matrix. Viral capsids of herpes simplex virus type 1 were seen attached to isolated matrices in electron micrographs suggesting a role for the matrix in either capsid formation or the encapsidation process (Bibor-Hardy et al., 1982). Also some of the viral proteins found to be associated with the matrix are nonstructural proteins required for viral replication or transcription, such as the T antigens of polyoma (Buckler-White et al., 1980) and SV40 (Deppert, 1978; Staufenbiel and Deppert, 1983; Verderame et al., 1983), and the $E1A_a$ protein of adenovirus (Feldman and Nevins, 1983).

The nuclear matrix has been established as a site for nuclear functions. Whether its role is active or passive has yet to be determined. Its contractile ability and the phosphorylation of its

proteins during different stages of the cell cycle suggest that the matrix could control the associated functions through its conformation (Berezney, 1979).

B. Parvoviruses

1. Characteristics

Bovine Parvovirus is a member of the virus family Parvoviridae, a group of small animal viruses about 20 nm in size that replicate in the nucleus of infected cells. The capsid is composed of three or four major proteins and lacks an envelope. The genome of BPV is a linear molecule, about 5500 basepairs (Snyder et al., 1982) that replicates through a double-stranded DNA intermediate.

2. Factors in Replication

The small size of the genome requires that most, if not all, of the factors in virus replication to be supplied by the host cell. Hence the cell must be in S phase for the virus to replicate. Infection of the host cell results in the conversion of the linear genome to a duplex replicative form (RF) DNA molecule. This parental RF replicates to produce progeny RF that then serve as templates for the synthesis of new viral genomes (Challberg and Kelly, 1982). While DNA polymerase gamma may play a role in BPV DNA replication (Kolleck et al., 1982), it is thought that DNA polymerase alpha is primarily responsible. The rate of viral synthesis correlates with the level of DNA polymerase alpha activity in the cell (Bates et al., 1978) and viral DNA synthesis is inhibited by aphidicolin, an inhibitor of DNA polymerase alpha, and is sensitive to anti-DNA polymerase alpha antibody (Pritchard et al.,

1981; Kolleck et al., 1981). Viral proteins and replicative forms of the parvoviruses LuIII (Gautschi et al., 1976) and MVM (Ben-Asher et al., 1982) have been shown to associate with the host cell chromatin. In vitro studies using nuclear lysate systems failed to produce viral products resembling those synthesized in vivo. This aberrant viral replication could be due to the disruption of the nuclear matrix during nuclear lysate preparation. Therefore the nuclear matrix should provide an alternative means to study BPV DNA replication. In turn the elucidation of parvovirus DNA replication may shed light on the processes that regulate gene expression in eucaryotes which thus far have been encumbered by the difficulties of working with large and complex genomes.

III. THE ASSOCIATION OF BOVINE PARVOVIRUS DNA AND PROTEINS WITH THE NUCLEAR MATRIX OF INFECTED CELLS

A. MATERIALS AND METHODS

1. Cell Cultures and Virus

Bovine Parvovirus (BPV) virus stocks were prepared as described (Parris and Bates, 1976). Bovine fetal lung (BFL) cells were seeded into roller bottles in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and were synchronized with 2 mM hydroxyurea (Parris et al., 1975). Following synchronization for 26-32 hr with hydroxyurea (HU), the cells were rinsed three times with Dulbecco's phosphate buffer and infected with BPV (5-10 PFU/cell). After a 1 hr adsorption period, MEM containing 10% FBS and 1 uCi/ml of [^{3}H]thymidine (ICN Pharmaceutical, 9 Ci/mMol) was added and the cells were labeled continously.

2. Isolation of Nuclei

At the times indicated, the cells were scraped into the media and centrifuged at 1000 x g for 10 min at 4° C. The cells were suspended and washed in phosphate-buffered saline (PBS) to which phenylmethylsulfonylfluoride (PMSF) was added immediately upon suspension to a final concentration of 1 mM. PMSF was added in this manner to all buffers throughout the isolation procedure (Barrack and Coffy, 1980). After centrifugation at 1000 x g for 10 min at 4° C, the

cells were suspended in hypotonic buffer (10 mM Hepes [pH 7.6], 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol) and allowed to stand on ice for 10 min. Nuclei were obtained after 200-250 strokes of mechanical homogenization using a Potter Elvehjem tissue grinder and centrifugation at 1000 x g for 10 min at 4° C. The pelleted nuclei were resuspended in phosphate-buffered saline (PBS) and counted using a hemacytometer chamber and centrifuged at 1000 x g for 10 min at 4° C.

3. Nuclear Matrix Preparation Procedures

Nuclear matrices were prepared by two methods. Freshly-isolated nuclei were suspended in their respective buffers at a concentration of 1 x 10^6 nuclei/ml.

Method (a) used for nuclear matrix preparation was the first method of Younghusband and Maundrell (1982), modified as described below. Isolated nuclei were suspended in NW buffer (0.25 M sucrose, 10 mM triethanolamine [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$ with 0.5% Nonidet P-40 [NP-40] and incubated with 2 ug/ml of DNase I (Worthington Biochem. Corp., Freehold, NJ) at 37° C. After 15 min the nuclei were incubated at 4° C and the concentration of NaCl was increased incrementally to 2 M over a 1 hr period. At this point the preparation was divided into aliquots for DNase I digestion. After a 30 min digestion at 37° C, EDTA was added to 10 mM and the mixtures were layered over a solution of 15% glycerol, 2 M NaCl, 10 mM triethanolamine (pH 7.4), 10 mM EDTA with 0.1% NP-40 and centrifuged at 5000 x g for 30 min at 4° C. The pellets were suspended in TE and

precipitated with 0.1 volume of sodium acetate and 2 volumes of ethanol.

Method (b) used for isolating matrices was the modified version of the Berezney and Coffy (1977) procedure described by Barrack and Coffy (1980). Nuclei were extracted with 1% Triton X-100 in 0.25 M sucrose containing 5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4 (TM buffer) for 10 min, centrifuged at 1000 x g for 10 min at 4° C, and resuspended in TM buffer, divided into aliquots, and then digested with various concentrations of DNase I (Worthington Biochem. Corp., Freehold, NJ) for 30 min at 37° C. After digestion, EDTA was added to 10 mM and the nuclei were centrifuged at 1000 x g for 15 min at 4° C. After centrifugation the nuclear pellet was resuspended in 0.2 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4 (LM buffer) for 15 min at 4° C. After centrifugation for 30 min at 4° C the nuclear pellet was resuspended in LM buffer, and in the course of two extractions for 30 min at 4°C the concentration of NaCl was increased from 0 to 2 M incrementally in LM buffer. After a final centrifugation of 1500 x g for 30 min at 4° C, the nuclear matrices were suspended in 10 mM Tris-HCl (pH 8) and 1 mM EDTA (TE) and precipitated with 0.1 volume sodium acetate and 2 volumes of ethanol.

4. Isolation of Matrix-Associated DNA

A modification of the procedure described by Hunt and Vogelstein (1981) was used to isolate DNA from nuclear matrices. Nuclear matrices were resuspended in TE containing 0.5% sodium dodecyl sulfate (SDS) and Proteinase K (E. Merck) at a concentration of 100 ug/ml. After

incubation for 24 hr at 60° C, the nucleic acids were extracted twice with chloroform/isoamyl alcohol (24:1) and ethanol precipitated. The samples were resuspended in TE containing boiled RNase A (Sigma) at a concentration of 100 ug/ml and incubated for 5 hr at 37° C, followed by another SDS-Proteinase K digestion. After organic extraction 0.1 volume of each sample was removed for 10% trichloroacetic acid precipitation (TCA) following the procedure in the Manual of Molecular Cloning (Maniatis et al., 1982) to determine the amount of DNA remaining with the matrix. Assuming that the DNA was uniformly labeled, the same number of counts in different samples would represent equal amounts of DNA. It was in this manner that equal amounts of DNA were applied to dot blots and agarose gels.

5. Nick Translation

Nick translation of BPV DNA or of matrix-associated DNA was performed according to the manufacturer's instructions using the Nick Translation Reagent Kit (Bethesda Research Labs) and [^{32}P]dCTP (Amersham) with a specific activity of 410 Ci/mMol.

6. Dot Blot Hybridization

Dot blot assays were performed using a Schleicher and Schuell 96-place microsample filtration manifold as described by Robertson et al. (1984). Briefly matrix DNA samples were denatured, neutralized, and quick-chilled on ice. Serial two-fold dilutions of each sample were made

in 25 mM NaPO4 (pH 6.5). The samples were spotted onto Gene Screen (NEN, Boston) and after air drying, the membrane was heated for 2 hr at 80° C, and prehybridized. Hybridization and exposure conditions were as described below. Purified BPV DNA was used as a standard.

7. Gel Electrophoresis

Matrix proteins were quantified by using the Bio-Rad Protein Assay kit. Samples were electrophoresed by the method of Laemmli (1970) on 10% polyacrylamide gels. The gels were stained with 0.2% Coomassie Brilliant Blue R in 50% methanol, 10% acetic acid and destained in 30% methanol, 10% acetic acid.

For agarose gel electrophoresis equal amounts of DNA were suspended in water and adjusted to 10% formamide and 0.1% bromophenol blue (Robertson et al., 1984). Electrophoresis was performed on submerged horizontal 1.4% agarose gels (4 mM thickness) with 40 mM Tris-acetate (pH 8.3), 20 mM sodium acetate, and 2 mM EDTA as running buffer and run at 80 volts for 4 hr (Robertson et al., 1984).

8. Southern Blot Analysis

Gene Screen membranes (New England Nuclear, Boston, MA) and the modifications of the Southern method (Southern et al., 1975) reported in the Gene Screen instruction manual were used for blot analysis of DNA.

Prehybridization and hybridization were performed using method 1

as reported in the Gene Screen instruction manual. Dot blots were probed with 1 x 10^6 dpm of nick-translated BPV DNA (sp. act. 2.5-3.7 x 10^7 dpm/ug) while blots of gels were probed with 3 x 10^6 dpm of BPV probe of the same specific activity. After hybridization, the blots 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.

B. RESULTS

1. Comparison of protein composition of nuclear matrices isolated by two procedures. Nuclear matrices are the residual structures after sequential treatment of nuclei with non-ionic detergents, low and high salt buffers, and nucleases (Shaper et al., 1979). Two procedures were used in matrix isolation as even minor changes in the conditions employed and/or the sequence of exposure of the nuclei to the above treatments during the extraction procedure can influence the composition and the morphology of the matrix isolated (Shaper et al., 1979; Kauffman et al., 1981; Basler et al., 1981). SDS-polyacrylamide gel electrophoresis of the matrix proteins isolated from both BPV-infected and mock-infected BFL cells prepared at 16 hr post-infection were comparable in polypeptide composition for the two procedures (FIG. 1). The presence of major proteins possessing molecular weights in the 40,000-70,000d range and the absence of lower molecular weight proteins in these matrix preparations are characteristic of matrices prepared from other sources (Shaper et al., 1979). In addition the polypeptide profiles of BPV-infected and mock-infected cells were similar with the exception of the presence of viral proteins (FIG. 1).

2. Comparison of BPV DNA associated with nuclear matrices isolated by two procedures. The procedures differed primarily in the order in which the nuclei were exposed to high salt conditions and nuclease digestion. Treatment with DNase I before exposure to high salt could result in the enrichment or removal of specific sequences associated

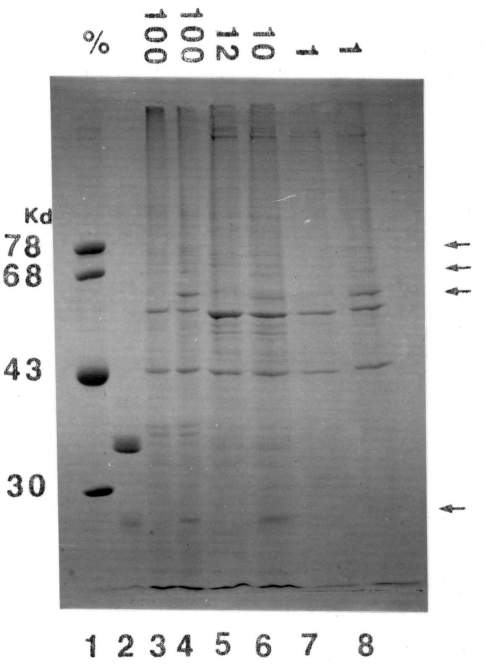

FIG. 1

SDS-polyacrylamide gel electrophoresis of nuclear and matrix proteins from BPV-infected and mock-infected BFL cells. Each protein sample (20 ug) was loaded, electrophoresed on a 10% resolving gel at 35 mA for 2 hr and then stained with Coomassie Brilliant Blue R. Indicated above each lane is the percentage of total DNA remaining with the matrix after DNase I digestion. Lane 1, molecular weight markers: human transferrin (76,000); bovine serum albumin (68,000); ovalbumin (43,000); and carbonic anhydrase (30,000). Lane 2, DNase I (31,000). Lane 3, untreated nuclei from mock-infected BFL cells. Lane 4, untreated nuclei from BPV-infected cells. Lane 5, matrix proteins (procedure a) from mock-infected BFL cells. Lane 6, matrix proteins (procedure a) from BPV-infected BFL cells. Lane 7, matrix proteins (procedure b) from mock-infected BFL cells. Lane 8, matrix proteins (procedure b) from BPV-infected BFL cells. Matrix proteins (lanes 5, 6, 7, and 8) were isolated from matrices prepared with 100 ug/ml of DNase I. Viral proteins are indicated by the arrows.

with the matrix as the presence of histones and related proteins could hinder DNase I digestion (Basler et al., 1982; Small et al., 1982). Serial dilutions of equal amounts of DNA, isolated from matrices prepared by both procedures using increasing concentrations of DNase I (0, 5, 50, 75, and 100 ug/ml) were spotted onto Gene Screen, and probed with nick-translated ^{32}P-labeled BPV DNA. Comparison of digested matrix samples to the undigested matrix samples or untreated nuclei show not only an association of BPV DNA with the matrix but an enrichment as well. The extent of digestion as indicated by the percentage of DNA remaining with the matrix correlates with the intensity of the dots observed in FIG. 2. The protein and DNA composition of the two matrix types were similar. Procedure (b) was selected for most of the remaining experiments due to the discrepancy between the amount of DNase I added and the amount of digestion observed (FIG. 2) and the lack of reproducibility with procedure (a).

3. Association of BPV DNA with the nuclear matrix through the infection cycle. To determine when BPV DNA associates with the nuclear matrix, matrices were prepared at the times post-infection indicated in FIG. 3. These times were chosen on the basis of the BPV replication cycle: 2 hr post-infection is before the conversion of single-stranded DNA to a double-stranded replicative form (RF); 8 hr is at or near the time of the conversion; and late in infection at 16 hr (70% CPE) there is production of RI and progeny strands and encapsidation (Robertson et al., 1984). Serial dilutions of equal amounts of purified DNA from matrices isolated with progressive DNase I digestion, were

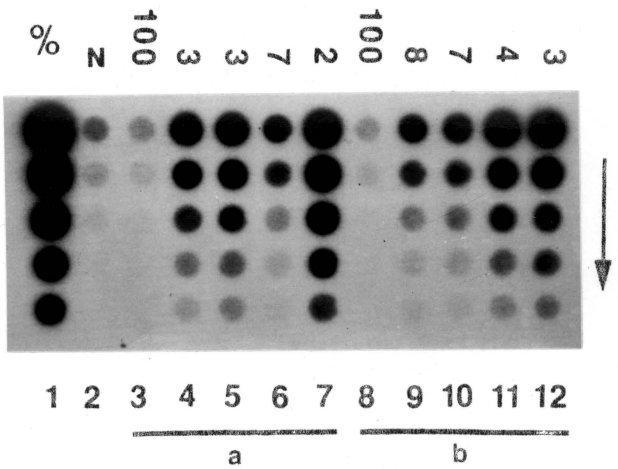

FIG. 2

Dot blot assay for matrix-associated BPV DNA. Serial two-fold dilutions (↓) of equal amounts of purified DNA from untreated nuclei and matrices isolated by procedures (a) and (b) at 16 hr post-infection were spotted onto Gene Screen, and probed with nick-translated ^{32}P-labeled BPV DNA. Indicated above each lane is the percentage of total DNA remaining with the matrix after DNase I digestion. Lane 1, 0.5 ug BPV DNA. Lane 2, untreated nuclei(N). Lanes 3-7, nuclear matrices (procedure a) prepared with 0, 5, 50, 75, and 100 ug/ml of DNase I, respectively. Lanes 8-12, nuclear matrices (procedure b) prepared with 0, 5, 50, 75, and 100 ug/ml of DNase I, respectively.

spotted onto Gene Screen, and probed with nick-translated ^{32}P-labeled BPV DNA. Dot blot analysis (FIG. 3) shows that BPV DNA was associated with the nuclear matrix at each of the selected points in the replication cycle. Futhermore the amount of BPV DNA associated with the matrix increased with time post-infection (FIG. 4A). When the data was plotted as the percentage of BPV DNA associated with the digested matrix as to that associated with the undigested matrix one sees a decrease from 21% at 2 hr post-infection to 7% at 8 hr with a subsequent rise to 13% at 16 hr (FIG. 4B). This profile of BPV DNA is similar to that seen for polyomavirus DNA during its replication cycle in infected cells (Buckler-White et al., 1980).

4. Demonstration of the BPV DNA species and the nature of their association with the nuclear matrix. The experiments described above demonstrated that BVP DNA is enriched in the nuclear matrix. To determine if discrete BPV DNA species were associated with the matrix following extensive DNase I digestion, matrix samples were analyzed by the blotting procedure of Southern (1975). The species of BPV DNA associated with the nuclear matrix isolated at 16 hr post-infection are shown in FIG. 5A. They consist of double-stranded, single-stranded, and smaller than single-stranded forms of BPV DNA. Replicative species larger than double-stranded DNA were absent. To determine if the attachment of the BPV DNA species to the nuclear matrix was through a specific sequence, DNA was isolated from matrix samples prepared by procedure (b) at 2, 8, and 16 hr post-infection. The samples were digested with Hinc II and the products electrophoresed,

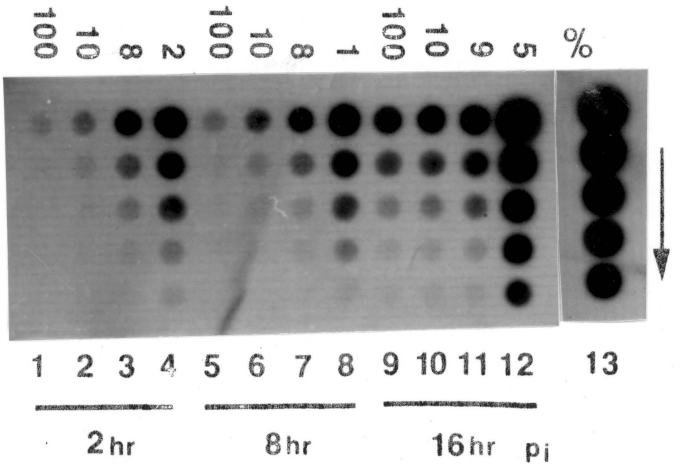

FIG. 3

Dot blot assay of BPV DNA associated with the nuclear matrix through the infection cycle. Serial two-fold dilutions (↓) of equal amounts of purified DNA from matrices prepared by procedure (b) at (a) 2 hr, (b) 8 hr, and (c) 16 hr post-infection were spotted onto Gene Screen, and probed with nick-translated ^{32}P-labeled BPV DNA. The matrix preparations were digested with 0, 0.5, 5, and 100 ug/ml of DNase I, respectively. Indicated above each lane is the percentage of total DNA remaining with the matrix after DNase I digestion. Lanes 1-4, matrix samples isolated at 2 hr post-infection. Lanes 5-8, matrix samples isolated at 8 hr post-infection. Lanes 9-12, matrix samples isolated at 16 hr post-infection. Lane 13, 0.5 ug BPV DNA.

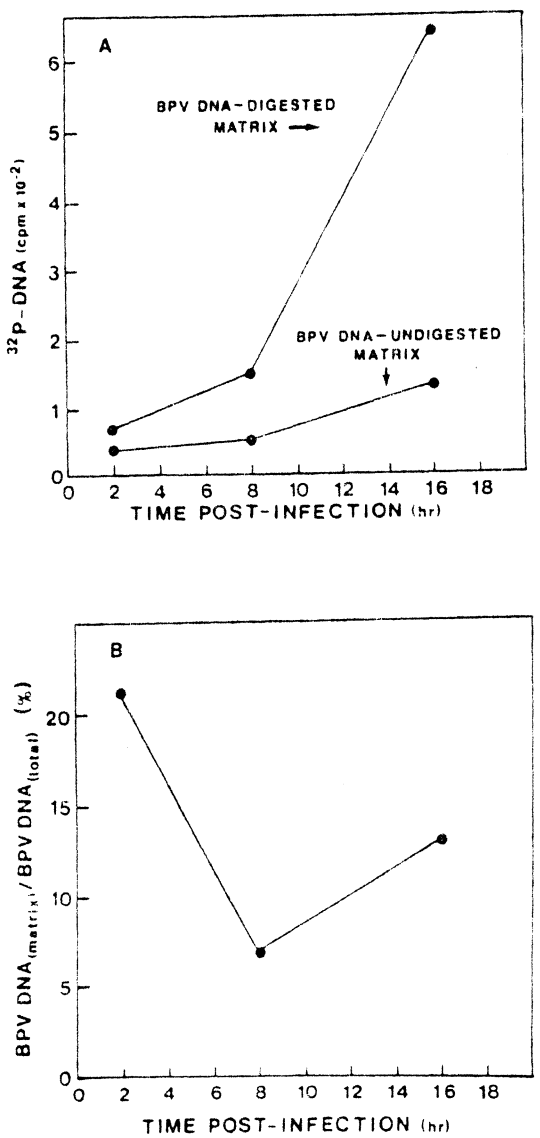

FIG. 4

Comparison of the amount of BPV DNA associated with undigested matrix DNA and with digested matrix DNA through the infection cycle. The dots of matrix samples from FIG. 3, prepared by method (b) at 2 hr, 8 hr, and 16 hr post-infection with and without DNase I digestion , were punched out, and the amount of radioactivity in each sample was determined. Panel A shows enrichment of BPV DNA with the digested matrix. Panel B shows the percentage of the viral DNA that is associated with digested matrix DNA compared to the percentage of viral DNA that is associated with the undigested matrix DNA.

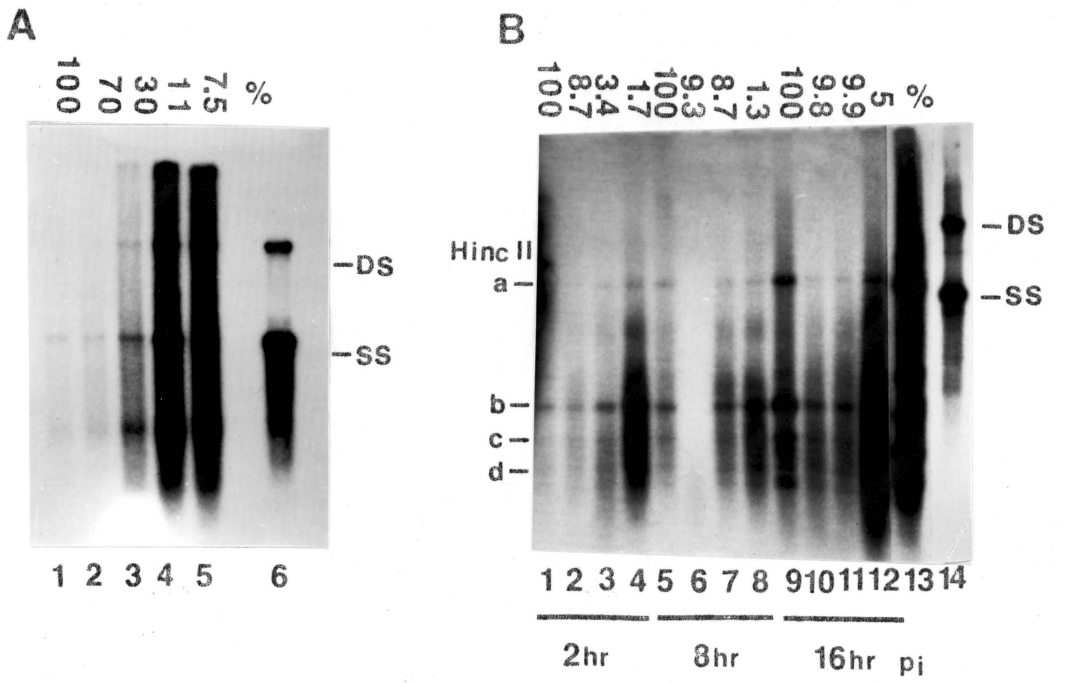

FIG. 5

Demonstration of BPV DNA species and their association with the nuclear matrix. Nuclear matrices were prepared by procedure (b) at (a) 2 hr, (b) 8 hr, and (c) 16 hr post-infection. Matrix DNA was purified, loaded based on equal amounts of DNA, and electrophoresed directly (Panel A) as described in Materials and Methods, or the matrix DNA was purified, digested with Hinc II, and electrophoresed in the same manner (Panel B). After electrophoresis the DNA was transferred to Gene Screen, probed with nick-translated ^{32}P-labeled BPV DNA, and autoradiographed. Indicated above each lane is the percentage of total DNA remaining with the matrix after DNase I digestion. Panel A shows purified uncut DNA isolated from matrices prepared at 16 hr post-infection. Lanes 1-5, matrices prepared with 0, 0.5, 5, 50, and 100 ug/ml of DNase I, respectively. Lane 6, marker single- and double-stranded BPV DNA. Panel B shows Hinc II fragments of BPV DNA isolated from matrices prepared at 16 hr post-infection using 0, 0.5, 5, and 100 ug/ml of DNase I, respectively. Lanes 1-4, matrices isolated at 2 hr post-infection. Lanes 5-8, matrices isolated at 8 hr post-infection. Lanes 9-12, matrices isolated at 16 hr post-infection. Lane 13, Hinc II digest of marker BPV DNA. Restriction fragments are indicated (Burd et al., 1983). Lane 14, marker single- and double- stranded BPV DNA.

blotted, and probed as described in Materials and Methods. The autoradiograph (FIG. 5B) shows that all fragments of the BPV genome were represented proportionally at all levels of digestion at each time point examined during the replication cycle. The 2 hr sample should not show restriction fragments since the DNA at that time is still single-stranded and thus won't be cleaved. The fragments which were observed are due to the cleavage of annealed + and - strands from infecting capsids. To ensure that there was no enrichment or absence of any one fragment, matrix samples, prepared by both procedures with and without DNase I digestion, were nick-translated, and used to probe blots of Hinc II and Bgl II digests of BPV DNA. The probes prepared with digested and undigested matrix DNAs hybridized to each restriction fragment in the same proportion as did the BPV DNA probe. This confirmed that all sequences of the BPV genome were equally represented in matrix association.

5. Demonstration of viral proteins associated with the nuclear matrix. Coomassie Brilliant Blue staining of polyacrylamide gels showed the overall protein composition of nuclear matrices (FIG. 1). In such a gel BPV proteins were observed in matrix preparations, however the extent of the association with the matrix was not readily apparent. Therefore, cells were infected with BPV and labeled with [^{35}S]methionine. Shown in FIG. 7 is a fluorograph of proteins isolated from matrices prepared by procedure (b) with increasing amounts of DNase I. When the protein samples were loaded based on equal amounts of DNA, an increase of BPV protein species with increasing digestion

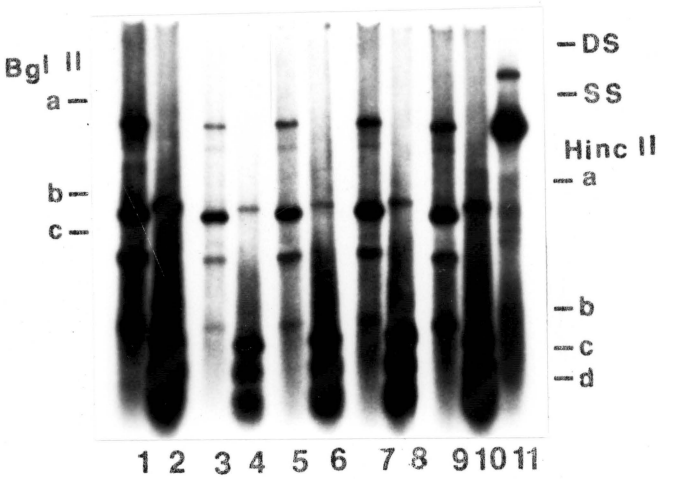

FIG. 6

Restriction digests of BPV DNA probed with nick-translated ^{32}P-labeled matrix DNA. The DNA was isolated from matrices prepared by procedures (a) and (b) at 16 hr post-infection with and without DNase I digestion. It was nick-translated and ^{32}P-labeled. Odd-numbered lanes contain Bgl II digests of BPV DNA and even-numbered lanes contain Hinc II digests of BPV DNA. Lanes 1 and 2, BPV DNA used as a probe. Lanes 3 and 4, matrix DNA prepared by procedure (a) without DNase I, used as a probe. Lanes 5 and 6, matrix DNA prepared by procedure (a) with 100 ug/ml DNase I, used as a probe. Lanes 7 and 8, matrix DNA prepared by procedure (b) without DNase I, used as a probe. Lanes 9 and 10, matrix DNA prepared by procedure (b) with 100 ug/ml of DNase I, used as a probe. Lane 11, marker single- and double- stranded BPV DNA. Restriction fragments are indicated (Burd et al., 1983).

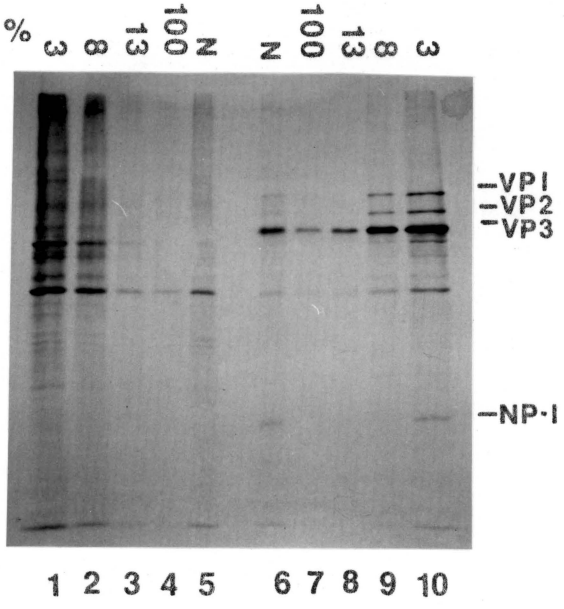

FIG. 7

An SDS-polyacrylamide gel fluorograph of proteins isolated from matrices prepared from both BPV-infected and mock-infected BFL cells. At 6 hr post-infection the medium was replaced with low methionine medium supplemented with 10% dialyzed FBS. At 10 hr post-infection 1 uCi/ml of [^{35}S]methionine (Amersham, 1300 Ci/mMol) was added. The cells were harvested at 17 hr post-infection and matrices prepared by procedure (b) with increasing concentrations of DNase I. Indicated above each lane is the percentage of total DNA remaining with the matrix. Isolated matrices were suspended in Laemmli buffer, loaded based on equal amounts of DNA, and electrophoresed on a 10% SDS-polyacrylamide resolving gel for 3.5 hr at 35 mA. The gel was stained with Coomassie Brilliant Blue R, destained, washed first in Enhance (New England Nuclear), and then washed in water. The gel was dried and exposed to Kodak SB-5 x-ray film with an intensifying screen at -80° C. Numbers represent molecular weight standards seen by Coomassie Blue staining (m). Lane 1, untreated nuclei from mock-infected BFL cells.

Lanes 2-5, matrix proteins from matrices prepared from mock-infected BFL cells using 100, 5, 0.5, and 0 ug/ml of DNase I, respectively. Lane 6, untreated nuclei from BPV-infected BFL cells. Lanes 7-11, matrix proteins from matrices prepared from BPV-infected BFL cells with 0, 0.5, 5, and 100 ug/ml of DNase I, respectively. Viral proteins are indicated (Lederman et al., 1983, 1984).

was observed. This includes the major capsid proteins VP1, VP2, and VP3, and the noncapsid protein, NP-1 (Lederman et al., 1984). However when equal amounts of each protein sample were loaded the amounts of viral protein seen did not appear to increase or decrease (data not shown). These experiments demonstrate the intimate association of BPV proteins with the nuclear matrix.

C. DISCUSSION

The nuclear matrix as a site for various nuclear functions has been established (Berezney, 1979; Shaper et al., 1979). Previous studies of matrices isolated from virus-infected cells have shown that viral components such as DNA, RNA, and proteins of several viruses to be associated with the nuclear matrix (see introduction). Since other DNA viruses that replicate in the nucleus are associated with the matrix, it is likely that parvoviruses would also be associated with the matrix especially when one considers their small genome size, 5.5 kilobases (Snyder et al., 1982). This minimal genome demands that they rely heavily on the host cell for factors required for viral replication. In particular an unknown S phase function is required for replication (Challberg and Kelly, 1982). It is likely that both enzymatic (functional) and structural factors are supplied by the host nucleus. These factors could be partially or totally fulfilled by matrix association.

This study was initiated to determine if BPV DNA is associated with the matrix during its replication cycle and then to determine the nature of that association. BPV DNA was shown to be preferentially associated with the nuclear matrix regardless of the procedure used in matrix isolation (FIG. 2) as indicated by enrichment of viral DNA with progressive DNase I digestion. An artifactual association is unlikely as procedures used in matrix isolation should remove unassociated viral DNA. This was confirmed with reconstruction experiments where BPV

DNA was added at different steps during the isolation procedure of matrices from mock-infected cells without retention of BPV DNA (data not shown).

BPV DNA is associated with the matrix through the infection cycle (FIG. 3) and increases in its association over time (FIG. 4A). Yet the percentage of BPV DNA associated with the matrix decreases from 21% at 2 hr post-infection to 7% at 8 hr with a subsequent rise to 13% at 16 hr post-infection (FIG. 4B). A similar profile was observed for polyomavirus DNA in its replication cycle (Buckler-White et al., 1980). When one considers the BPV replication cycle and the DNA species seen at 16 hr (FIG. 5A) the decrease of association is not surprising. No BPV DNA species larger then double-stranded was observed to be associated with the matrix. It could be that matrix association is required only for the initial conversion of single-stranded DNA to double-stranded DNA. This step is shown to utilize DNA polymerase alpha (Robertson et al., 1984), an enzymatic activity found to be associated with the matrix (Smith and Berezney, 1980; Jones and Su, 1982). Once the replication complex is attached, further BPV replication (RF to RI) could proceed without matrix association. This would not be surprising when one considers that progeny single-stranded DNA is produced by a strand displacement mechanism while host cell DNA uses a bidirectional mode of replication (Pardoll et al., 1980). This is in contrast to adenovirus which also replicates via a strand displacement mechanism, yet whose percentage of viral DNA associated with the matrix continues to increase through the infection

cycle. This may reflect genome size differences (35 to 5.5 kilobases) or more probably, reflects the species of DNAs involved in their respective replication cycles.

Yet BPV DNA is similar to adenovirus in that its association with the matrix is not determined by a specific sequence. This was determined by direct restriction enzyme digestion of matrix DNA (FIG. 5B) and by using nick-translated ^{32}P-labeled matrix DNA to probe blots of BPV DNA digests (FIG. 6). No sequence specificity was seen by either method as was the case for adenovirus (Younghusband and Maundrell, 1982). Various cell genes have been shown to be preferentially associated with the matrix but also random sequence association has been demonstrated. These discrepancies could be due to the procedure used in matrix isolation (Small et al., 1982). To rule out this possibility, matrices were prepared by two procedures and lack of sequence specificity was observed with both procedures (FIG. 6). This lack of sequence specificty could be due to a different association than that observed for cell DNA or the level of detectability was not sensitive enough to determine sequence specificity.

The increased enrichment of BPV DNA observed at 16 hr post-infection could be a result of encapsidation of single-stranded progeny DNA occurring on the matrix which would concur with the finding of viral capsid proteins associated with the matrix at 16 hr post-infection (FIG. 1 and FIG. 7). Gautschi et al. (1976) using a chromatin isolation procedure also observed viral proteins of the parvovirus LuIII to be associated with this material. They used 0.5 M NaCl as opposed to the

2 M NaCl which was observed to make a difference in whether adenovirus proteins were associated with the matrix (Younghusband and Maundrell, 1982). Viral capsids of herpesvirus attached to the nuclear matrix have been seen in electron micrographs (Bibor-Hardy et al., 1982), hence the idea of a viral assembly factory occurring on the matrix is feasible as both viral DNA and proteins have been shown to be associated with the matrix.

NP-1, a nonstructural BPV protein can also be detected in association with the matrix. While its function has yet to be defined it would not be the first nonstructural protein required for viral replication or transcription to be found associated with the nuclear matrix. The T antigens of SV40 (Depppert, 1977; Staufenbial and Deppert, 1983) and of polyomavirus (Buckler-White et al., 1980) as well an HSV DNA polymerase activity (Bibor-Hardy et al., 1982) have all been found in the matrix, including the $E1A_a$ protein of adenovirus required for viral transcription (Feldman and Nevins, 1983). In addition NP-1 is known to be phosphorylated (Lederman, et al., 1984) and matrix proteins have been shown to be phosphorylated to a maximal level immediately prior to the onset of DNA replication. This observation might implicate a role for NP-1 in BPV replication.

This study demonstrated an association of BPV DNA and proteins with the nuclear matrix through the infection cycle. The structural and/or functional aspects of the nuclear matrix could provide the basis for BPV replication, but further studies emphasizing replicating BPV DNA will have to be pursued to determine if the matrix plays a role in

parvovirus replication.

IV. CONCLUDING REMARKS

The nuclear matrix as a site for various nuclear functions has been established (for reviews see Berezney, 1979 and Shaper et al., 1979). Previous studies of matrices isolated from virus-infected cells have shown that various viral components, such as DNA, RNA, and proteins of several viruses to be associated with the nuclear matrix during their infection cycle (see literature review). This implies at the very least a structural role for the matrix in viral replication, especially as its composition is altered with the addition of the viral components.

This study was undertaken to determine if any components of BPV are associated with the nuclear matrix during its replication cycle. Both viral DNA and proteins were found to be associated with the matrix and examination of the associated viral DNA showed that this association was not sequence specific. Such an intact nuclear matrix could be required for normal viral replication. Therefore the unusual BPV DNA species synthesized by in vitro nuclear lysate systems could result from the disruption of the nuclear matrix during nuclear lysate preparation.

Hence, it should be possible to prepare nuclear matrices without the use of DNase I from cells to which various inhibitors of DNA replication, transcription, and translation would be added. The effects of the inhibitors on the different processes would be assessed by Southern blot analysis and SDS-polyacrylamide gel electrophoresis of the

viral products.

Finally, the small size of the BPV genome confers a strong dependence on the host cell for its replication. Thus, this virus model could shed light on any functional roles provided by the matrix as well as any factors required in eucaryotic gene expression that thus far have been encumbered by the difficulties of working with large and complex genomes.

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