CELLULAR FACTORS AND VIRAL ELEMENTS FOR PARVOVIRUS REPLICATION

by

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Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

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April, 1994

Blacksburg, Virginia

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(ABSTRACT)

Autonomous parvoviruses, such as bovine parvovirus (BPV), need a factor present at the S-phase of the cell cycle for a productive infection, while dependent parvoviruses, the adeno-associated viruses (AAVs), require a helper virus to complete an infectious cycle. However, AAV can replicate autonomously in synchronized cells, suggesting that an S-phase factor substitutes for the helper virus. To investigate the nature of the cellular S-phase factor, we performed DNA retardation assays with uninfected nuclear extracts of S-phase cells, synchronized by hydroxyurea pretreatment, and radiolabeled parvoviral termini in their hairpinned conformation. We observed that proteins in HeLa cells, a tissue culture host for AAV, specifically interacted with the terminal sequences of this virus, which act as origins of replication (*ori*s). These assays also showed specific binding between S-phase cellular proteins and termini (*ori*s) of heterologous parvoviruses, for which the cells are not a natural host. For example, proteins from bovine fetal lung (BFL) cells, a tissue culture host for BPV, were able to bind to an AAV terminus and HeLa cell proteins interacted with both termini of the BPV genome. All DNA-protein complexes investigated appeared to be specific for S-phase synchronized cells. In order to begin to characterize the protein(s) involved in the complex formation, we performed SDS-PAGE electrophoresis of some retarded complexes. We report that a 54 kd protein was contained in the complex formed with the BPV left terminus and BFL cell extract. [Binding of BFL cell proteins

to a BPV left terminus has been reported earlier]. Using a similar technique, we observed that two phosphoproteins of 55 and 90 kd were present in the retarded complex formed between a BPV left terminus and HeLa cell extract. An antibody directed against human p53, an anti-oncoprotein, was shown to compete binding of BFL cell extract and HeLa cell extract to the BPV left terminus. This antibody also competed the binding of HeLa cell extract to the AAV terminus. Our data suggest that proteins with similar characteristics, most probably among which is p53, are involved in the *ori*-binding complexes, possibly exerting a role as positive regulator of parvoviral replication.

The secondary structure of the viral ends is remarkably conserved among parvoviruses. Of particular interest is the presence of mismatched/unpaired nucleotides, forming a bubble, in the stem of the left hairpin of almost all autonomous parvoviruses. To analyze the possible role of these unpaired/mismatched nucleotides in the BPV life cycle, two mutants clones lacking the bubble region were constructed and their replicative properties were analyzed after electroporation in permissive cells. Infectivity of the mutant clones was determined by three techniques: observation of cytopathic effect, detection of virally-coded proteins by indirect immunofluorescence, and transient DNA replication assays. We report that the mutant clone containing duplicate sequences of the (mismatched) nucleotides numbered 46 to 57 (BLOP) was defective for replication. The other bubbleless clone (BLOM), that contains duplicate sequences of the (mismatched) nucleotides 99 to 105, was able to replicate. The later clone produced monomer-length viral DNA at about 20% of the level of the infectious genomic clone of BPV, when electroporated as a linear excised sequence. This clone was infectious since it could be propagated by subsequent passage. Expression of viral structural proteins was seen by an indirect immunofluorescence assay using anti-capsid antibodies. Our results suggest that the bubble in the left hairpin of BPV is not required for the viral life cycle, but that specific sequences within the mismatched/unpaired region are necessary for viral replication.

ACKNOWLEDGEMENTS

I am grateful to my advisor Dr Muriel Lederman, for her time, help and support throughout my studies. Her concern for me and my work will be remembered forever. I wish to thank my committee members: Drs Robert Bates, Patricia Rogers, Charles Rutherford, Ernest Stout, Sue Tolin, and Bruce Turner for their time and suggestions.

Special thanks to Dr Katherine Chen and Dr Laura Via for their advice and friendship. Thanks also to my others labmates: Ginger Braddon, Tom Freeman, and to Margarita Correa, Selester Bennett, Mara Sabre, Hélène Cornelis. I would also like to thank all my friends in Blacksburg who shared this American experience with me.

Most of all, I thank my parents Ghislaine and Jean Deville, my family, and my future husband Yves Mainguy, for encouraging me, especially during the times when home felt more than an ocean away.

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CHAPTER I

INTRODUCTION

Parvoviruses are among the smallest animal DNA viruses. They are non-enveloped icosahedral particles averaging 22 nm in diameter, which encapsidate a linear single-stranded DNA genome of 4.5 to 6 kb in size (Siegl *et al.*, 1985). The family *Parvoviridae* is divided into three genera depending on the infected host and on the virus requirements for replication. Densoviruses grow only in larvae of insects, whereas Dependoviruses and Parvoviruses infect vertebrate hosts. Dependoviruses, for example adeno-associated viruses (AAV), are generally defective and replicate with a helper virus. The autonomous parvoviruses, although capable of autonomous multiplication, depend upon host cell functions expressed transiently during the S-phase of the cell cycle for their replication. All parvoviruses replicate and assemble in the nucleus of the infected cells.

CHARACTERISTICS OF PARVOVIRUSES

Biology

Parvoviruses are distributed worldwide, infecting a wide variety of animals. The pathogenic potential of vertebrate parvoviruses differs greatly depending upon the virus species

and serotype, as well as on the host species. Autonomous parvoviruses have been associated with a number of diseases (reviewed in Pattison, 1990). They show a preference for fetal or neonatal tissues, but also infect adult tissues that divide rapidly. Infection with most of the autonomous parvoviruses, such as bovine parvovirus (BPV), porcine parvovirus (PPV), the rodent parvoviruses such as minute virus of mice (MVM), rat virus (RV), and H-1, often results in fetal abortion or teratogenesis. Canine parvovirus (CPV) and feline panleukemia virus (FPV) are highly pathogenic in young animals, leading to a rapid death by enteritis and myocarditis. B19 is the only human pathogenic parvovirus discovered to date: it causes erythema infectiosum in children, aplastic crises in immunodeficient adults, and fetal hydrops. The AAVs, in contrast, have not yet been identified as etiological agents of any disease (Blacklow, 1988). Both AAVs and autonomous parvoviruses have been isolated from neoplastic tissues, but are not oncogenic; on the contrary, they can be oncosuppressive (reviewed in Rommelaere and Tattersall, 1990).

Infections with parvoviruses are generally acute, productive, and short-term in newborn or young animals. However, parvoviruses can cause non-productive infections, such as abortive, latent, or cryptic infections (reviewed in Berns, 1990, Cotmore and Tattersall, 1987). Cryptic infections are associated with a low level of virus multiplication and excretion and probably occur when autonomous parvoviruses infect resting cells. AAV can establish a latent infection, by integrating into the host chromosome (Cheung *et al.*, 1980, Kotin *et al.*, 1990, reviewed in Berns, 1990). Infection with Aleutian disease virus (ADV) can also lead to a persistent infection, which is characterized by an immune complex pathology. Transmission of parvoviruses can occur vertically or horizontally, either by close contact or by contaminated materials or biological fluids.

Autonomous parvoviruses are highly host specific, infecting only one animal species and sometimes only a specific cell type within an animal host. BPV, for example, has only been shown to replicate in bovine cells (Bates, 1990). Specific cell-type tropism is best illustrated by

the two variants of MVM: MVM(p), the prototype strain, replicates only in fibroblasts, while MVM(i), the lymphotropic variant, infects only T-lymphocytes (Tattersall and Bratton, 1983). Host range variants of autonomous parvoviruses, such as the recently emerged CPV, have arisen by mutation of the viral genome. The genetic variation responsible for host cell or host range variation affects the capsid or/and the nonstructural parvoviral proteins (Ball-Goodrich and Tattersall, 1992, Vasudevacharya and Compans, 1992). In contrast, AAVs have a wide host range and can replicate in cells of various organisms if a host-specific helper virus is provided (reviewed in Cukor *et al.*, 1984).

Parvoviruses are highly stable viruses, which can resist high temperature, dehydration, and pH values of 3 to 9 (Arella *et al.*, 1990). The persistence of these viruses in natural ecosystems is presumably due to this resistance to environmental extremes.

Virion structure

Infectious virion particles have a buoyant density of 1.41 g/cm^3 in CsCl gradients, a sedimentation coefficient of 105 to 145 S, and a particle/infectivity ratio of 1/50 to 1/400 (Siegl *et al.*, 1985). Parvovirus particles do not contain enzymes of cellular or viral origin. The linear viral DNA does not appear to be complexed with histone-like proteins or contained in a core inside the capsid (Siegl *et al.*, 1985).

The three dimensional structure of CPV has been elucidated recently by X-ray diffraction (Tsao *et al.*, 1991). Infectious virions show a triangulation number (T) of 1 and contain 60 capsid subunits. The CPV capsid shows two depressions: a deep circular "canyon" around the 5-fold axis, and a "dimple" on the 2-fold axis of symmetry. By analogy to rhinoviruses, the canyon may be the site of receptor attachment for cell entry. In CPV, protein sequences adjacent to the dimple confer the hemagglutinating properties (Barbis *et al.*, 1992). A protrusion or "spike", formed by 3 loops of the major CPV coat protein exists on the 3-fold axis of symmetry. This spike contains

the most exposed amino acids of the viral capsid, which are associated with antigenic properties (Langeveld *et al.*, 1993). Neutralizing epitopes have similarly been mapped to this region in B19 (Yoshimoto *et al.*, 1991).

Mature infectious virions of AAV and of most of the autonomous parvoviruses contain 3 non-glycosylated capsid proteins, VP1, VP2 and VP3 (Matsunaga and Matsuno, 1983, McPherson and Rose, 1983, Ozawa and Young, 1987, Tattersall *et al.*, 1977). The average sizes of these coat proteins are 80 to 86 kd for VP1, 64 to 75 kd for VP2, and 60 to 62 kd for VP3. The different capsid proteins share common amino acid sequences, since they are transcribed from overlapping genes. The smallest protein is generally the most abundant in the parvoviral capsid. In AAV, all three capsid proteins originate from alternatively spliced mRNAs, while in the autonomous parvoviruses, only VP1 and VP2 are directly translated from mRNAs. VP3 is a proteolytic digestion product of VP2 and is only present in mature virions. BPV differs from the other parvoviruses and contains four capsid proteins (Lederman *et al.*, 1983). Three are coded, while the smallest is a proteolysis product. ADV grown on cell culture only contains two structural proteins (Bloom *et al.*, 1982), but can contain as many as four when isolated from infected mink (Shahrabadi *et al.*, 1977).

STRUCTURE AND TRANSCRIPTION OF THE VIRAL GENOME

Genome organization

All autonomous parvoviruses, with the exception of B19 and LuIII, package predominantly, if not exclusively, the minus strand of the viral genome. AAVs, B19 and LuIII encapsidate plus or minus viral DNA strands with equal frequency. Several parvoviruses have been sequenced entirely (Astell *et al.*, 1983, Astell *et al.*, 1986, Bloom *et al.*, 1988, Chen *et al.*, 1986, Diffoot *et al.*, 1993, Kariatsumari *et al.*, 1991, Rhode and Paradiso, 1983, Shade *et al.*, 1986, Srivastava *et al.*, 1983). The AAVs have the smallest genome with 4675 nt and BPV the

largest with 5517 nt. Hybridization experiments and sequence analysis show that the "rodent-like" parvoviruses (MVM, H-1, RV, LuIII, KRV, MEV) have a high degree of homology among themselves, but not with the other autonomous parvoviruses such as B19 and BPV. AAV is also distant at the nucleotide level from the autonomous parvoviruses (reviewed in Rhode and Iversen, 1990).

The coding sequences span almost the entire parvoviral genome and are bracketed at each extremity by non-coding palindromic sequences, which can fold back on themselves to form duplex hairpin structures. B19 and AAVs have identical inverted repeats, which can assume a T-shaped secondary structure, at both 5' and 3' ends of their genome (Deiss *et al.*, 1990, Lusby *et al.*, 1980) (Fig. 1). The other vertebrate parvoviruses possess different sequences at their left and right termini. By convention, the left end of the parvoviral genome is defined as the plus strand 5' end, and also, as the minus strand 3' end, since the plus, coding strand, of the genome is drawn left to right, 5' to 3' (Armentrout *et al.*, 1978). The right end of the genome therefore corresponds to the 3' end of the plus strand and to the 5' end of the minus strand. Termini vary in length irrespective of the genome size, from 115 nt for the MVM left terminus, to 383 nt for the B19 terminal repeats. *In vitro* experiments, such as cleavage by single-strand nucleases or restriction enzymes, show, in accordance with secondary structure predictions, that the left end of autonomous parvoviruses can assume a Y- or T-shape, while the right end can fold into a U-shaped structure (Fig. 1) (reviewed in Astell, 1990). The terminal sequences of autonomous parvoviruses are not perfectly symmetrical palindromes; thus, some nucleotides remain unpaired when the DNA is in its maximally hydrogen-bonded structure. These nucleotides form a "bubble" in the stem of the hairpin, that is otherwise a duplex (Fig. 1). The termini of parvoviruses can be found in two alternative sequence orientations, inverted complements of each other, named "flip" and "flop". The right end of all vertebrate parvoviruses can exist, at a ratio specific for each virus, in either of the two orientations (Astell *et al.*, 1979, Chen *et al.*, 1988, Deiss *et al.*, 1990, Diffoot

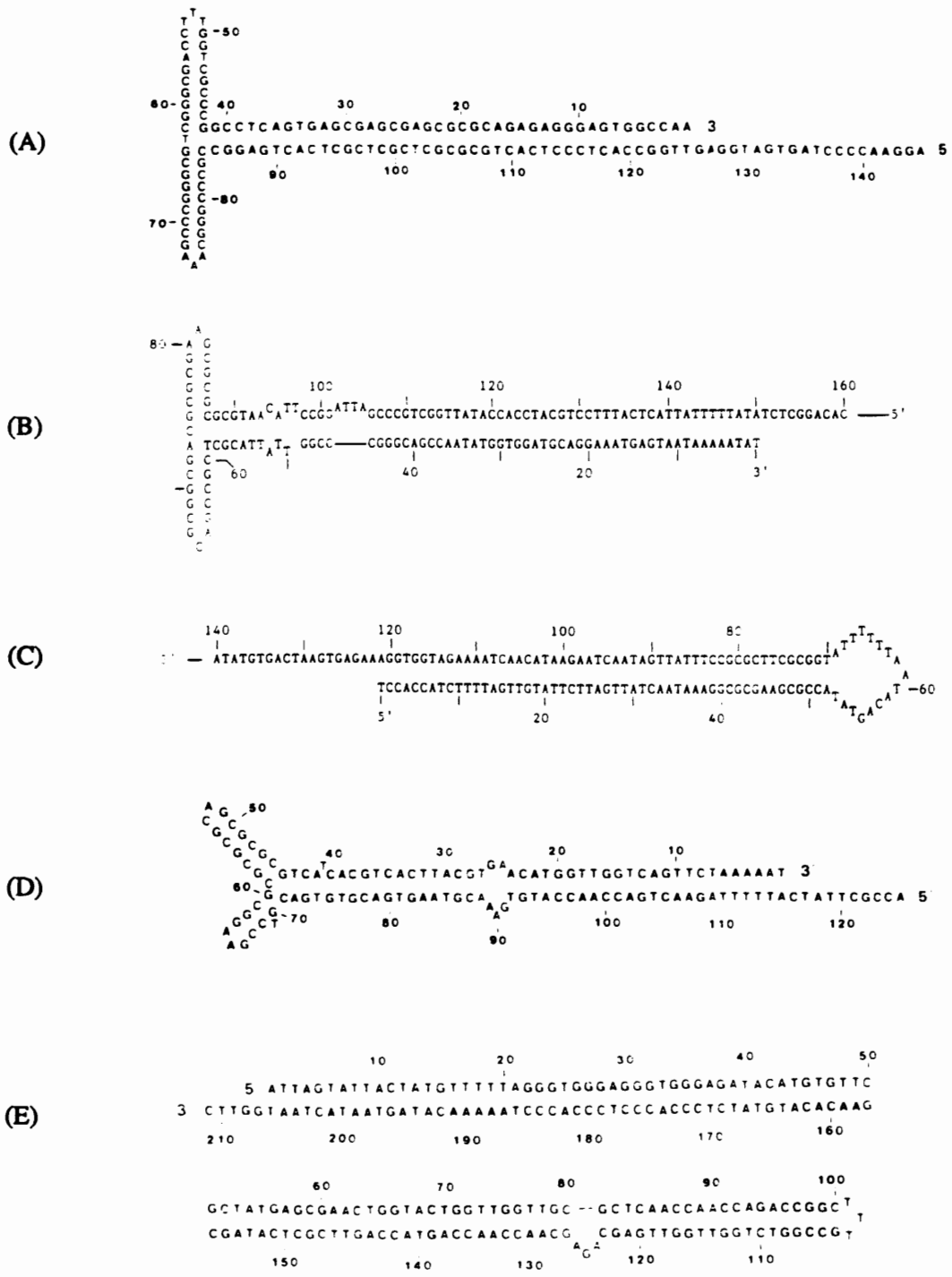


Figure 1. Nucleotide sequence and predicted secondary structure of terminal hairpins of parvoviruses (minus strands).
(A), Left and right ends of AAV-2; (B), left end of BPV; (C), right end of BPV; (D), left end of MVM(p); and (E), right end of MVM(p). The sequences shown correspond to the flip orientation Reprinted from Astell., 1990 and Chen *et al.*, 1986.

et al., 1989, Lusby *et al.*, 1980), but the left termini of rodent-like parvoviruses have only been found in one sequence orientation (Astell *et al.*, 1979). The flip and flop conformations are found with the same frequency in the AAV and B19 termini (Deiss *et al.*, 1990, Lusby *et al.*, 1980). Despite the sequence differences, the role of these termini in DNA replication (Rhode and Klassen, 1982, Salvino *et al.*, 1991, Senepathy *et al.*, 1984, Shull *et al.*, 1988, Snyder *et al.*, 1990 (b)), encapsidation (McLaughin *et al.*, 1988), excision of viral DNA from plasmid (Samulski *et al.*, 1983), and integration for AAV (Samulski *et al.*, 1989) is identical for all parvoviruses.

The genomes of autonomous and dependent parvoviruses are similarly organized and contain two main non-overlapping genes on the plus viral strand only (Astell *et al.*, 1983, Bloom *et al.*, 1988, Chen *et al.*, 1986, Diffoot *et al.*, 1993, Kariatsumari *et al.*, 1991, Rhode and Paradiso, 1983, Shade *et al.*, 1986, Srivastava *et al.*, 1983). A major open reading frame (ORF), located in the left half of the genome, codes for the nonstructural proteins (NS), while a second ORF, on the right side of the genome encodes the structural polypeptides (Cotmore and Tattersall 1986, Rhode and Paradiso, 1983). BPV, in addition, has a smaller middle ORF, whose gene product(s) is yet unknown (Chen *et al.*, 1986). Similarly, other viruses, such as B19, have several small ORFS of unknown significance (Luo and Astell, 1993). Parvoviruses have 2 or 3 TATA-based promoters. One promoter, generally the strongest, is found at the extreme left of the genome, m.u. 3 to 6, and a second promoter is present at m.u. 36 to 40. BPV and AAV have a third promoter at m.u. 13 and 19, respectively.

Viral nonstructural proteins

The viral left ORF codes for two major nonstructural proteins (NS1 and NS2) in rodent parvoviruses (Cotmore *et al.*, 1983), or four in the case of AAV-2 (Rep 78, 68, 52 and 40) (Mendelson *et al.*, 1986). B19, BPV, MVM, and LPV code for additional smaller non structural proteins (Cotmore *et al.*, 1983, Lederman *et al.*, 1984, 1987 (a), Matsunago and Matsuno, 1983,

Ozawa and Young, 1987). Sequence similarity has been found in the NS proteins of the different parvoviruses (reviewed in Iversen and Rhode, 1990). The larger nonstructural proteins are located in the nucleus and are phosphorylated (Hunter and Samulski 1992, Lederman *et al.*, 1987 (a)). The nonstructural proteins have an ubiquitous role in the parvoviral life cycle. They are involved in the regulation of transcription (Doerig *et al.*, 1990, Rhode, 1985, Tratschin *et al.*, 1986), in the DNA replication process (Tratschin *et al.*, 1984, Hermonat *et al.*, 1984), in proviral rescue (Hermonat *et al.*, 1984, Rhode, 1989), and suppression of oncogene-mediated cellular transformation (Hermonat, 1989).

Gene expression

In spite of a similar genome organization, gene expression of vertebrate parvoviruses differs. Rodent-like parvoviruses use their two promoters, one in front of each ORF, to initiate transcription of the structural and nonstructural proteins. Alternate splicing is further used to generate the different viral proteins. All transcripts co-terminate at the polyA signal located at the right end of the genome (Alexandersen *et al.*, 1988, Pintel *et al.*, 1983). In AAV, all three promoters are used, and the two different classes of AAV Rep proteins (Rep 78 and 68, and Rep 52 and 40) are generated by use of two different promoters, p4 and p19 (Green and Roeder, 1980). Temporal differences in expression of the viral proteins have been observed for BPV and the rodent-like parvoviruses: the nonstructural products appear prior to the capsid proteins (Lederman *et al.*, 1987 (a), Schoborg and Pintel, 1991). On the other hand, all three of the AAV promoters appear active at the same time. For autonomous and dependoparvoviruses, transcription regulation is modulated by the strength of the promoters and by NS1 or Rep, which regulates both its own and the downstream promoters (Beaton *et al.*, 1989, Doerig *et al.*, 1990, Hanson and Rhode, 1991, Rhode, 1985). Although NS1 has been shown to regulate the late promoter expression via a *trans*-activation responsive element (*tar*), located upstream of the late

promoter, other *cis*-acting upstream elements appear required for *trans*-activation of p38 in MVM and LuIII (Gu *et al.*, 1992). NS2 also affects autonomous parvovirus gene expression in a positive way (Li and Rhode, 1991). Regulation of the relative amounts of VP1 and VP2 is obtained by alternate splicing of the RNAs that originate from the right ORF.

B19 has a very different transcription pattern, since all mRNA transcripts originate from the left promoter, are extensively processed, and do not coterminate at the right end of the genome (Ozawa *et al.*, 1987). Cell-specific temporal expression of B19 proteins appears therefore independent of promoter strength and is controlled by transactivation by NS1, by upstream *cis* signals, and also possibly by RNA processing events, or protein stability (Doerig *et al.*, 1990, Liu *et al.*, 1991, 1992).

The expression pattern of BPV appears, so far, much closer to that of B19 than to that of any of the other parvoviruses, since only the left promoter directs transcription (Diffoot, 1992). The mechanism of regulation of the BPV transcription is yet unknown; it may not occur through a *tar* sequence, since none similar to those identified in other parvoviruses have been found in the genome (Chen *et al.*, 1986).

VIRAL REPLICATION

Viral host cell interaction

The early events involved in replication of parvoviruses are largely unknown. The cell-surface receptor of MVM appears to contain a sialic acid molecule (Cotmore and Tattersall, 1987). Viral entry occurs by endocytosis of uncoated pits and viral uncoating requires exposure to a low pH in the endosomal compartment (Basak and Turner, 1992). Replication studies with host range variants of CPV revealed that host cell restriction is not due to restrictive adsorption to the cell surface, as might be expected since these genomes differ in their capsid sequence, but rather is due to intracellular events (Horiuchi *et al.*, 1992).

Due to the limited coding capacity of their genomes, parvoviruses depend extensively on the host cell for replication. In fact, parvoviruses do not code for any replicative enzymes and must use cellular polymerase(s) for DNA replication. Although viral DNA replication is influenced by inhibitors of DNA polymerase α, β or δ (Pritchard *et al.*, 1981, Kolleck *et al.*, 1982), the cellular polymerase(s) involved in the *in vivo* replication of parvoviral DNA has not been identified yet (reviewed in Berns, 1990). Topoisomerase appears required for autonomous viral DNA replication (Gu and Rhode, 1991). Replication of viral DNA is therefore expected to be entirely dependent upon cellular functions, and particularly upon functions provided during the DNA synthesis phase. Indeed, autonomous parvoviruses require host factors present in the S-phase of the cell cycle for replication *in vivo* and *in vitro* (Rhode, 1973, Siegl and Gautschi, 1973, Tennant *et al.*, 1969). Since parvoviruses do not have the ability to induce resting cells to enter the S-phase, this requirement is provided *in vivo* by infecting actively dividing tissues (Pattison, 1990). Similarly, various treatments are used to synchronize the cell population for *in vitro* productive infections (Parris and Bates, 1976, Siegl and Gautschi, 1973). Experiments with cells synchronized at various phases showed that viral entry and intracellular accumulation can occur in asynchronous cells, whereas initiation of viral DNA synthesis and gene expression are S-phase dependent events (Cotmore and Tattersall, 1987, Rhode, 1973, Tattersall, 1972, Wolter *et al.*, 1980). The nature of the S-phase factor remains undetermined. There have been several reports of binding of cellular proteins to terminal fragments or complete genomes of autonomous parvoviruses (Avalosse *et al.*, 1989, Barrijal *et al.*, 1992, Chow *et al.*, 1986, Metcalf *et al.*, 1990, Willwand and Kaaden, 1990, Wobbe and Mitra, 1985). While two of these cellular proteins were identified as nucleolin (Avalosse *et al.*, 1989, Barrijal *et al.*, 1992) and nuclear lamin (Wobbe, 1984, Wobbe and Mitra, 1985), only the cellular protein detected by Metcalf (1990) was shown to be cell cycle regulated. Growth of MVM, and possibly of other rodent parvoviruses, also appears

dependent upon some undefined cellular factors that are developmentally regulated (Tattersall and Bratton, 1983, Spalholtz and Tattersall, 1983).

AAVs require the presence of a coinfecting helper virus -adenovirus, herpesvirus, or vaccinia virus- for a full infection (Atchison *et al.*, 1965, Buller *et al.*, 1981, Schlehoffer *et al.*, 1986). In the absence of a helper virus, AAV integrates, site-specifically, into the host chromosome 19, either as a single proviral copy or as a multiple viral element (Cheung *et* al, 1980, Kotin *et al.*, 1990). Under favorable conditions, such as viral coinfection, the integrated AAV is rescued, most probably by viral and cellular enzymes, and proceeds through a normal infection (Gottlieb and Muzyczka, 1988, Hermonat *et al.*, 1984). The requirement of AAV for a coinfecting virus is, however, not absolute since viral amplification has been described in some helper virus free cells lines that have been synchronized, UV-irradiated, or treated with chemical carcinogens prior to infection (Schlehofer *et al.*, 1986, Yakobson *et al.*, 1987, 1989, Yalkinoglu *et al.*, 1988, 1991). These experiments suggest that the helper virus functions are not directly involved in the AAV replication process. The adenovirus genes required for AAV replication are, in fact, not replicative genes (reviewed in Berns, 1990). On the other hand, a subset of herpes simplex virus replicative genes can provide AAV helper functions (Weindler and Heilbronn, 1991). Nevertheless, in view of the results of Schlehofer, Yakobson, and Yalkinoglu, the AAV-helper gene products of either viruses are likely to have an indirect effect on the cells that render them permissive for AAV replication.

Involvement of viral *cis* and *trans* elements

Two regions of the parvoviral genome are essential for DNA replication: the terminal repeats and the products of the left ORF. Mutational studies of MVM (Merchlinsky *et al.*, 1983, Salvino *et al.*, 1991), H-1 (Rhode and Klassen, 1982), AAV (Hermonat *et al.*, 1984, Samulski *et al.*, 1983, Senapathy *et al.*, 1984), LuIII (Rhode, 1989) and BPV (Shull *et al.*, 1988) indicate that

the termini act in *cis* for viral replication. It is proposed that, when folded back on itself, the 3' OH end of the viral DNA serves as primer for DNA synthesis. The secondary structure of the termini appears to be of major importance (Bohenzky *et al.*, 1988, Lefebvre *et al.*, 1984). An additional *cis* acting element that would function as an origin of replication (*ori*), in a species-specific manner has been identified in MVM(p), approximately at m.u. 94 (Salvino *et al.*, 1991). Similarly, AAV might contain an additional internal *ori* (Hong *et al.*, 1992). The AAV termini have the capacity of self repair if deleted, since deletion of one terminus allows rescue and replication of the AAV virus (Samulski *et al.*, 1983, Senapathy *et al.*, 1984). On the contrary, both termini, or two identical functional termini, must be present on the same DNA molecule to support replication of autonomous parvoviruses (Merchlinsky *et al.*, 1983, Salvino *et al.*, 1991, Shull *et al.*, 1988, Tam and Astell, 1993).

The products of the left ORF are required in *trans* for AAV and autonomous parvovirus replication (Hermonat *et al.*, 1984, Rhode, 1989, Senepathy *et al.*, 1984, Tratschin *et al.*, 1984). The current replication models (Astell *et al.*, 1985, Berns and Hauswirth, 1984, Chen *et al.*, 1989, Cotmore *et al.*, 1989, and reviewed in Berns, 1990, Cotmore and Tattersall, 1987) propose that resolution of viral DNA intermediates rely on a site-specific nickase. Such enzymatic properties have mainly been investigated for parvoviral nonstructural proteins, and it has been shown that Rep 78, Rep 68, and MVM NS1 have an ATPase and ATP-dependent helicase activity (Wilson *et al.*, 1991, Im and Muzyczka, 1990, 1992). In addition, Rep 78 and 68 proteins have been shown to bind to the AAV *ori* (Im and Muzyczka, 1989, 1992) and to nick AAV DNA site-specifically at the terminal resolution site (trs) *in vitro* (Im and Muzyczka, 1990, 1992, Snyder *et al.*, 1990 (b)). Functional domains studies of Rep proteins suggest that the amino terminus binds to the AAV terminus, while the central region stabilizes the binding (Yang and Trempe, 1993).

New data reveal that NS2 is required, in a cell specific manner, for replication of MVM and H-1. NS2 is apparently involved in the accumulation of viral DNA replication intermediates

(Cater and Pintel, 1992, Li and Rhode, 1991). Similar observations have been made for AAV Rep 52 and Rep 40 (Chejanovsky and Carter, 1989).

The capsid proteins, in addition to being required for encapsidation and infectivity, play a role in the viral DNA replication process by allowing the accumulation of progeny single-stranded viral DNAs (Hermonat *et al.*, 1984, Trastchin *et al.*, 1984, Tullis *et al.*, 1993).

DNA replication models

Parvoviruses, like others viruses with linear genomes, evolved a strategy to replicate their ends faithfully; these viruses use the terminal DNA sequences as primers, eliminating the need for RNA, protein primers, or primase. Due to the difference in encapsidation pattern and terminal sequences of the parvoviral genomes, several models have been proposed for their replication (Astell *et al.*, 1985, Berns and Hauswirth, 1984, Chen *et al.*, 1989, Cotmore *et al.*, 1989, and reviewed in Berns, 1990, Cotmore and Tattersall, 1987). Viral DNA replication is proposed to occur by continuous leading strand synthesis. In fact, no Okazaki fragments were detected during H-1 replication (Tseng *et al.*, 1978). All models are derived from the model proposed by Cavalier-Smith (1974), for the replication of eukaryotic chromosome ends. They all include hairpin transfer to resolve replicative intermediates.

The simplest model is the one proposed for the replication of AAV by Berns and Hauswirth (1984) (Fig. 2). Initiation of the first round of DNA synthesis occurs either from the 3' termini of the hairpinned genome (forms II or III). After elongation, nicking of the duplex linear intermediate obtained occurs site-specifically at the terminal resolution site (*trs*) on the parental strand. The terminal palindrome is then transferred to the progeny strand, and inverted during the transfer, changing from flip to flop or vice-versa. The new 3'OH end on the parental viral strand is used, as a primer, to repair the terminal sequence to a full length, open duplex configuration. A second round of replication can then take place. Evidence supporting this model has accumulated:

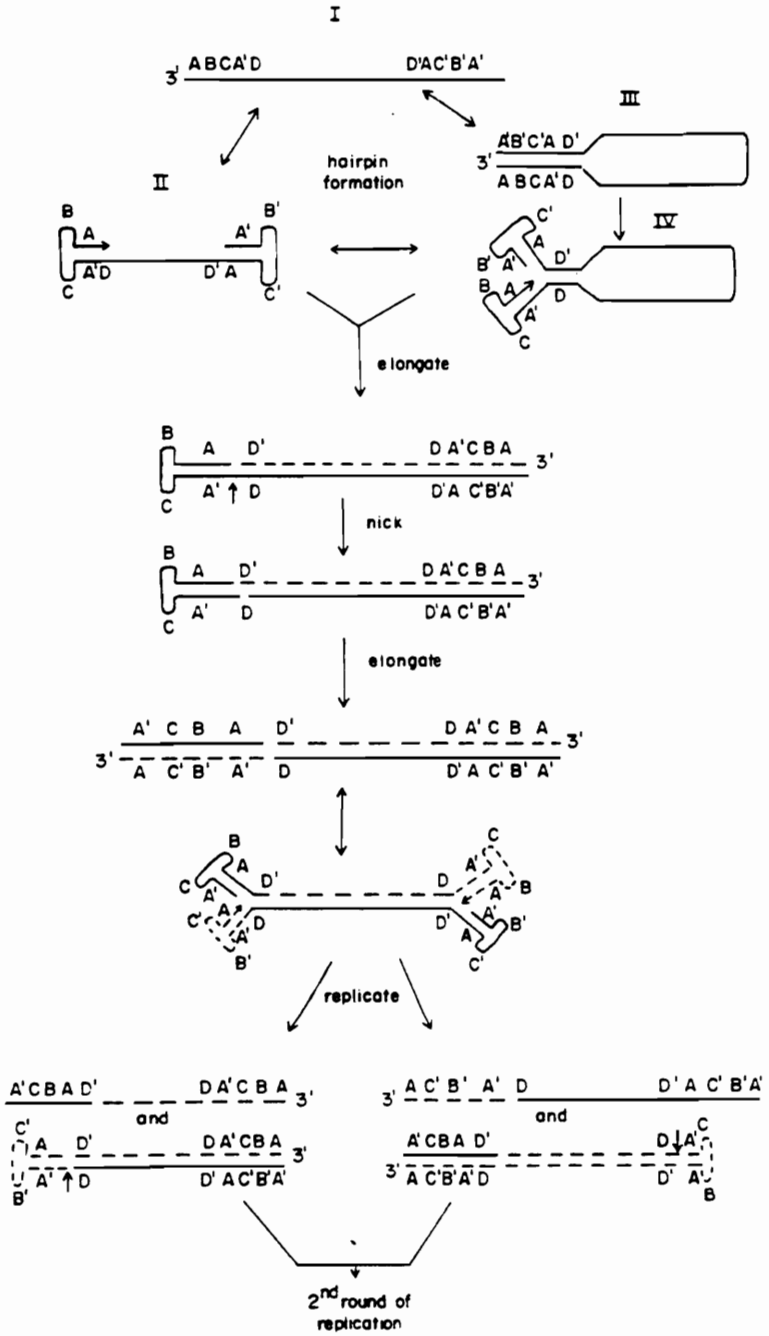


Figure 2. Model for AAV DNA replication.
The terminal sequence domains represented by primed letters are the complements of those represented by unprimed letters. Reprinted from Berns and Hauswirth (1984)

it has been shown that initiation can occur from the terminal structure of the AAV DNA (Srivastava, 1987), that a covalently joined dimeric intermediate is formed during *in vitro* replication (Hong *et al.*, 1994, Straus *et al.*, 1976). Rep 68 and Rep 78 proteins, which possess a nickase activity, can resolve the DNA site specifically at the *trs*, *in vitro*, and have been found attached to the 5' end of the nick (Im and Muzyczka, 1990, 1992, Snyder *et al.*, 1990 (a) and (b)).

A second model has been proposed for the replication of autonomous parvoviruses, based on data from the rodent-like parvoviruses (Astell *et al.*, 1985, Cotmore *et al.*, 1989, Cotmore and Tattersall, 1987)(Fig. 3). To account for the lack of sequence inversion at the 3' termini of rodent parvoviruses and for the uniqueness of the virion DNA polarity, the modified rolling hairpin model proposes that hairpin transfer occurs only at the right end. After initiation of replication from the viral minus strand (Fig. 3, step 1), the left end of the monomer duplex replicative form (RF) is not "resolved", rather the right hairpin is nicked, copied and displaced directly (steps 4, 5). Cotmore (1989) proposes that an interstrand ligation might occur at step 2. A rabbit-eared structure is then formed (step 6) and displacement leads to a dimer intermediate (steps 7, 8). This dimer is resolved by a nick proposed to occur solely at the right end of the parental strand (step 9). The nick created is then used for extension of the parental strand (step 10). Production of the progeny minus strand is proposed to occur by repetitive displacement synthesis from the plus strand template (steps 12, 13). In support of this model, it has been reported that the palindromic termini of the MVM RF can exist in the closed circular as well as in the extended forms, and that plasmids containing the MVM right palindrome could be resolved (Cotmore *et al.*, 1989, Cotmore *et al.*, 1992). NS1 of MVM is found covalently linked to the 5' end of the duplex RF and progeny single-strand DNA (Cotmore and Tattersall, 1988, 1989).

This model, however cannot explain replication of non rodent-like parvoviruses, such as BPV and LuIII, that have different genome characteristics. Chen *et al.* (1989) developed a simpler model, that can account for the replication of these viruses, as well as for the autonomous and

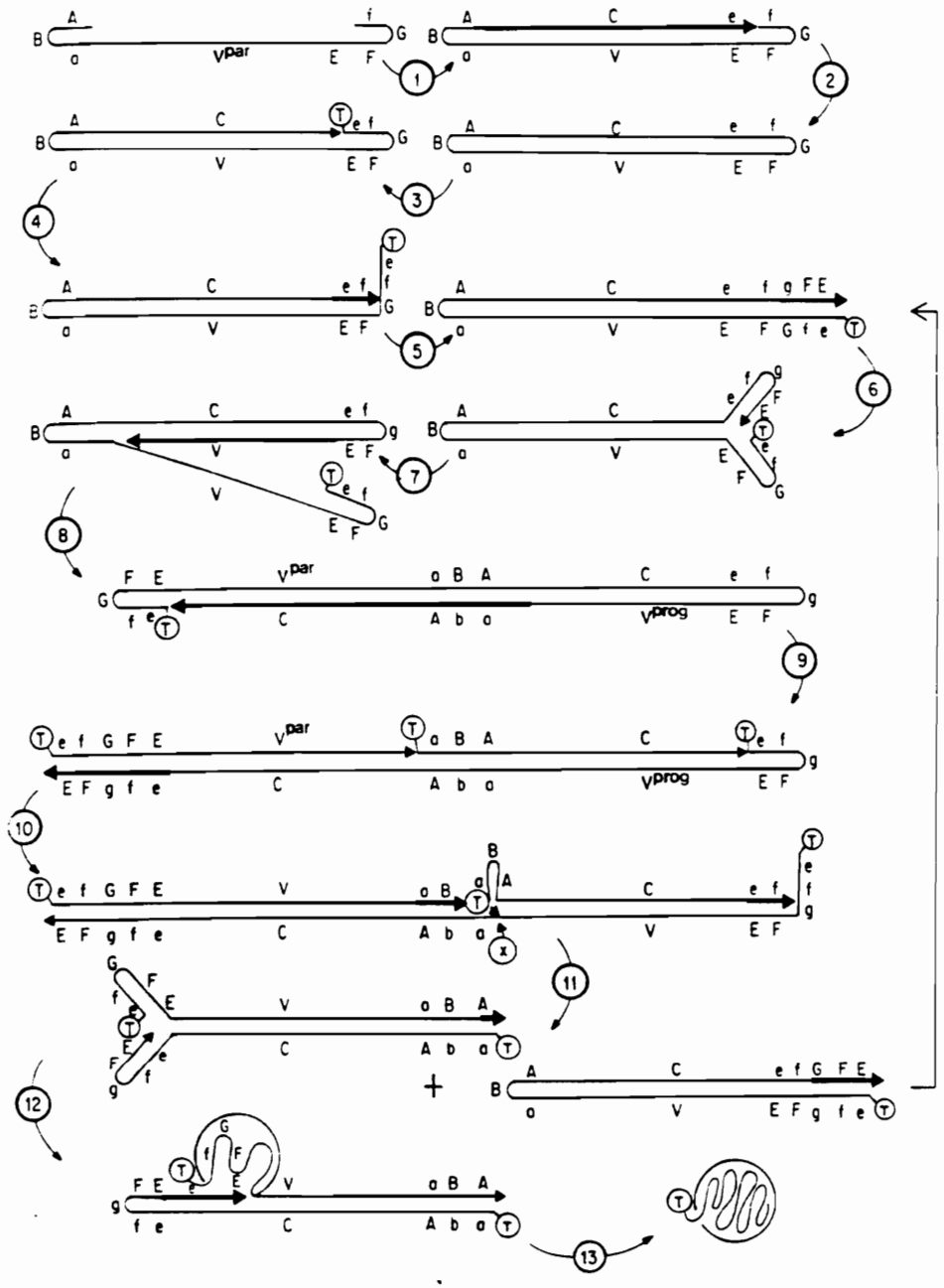


Figure 3. Modified "rolling hairpin" model for rodent parvoviruses replication.
Upper and lowercase letters denotes sequences complementary to one another. ABa, 3'-terminal palindrome of virion strand; FGf, 5'-terminal palindrome of virion strand; e, 18 to 26-nucleotide sequence present in replicative intermediate DNA; V, virion strand; V^{par}, parental virion strand; V^{prog}, progeny virion strand; C, complementary strand; T, site-specific nucleases; X, site of possible topoisomerase action. Reprinted from Berns (1990).

dependoparvoviruses. The kinetic model is based on the simple hairpin transfer model and proposes that DNA replication proceeds through four monomer double stranded RFs and that hairpin transfer occurs on the progeny as well as on the parental strand of the dimer intermediates. The model proposes that the hairpin transfer rate constants differ for each extremity, depending on the polarity of the DNA and on the primary and secondary structure of the hairpin.

Encapsidation

Encapsidation of progeny strands is proposed to be non-selective, since for several parvoviruses studied, RF polarity and sequence distributions are identical to the virion distributions (Astell *et al.*, 1985, Berns and Hauswirth, 1984, Chen *et al.*, 1988). Packaging signals are unknown, but experiments on AAV show that they reside in the *ori*s (McLaughin *et al.*, 1988). Encapsidation occurs in the nucleus and is driven by the capsid proteins, which have the ability to self-assemble (Kajigaya *et al.*, 1991). Structural proteins have been shown to bind to parvoviral termini (Lederman *et al.*, 1987 (b), Willwand and Kaaden, 1990, Willwand and Hirt, 1991) and encapsidation models postulate that the capsid proteins displace the progeny single-stranded viral DNA for direct encapsidation (Willwand and Kaaden, 1990). Neutralization of the DNA negative charges, during packaging, is proposed to occur through interaction with the basic amino terminal sequences of VP1 (Cotmore and Tattersall, 1987).

PARVOVIRUSES AND CELLULAR ONCOSUPPRESSION

AAVs and most autonomous parvoviruses can be recovered from tumor cell lines or naturally occuring tumors. Some of the rodent parvoviruses were actually originally isolated from tumor cells. Parvoviruses have tumor suppressive properties: they can inhibit spontaneous cell transformation, reduce metastasis formation *in vivo*, and prevent induced tumoregenesis *in vitro* (reviewed in Rommelaere and Tattersall, 1990). However, they do not have the ability to destroy

existing tumors. Association of parvoviruses with neoplastic cells most probably result from the requirement for active cell division, and, in the case of AAV, from the oncogenic nature of its coinfecting helper viruses. The mechanism of oncosuppression by parvoviruses is yet unknown. It has been shown that Rep 78 possess antiproliferative properties *in vitro* (Hermonat, 1989). AAV, in addition, has the ability to modify the course of infection of helper and heterologous non-helper viruses during coinfection (Antoni *et al.*, 1991, Rommelaere and Tattersall, 1990). Integrated AAV can also modulate the cellular phenotype and the cell cycle of the infected cells (Winoccour *et al.*, 1988, 1992). The oncosuppression mechanism of parvoviruses is likely to be complicated due to the very unique interaction of these viruses with their host cells.

The mechanisms of oncogenesis are similarly complex. Several viral and cellular oncogenes have been characterized and their mechanism of action, along with their interaction with various cellular proteins, have been extensively studied (for review, see Cooper, 1990). Several tumor suppressor genes, whose action counterbalances the action of proto-oncogenes, have been identified in normal cells, mainly because of their absence from the genome of tumor cells (reviewed in Weinberg, 1991). Among them, p53, which was first identified as a cellular protein bound to the SV40 T antigen in virus-transformed cells (Lane and Crawford, 1979), was initially thought to act as an oncogene. The tumor suppressor activity of wild type p53 became evident when its growth suppressive properties were shown *in vitro* (Baker *et al.*, 1990, Finlay *et al.*, 1989, Yin *et al.*, 1992), and when it was observed that the p53 gene was deleted or mutated in more than half of almost all human tumors (reviewed in Harris and Hollstein, 1993). Inactivation of p53 by somatic mutation (Sidransky *et al.*, 1991) has been linked to tumor development or/and progression. Association of p53 with cellular oncoproteins, viral oncoproteins, or mutant p53 proteins is similarly presumed to alter the growth suppression properties of p53 (Lane and Crawford, 1979, Momand *et al.*, 1992, Scheffner *et al.*, 1990, Symonds *et al.*, 1991, and reviewed in Levine 1990). p53 has a pleiotropic role in the cell (reviewed in Harris and Hollstein, 1993).

The anti-oncoprotein is involved in the control of the cell cycle and is able to restore the G1/S control point, when introduced as wild type in transformed cells (Yin *et. al.*, 1992). p53 is also implicated in DNA repair and synthesis. It appears to be part of the apoptosis and DNA-damage control pathways. Finally, p53 acts as a modulator of cell differentiation and genomic plasticity (reviewed in Ullrich *et al.*, 1992). However, p53-deficient mice are able to develop normally, but are subject to tumors earlier than normal mice, suggesting the p53 is dispensable for development but essential for preventing tumors (Donehover *et al.*, 1992). p53 has been shown to act biochemically as a transcriptional activator by binding to p53-specific binding sequences (Farmer *et al.*, 1992, Vogelstein and Kinzler, 1992, Zambetti *et al.*, 1992). Interestingly, p53 can also repress transcription from TATA boxes, by binding to TATA binding protein (TBP)(Mack *et al.*, 1993, Seto *et al.*, 1992). p53 might also have a direct role in DNA replication. It has been shown recently to inhibit DNA replication by inactivating a cellular replication factor, the single-stranded DNA-binding protein complex RPA (Dutta *et al.*, 1993). The activity of p53 must presumably be modulated through the cell cycle in order to take part in so many cellular processes. Regulation most likely occurs by conformational change, post-translational modifications (e. g., phosphorylation), and interaction with other proteins (reviewed in Ullrich *et al.*, 1992).

PURPOSE

Understanding the replication mechanism(s) and cellular interactions during infection is a challenging part of the research on parvoviruses. The small size of these viral genomes results in a rather complex utilization of the coded information and an exquisite dependence on the cellular machinery. As a result, some encoded viral proteins and particular DNA sequences, such as the termini, appear to have an ubiquitous role in the life cycle of all parvoviruses. Some of the viral factors or DNA sequences required for replication are now well identified. *In vitro* replication assays have been developed, and the different replication models are being tested for accuracy. Much less is known, however, about the cellular factors involved in the replication process. My dissertation will try to provide new insights on some cellular factors and viral elements that might be involved in the replication process.

The first area of research will focus on cellular factors that bind to the origins of replication of parvoviruses. Proteins from different cell types have been shown to interact with the termini of several autonomous parvoviruses. Although most of these proteins are yet unidentified, they probably have a precise function in the viral life cycle, given the role of the parvoviral termini. Initiation of autonomous parvoviruses DNA replication, which occurs through the termini, requires cellular factor(s) present in the S-phase of the cell cycle. Recent experiments

showing helper-independent replication of AAV, suggest that the AAVs might also depend on particular cellular factors expressed during the DNA synthesis phase for their replication. In order to gain more insight onto the nature of this cellular S-phase factor, I will use mobility shift assays to detect binding of S-phase cellular proteins to the origins of replication of homologous and heterologous parvoviruses. If any proteins are detected, I will begin to characterize them.

The second research project will study the role in replication of some sequences within the BPV termini. A conserved feature of all parvoviral termini is their ability to fold into a hairpin, of either a U- or T-shape, providing a free 3' OH end for initiation of DNA replication. In addition, the terminal palindromic sequences of almost all autonomous parvoviruses contain mismatched/unpaired nucleotides, forming a bubble, in the stem of their hairpin. To investigate the function of this bubble, mutant BPV clones that lack this region will be constructed and assayed for replication.

Specifically, my dissertation will address the following questions:

1. Do cellular protein(s) interact with the origins of replication (*oris*) of AAV? If so, are these proteins or proteins from other cell types capable of binding to *oris* of heterologous parvoviruses?
2. What is the size of these *ori*-binding cellular proteins and do they share common characteristics with other known proteins?
3. Are the mismatched/unpaired nucleotides forming the bubble of the BPV left terminus essential to the viral life cycle?

CHAPTER II

BINDING OF CELLULAR PROTEINS OF DIFFERENT CELL TYPES TO PARVOVIRAL *ORIS*

INTRODUCTION

The genome of parvoviruses is a linear single-stranded DNA molecule of about 5000 bases, with imperfect palindromic sequences at both ends (Siegl *et al.*, 1985). Both the virally-coded non-structural proteins and the terminal repeats, which function as origins of DNA replication (*oris*), are required for parvoviruses replication (for reviews see Astell, 1990, Berns, 1990, Cotmore and Tattersall, 1987). Due to the limited coding capacity of parvoviral genomes, almost all genes have been identified and the functions necessary for replication that are not virally encoded must be provided by the cellular machinery.

Autonomous parvoviruses are dependent on an S-phase host factor, provided by actively growing cells, for a productive infection both *in vitro* and *in vivo* (Rhode, 1973, Siegl and Gautschi, 1973, Tennant *et al.*, 1969). The use of synchronized cell culture systems showed that initiation of viral DNA synthesis and gene expression are the two S-phase dependent events in the parvovirus infectious cycle (Cotmore and Tattersall, 1987, Wolter *et al.*, 1980). Parvoviruses from the dependent group, adeno-associated viruses (AAVs), were previously considered to

absolutely require coinfection with a helper virus - adenovirus, herpesvirus, or vaccinia virus - for a full infection (for review, see Carter, 1990). However, AAV amplification without a helper has been described in some cells lines that have been synchronized, UV-irradiated, or treated with chemical carcinogens prior to infection (Schlehofer *et al.*, 1986, Yakobson *et al.*, 1987, 1989, Yalkinoglu *et al.*, 1988, 1991). The cellular functions that supported helper-independent AAV replication were thought to result primarily from DNA damage, but experiments (Yakobson *et al.*, 1987, 1989) suggested that some of them are linked to S-phase gene expression. This suggests that AAV, like the autonomous parvoviruses, might also depend on a specific event(s) in the S phase of the cell cycle for its replication.

Cellular proteins were shown to bind to the complete genome or terminal fragments of four autonomous parvoviruses: Kilham rat virus (KRV) (Wobbe and Mitra, 1985), minute virus of mice (MVM) (Avalosse *et al.*, 1989, Barrijal *et al.*, 1992, Chow *et al.*, 1986), bovine parvovirus (BPV) (Metcalf *et al.*, 1990), and Aleutian disease virus (ADV) (Willwand and Kaaden, 1990). The BPV DNA-binding protein identified by Metcalf (1990) appeared to be cell cycle-regulated, with its concentration or/and binding affinity for the BPV left *ori* (the *ori* for plus strand synthesis) increased in actively dividing cells compared to contact-inhibited cells, making it a candidate for the S-phase factor.

I carried out DNA retardation assays to determine if cellular proteins prepared from S-phase synchronized HeLa cells would be capable of binding to AAV native *oris* in view of the experiments of Yakobson *et al.* (1987), which revealed helper-independent AAV replication in S-phase synchronized cells. I also investigated interactions between the *oris* of parvoviruses and protein extracts from cells that are not derived from a natural host.

MATERIALS AND METHODS

Cell cultures

Primary bovine fetal lung (BFL) cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT) as previously described (Parris and Bates, 1976). BFL cells were synchronized by hydroxyurea (HU) (Sigma, St Louis, MO) as described by Parris *et al.* (1975). The cells were either released from the drug, after 24 hours, by three washes, grown in complete medium for 4 hours and harvested while in the S-phase (Parris, 1975, Parris *et al.*, 1975), or harvested at the end of the HU block while in the G1/S transition. Contact-inhibited BFL cells were harvested 2 days after confluence (7 days post-seeding).

Substrate-adapted HeLa cells (obtained from G. Attardi) were grown in Eagle's minimum essential medium with 5% bovine calf serum (Hyclone, Logan, UT). The cells were synchronized with HU following the conditions of Yakobson *et al.* (1987). HeLa cells were released from the drug, after 40 hours, by three washes, and grown in complete medium for either 4 or 24 hours before being harvested in S-phase (Pfeiffer and Tolmach, 1967), or at an asynchronous stage, respectively. HeLa cells, for some experiments, were harvested at the end of the HU block. The cell-cycle compartmentalization of the HU-treated HeLa cells was determined by flow cytometry analysis (Konchuba et al., 1989), after staining with propidium iodide (Sigma, St Louis, MO). Some HeLa cells were harvested while in the HU block.

Nuclear extracts

BFL nuclear extracts were prepared as described by Metcalf *et al.* (1990). HeLa cells were lysed using the technique described by Attardi and Attardi (1967) with the exception that homogenization was performed in a Dounce homogenizer, pestle A, with approximately 40 strokes. HeLa cell nuclei were then processed identically to the BFL cell nuclei to obtain nuclear proteins soluble in 1 M NaCl.

Preparation of radiolabeled DNAs

Restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN), and used as directed by the manufacturer. ^{32}P nucleotides triphosphate (3,000 Ci/mmol) used for labeling were from Amersham (Arlington Heights, IL). The BPV left and right termini were obtained from clone pVTC350 (Metcalf *et al.*, 1990) that contains an 809 bp BPV insert with a ClaI site internal to each terminus. Distinct restriction endonuclease sites located outside the BPV insert allowed strand-specific labeling of the viral termini. The BPV left terminus was labeled at the HindIII site on the 3'OH end of the negative strand (flop conformation), while the BPV right terminus was labelled at the BamHI site on the 3'OH end of the positive strand (flip conformation). To obtain a labeled BPV left terminus, pVTC350 was digested with HindIII and the ends were filled in with Klenow enzyme, [α-^{32}P]dCTP, [α-^{32}P]TTP, dGTP and dATP. The BPV clone was then digested with ClaI in order to excise the radiolabeled 191 bp BPV left terminus (nt 1 - 170). The labeled fragment was then purified by low-melting-point agarose gel electrophoresis, eluted by ultracentrifugation (239,000 g for 30 min) and passed over a QuickSpin G-50 column (Boehringer Mannheim, Indianapolis, IN). The BPV right terminus was prepared identically, except that the pVTC350 clone was first digested and labeled at the BamHI site. The labeled BPV right terminus was obtained as a 200 bp fragment (nt 5329 - 5517).

Clone psub201(+) of AAV type 2 (Samulski *et al.*, 1987) was used to prepare the radiolabeled AAV terminus, which was equally labeled on the 3'OH ends of the plus and minus strands. psub201(+) was first digested with XbaI and end-labeled with Klenow enzyme, [α-^{32}P]dCTP, [α-^{32}P]TTP, dGTP and dATP. The clone was further digested with PvuII to excise the 182 bp labeled AAV terminus (nt 4484 - 4652). The radiolabeled fragment was purified identically to the BPV termini.

To form the hairpinned conformation, all radiolabeled DNAs were boiled and quick-chilled immediately prior to use.

Preparation of competitor DNAs

The 210 bp fragment of pUC8 (nt 449 - 659) was obtained by restriction digestion of the plasmid with HindIII and PvuII. The BPV left and right unlabeled termini were obtained after double digestion of pVTC350 with HindIII and ClaI, or BamHI and ClaI, respectively. The AAV competitor DNA was obtained as a XbaI-PvuII fragment of the psub201(+) clone. The competitor DNAs were purified by low-melting-point agarose gel electrophoresis and electroelution. The 123 bp DNA ladder (Gibco BRL, Gaithersburg, MD) was used without any further treatment. Competitor fragments were either boiled and quick-chilled or used in their linear conformation.

DNA retardation assays

DNA retardation assays were performed as described by Metcalf *et al.* (1990). Nuclear extracts (9 µg of protein) were first incubated with 2 to 6 µg of sheared, heat-denaturated salmon sperm DNA, as a non-specific competitor, for 30 min at room temperature in 25 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 2% glycerol, 0.01% Nonidet P-40, 2.6 µg bovine serum albumin. When competition experiments were performed, specific DNA competitor was added after 15 min of incubation with the non-specific competitor and incubation was continued for 15 min. The end-labeled ^{32}P termini (1 to 3 ng, ~2 x 10^4 cpm) were then added to the reaction and incubated for 30 min at room temperature. The binding reactions were immediately electrophoresed at 28 mA on a 4% non-denaturing polyacrylamide gel of 26 cm, in TBE running buffer (0.089 mM Tris, 0.089 mM boric acid, 0.2 mM EDTA [pH 7.7]), at room temperature.

RESULTS

Retardation of the AAV *ori*

Nuclear extracts from HU-synchronized HeLa cells, harvested in the S-phase, were tested in DNA retardation assays for their ability to interact with the hairpinned labeled AAV terminus. HeLa cell nuclear proteins were able to bind the AAV *ori* in a specific manner, as determined by competition experiments (Fig. 4, lane 2). The DNA-protein complex was abolished with a 100-fold excess of unlabeled homologous competitor in the hairpinned or linear conformation (lanes 3, 4). Size-matched DNAs of unrelated sequence such as a 210 bp pUC fragment (HindIII-PvuII fragment)(lane 6 compared to lane 5), or 123 bp-ladder DNA (lane 7 compared to lane 5) were unable to compete at a 50-fold molar excess after being heated and quick-chilled.

To determine if the complex formation was dependent on S-phase proteins, I prepared extracts from asynchronous HeLa cell cultures and from HU-treated cells harvested either 24 hours after release (returned to asynchronous culture), or unreleased (arrested in the G1/S transition). The cell-cycle compartmentalization of the HU-treated cells was verified by flow cytometry analysis (data not shown). No complex was formed with the two asynchronous extracts (Fig. 4, lanes 8, 9), indicating that the bound protein(s) is controlled during the cell cycle. However, a complex of reduced intensity was observed with extracts of HU-blocked cells (lane 11 compared to lane 10). This complex did not disappear with addition of a 100-fold excess of hairpinned, unlabelled AAV terminus, indicating a non-specific complex (lane 12 compared to lane 11).

A slower migrating DNA species can be observed in lane 1 (unbound AAV probe). This band may correspond to a higher molecular weight species formed between hairpinned termini, since it was not observed when unboiled termini were electrophoresed, and since the homogeneity of the labeled terminus was confirmed by electrophoresis on sequencing gels (data not shown).

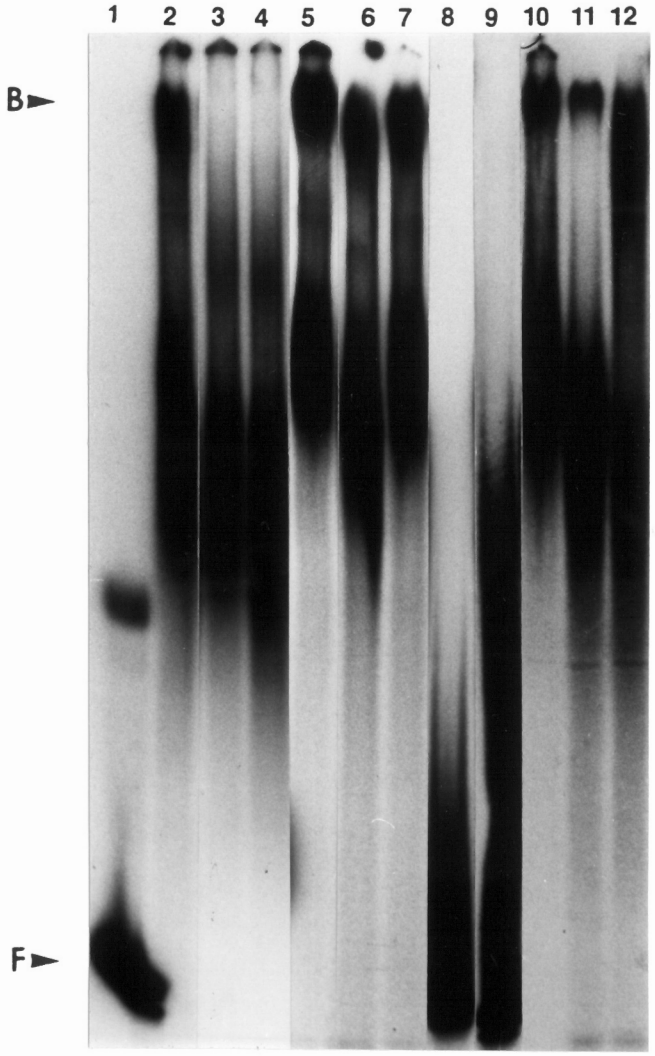


Figure 4. Interaction of HeLa cell proteins with the AAV terminus.
DNA retardation assays using hairpinned AAV terminus were carried out in the absence (lane 1), or presence (lanes 2 to 12) of nuclear extract from HeLa cells. In lane 2 to 7 and 10, the extract was from HU-treated HeLa cells harvested at 4 hours after release, in lane 8 from asynchronous HeLa cells cultures, in lane 9 from HU-treated HeLa cells harvested at 24 hours after release, and in lane 11 and 12 from HU-treated HeLa cells that were harvested at the end of the hydroxyurea-block. Competitors used were the hairpinned AAV terminus at a 100-fold excess (lane 3), the linear double-stranded AAV terminus at a 100-fold excess (lanes 4), a 210 bp fragment of pUC8 at a 50-fold excess (lane 6), the 123 bp DNA ladder at a 50-fold molar excess (lane 7), or the hairpinned AAV terminus at a 100-fold excess (lane 12). F: free probe, B: bound probe.

Interaction of parvoviral *ori* with nuclear extracts of non natural host cells.

Metcalf (1990) and Braddon (submitted) reported that proteins from primary bovine fetal lung cells specifically interacted with the left and right *ori* of BPV, respectively. These data and Fig. 4 showed that proteins from one cell type can recognize the *oris* of the parvovirus for which they are permissive. Although autonomous parvoviruses have a very limited host range, I wondered if these cellular proteins could be conserved across species. I carried out DNA retardation assays to detect the interaction of nuclear extracts from S-phase synchronized BFL and HeLa cells with the *oris* of heterologous parvoviruses, for which these cells are not natural hosts. Fig. 5 (lane 2) shows that BFL cellular proteins bind to a labelled AAV terminus. As previously observed for the AAV/HeLa cell complex (Fig. 4), both the hairpinned and extended forms of the AAV *ori* competed for the binding at a 100-fold excess (Fig. 5, lanes 3, 4). Unrelated DNAs were also tested and did not compete (lanes 6, 7 compared to lane 5). This DNA-protein interaction also appeared to be restricted to nuclear extracts prepared from S-phase synchronized cells, as indicated by the absence of binding using extracts from contact-inhibited (lane 8) or HU-treated but unreleased BFL cells (lane 9).

I then tested the ability of proteins from HeLa cells to interact with the two origins of replication of BPV. Figs. 6 and 7 show that cellular factors in S-phase synchronized HeLa cells also have the ability to recognize the BPV right (Fig. 6, lane 2) and left (Fig. 7, lane 2) termini, which differ greatly both in sequence and secondary structure (Chen et al., 1986). The specificity of the shifted bands was investigated by competition experiments: addition of unlabeled BPV right terminus, at a 130-fold excess, in hairpinned or linear conformation, caused the disappearance of the BPV right terminus/HeLa cell complex (Fig. 6, lanes 3 and 4). The linear form of the BPV left terminus appeared to be a more efficient competitor than its hairpinned homolog for the interaction between the BPV left *ori* and the HeLa cell extract (Fig. 7, lanes 3, 4). Other DNAs were used to demonstrate the specificity of complex formation by lack of

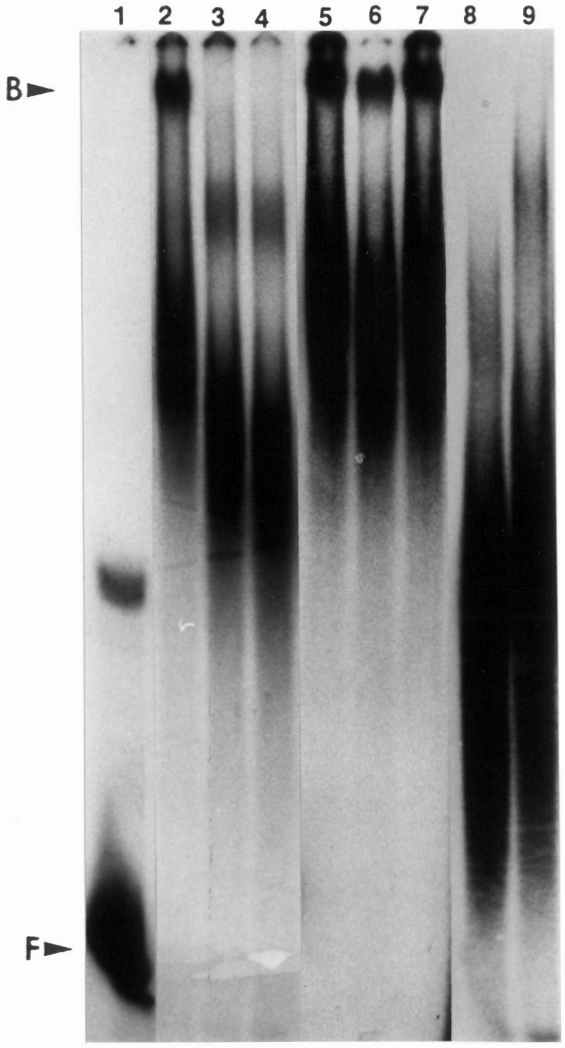


Figure 5. Binding of bovine cell proteins to the AAV terminal sequences.
The AAV terminus was electrophoresed alone (lane 1), or after incubation with nuclear extract of BFL cells (lane 2 to 9). The nuclear extract was prepared from S-phase, HU-treated BFL cells (lanes 2 to 7), from contact-inhibited BFL cells (lane 8), or from HU-treated BFL cells that were harvested unreleased (lane 9). Unlabeled homologous terminus was added, at a 100-fold excess over the probe, in its hairpinned (lane 3), or linear double-stranded conformation (lane 4). A 210 bp fragment of pUC8 (lane 6), or the 123 bp DNA ladder (lane 7) was added to the binding reaction as competitor at a 50-fold molar excess over the probe. F: free probe, B: bound probe.

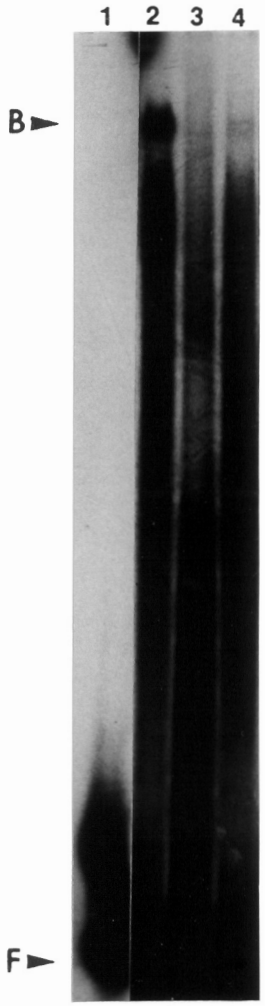


Figure 6. Recognition of the BPV right terminus by HeLa cell proteins.
The labeled BPV right terminus was incubated without (lane 1), or with (lanes 2 to 4) nuclear extract from S-phase, HU-synchronized HeLa cells. Unlabeled AAV terminus DNA was added at a 130-fold excess over the probe, in its hairpinned (lane 3), or linear double-stranded conformation (lane 4). F: free probe, B: bound probe.

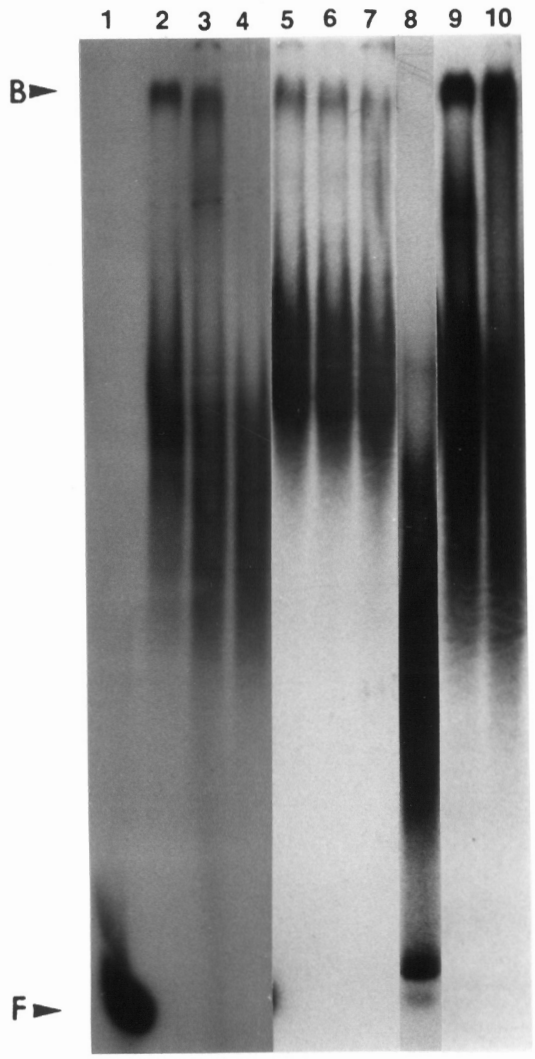


Figure 7. Recognition of the BPV left terminus by HeLa cell proteins.
The BPV left terminus was electrophoresed alone (lane 1), or after incubation with nuclear extract of HeLa cells (lanes 2 to 10). The nuclear extract was prepared from S-phase, HU-treated, HeLa cells (lanes 2 to 7), from asynchronous HeLa cells (lane 8), or from HU-treated HeLa cells harvested unreleased (lanes 9 and 10). Competitors used were the hairpinned BPV left terminus at a 100-fold excess (lane 3), the linear double-stranded BPV left terminus at a 100-fold excess (lanes 4), a 210 bp fragment of pUC8 at a 50-fold molar excess (lane 6), the 123 bp DNA ladder at a 50-fold molar excess (lane 7), or the hairpinned BPV left terminus at a 100-fold excess (lane 10). F: free probe, B: bound probe.

competition (Fig. 7 lanes 6, 7, compared to lane 5). No retardation of the BPV left terminus occurred when nuclear extracts were prepared from asynchronous HeLa cells (Fig. 7, lane 8). However, a complex was seen when nuclear extracts were prepared from HeLa cells harvested at the end of the HU treatment (lane 9), but this complex is nonspecific as a 100-fold excess of terminus did not compete (lane 10).

Competition for parvoviral *ori*-protein complexes by heterologous *oris*

The three unlabelled termini were tested for their ability to compete, in their hairpinned form, which each of the complexes previously observed at excesses of 150 or 200 fold (Fig. 8). The complex formed between BFL cells extract and the AAV *ori* was not competed by an excess of either BPV left (Fig. 8A, lane 3), or right (Fig. 8A, lane 4) terminus.

Fig. 8B shows that the DNA-protein complex formed between the AAV *ori* and HeLa cells proteins was also unaffected by excess unlabeled BPV left (lane 3) or BPV right (lane 4) terminus. These assays also revealed that the BPV left and right termini did not compete with each other in complexes formed with HeLa cell nuclear extracts (Fig. 8C, lane 4, and 8D, lane 3). Finally, the AAV terminus competed for binding to HeLa cell proteins, with the BPV left terminus (Fig. 8D, lane 4) but not with the BPV right terminus (Fig. 8C, lane 3).

DISCUSSION

I present evidence that proteins from nuclear extracts of S-phase HeLa cells, obtained after release from hydroxyurea (HU) synchronization, specifically interacted with the terminal sequences of AAV. DNA retardation assays also revealed specific binding between cellular proteins from HU-synchronized BFL and HeLa cells and *oris* of heterologous parvoviruses, for which the cells are not a natural host. BFL cell proteins were able to bind to an AAV terminus and HeLa cell proteins interacted with both *oris* of the BPV genome. All DNA-protein complexes investigated appeared to be specific for S-phase synchronized cells.

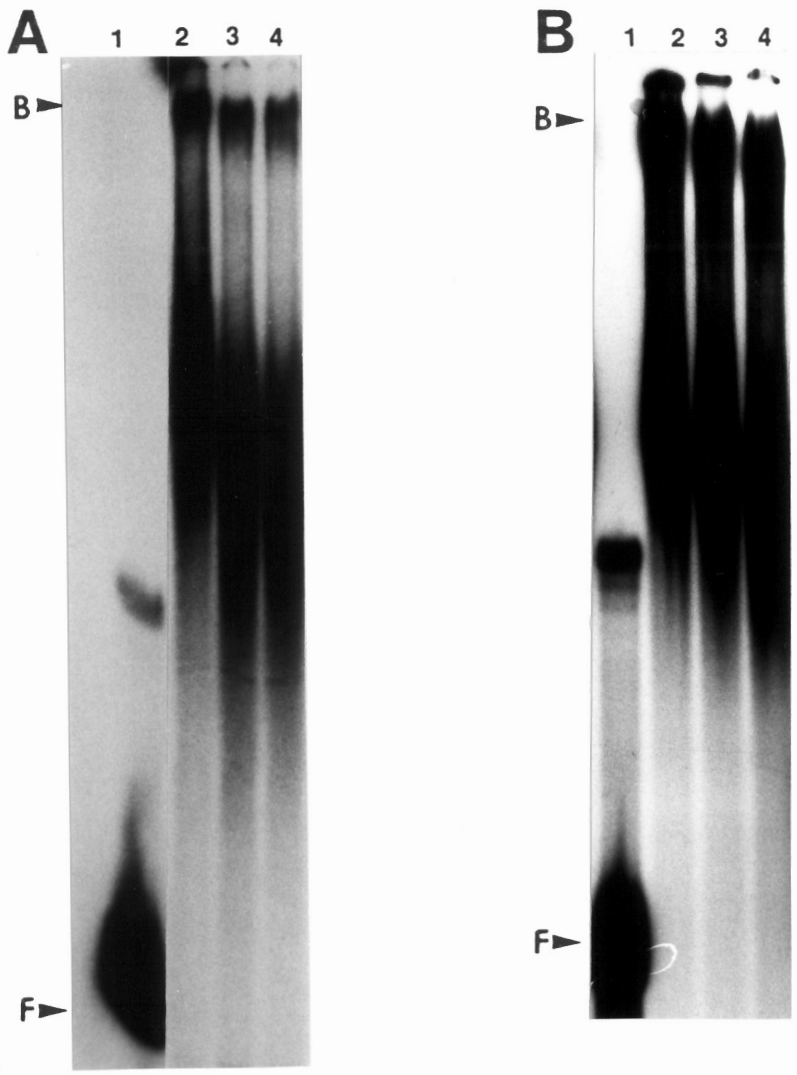


Figure 8. Competition of the DNA-protein complexes by viral heterologous *oris*.
DNA retardation assays were carried out on the complexes formed with the (A) AAV terminus and BFL cell nuclear extract, (B) AAV terminus and HeLa cell nuclear extract, (C) BPV right terminus and HeLa cell nuclear extract, and (D) BPV left terminus and HeLa cell nuclear extract. In each panel, lane 1 shows the labeled parvoviral terminus with no nuclear extract added, lane 2 is the basic mobility shift reaction, and lanes 3 and 4 contain the heterologous competitor DNAs. Competitors DNAs were used in their hairpinned form at a 200-fold excess, except for panel C where a 150-fold excess was used for both competitors. Heterologous competitors were in the following order for lanes 3 and 4 of each panel: Panel (A) BPV left terminus, BPV right terminus; Panel (B) BPV left terminus, BPV right terminus; Panel (C) AAV terminus, BPV left terminus; Panel (D) BPV right terminus, AAV terminus. F: free probe, B: bound probe.

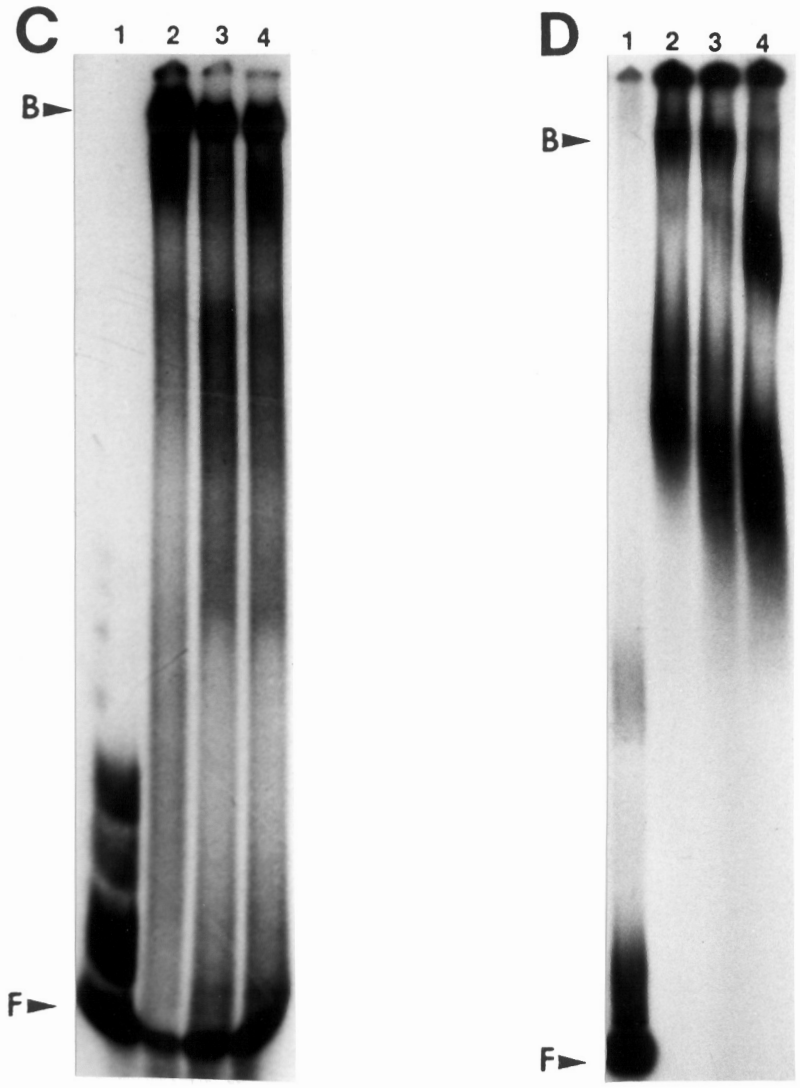


Figure 8. Competition of the DNA-protein complexes by viral heterologous *oris*. Continued

Nuclear proteins from asynchronous or contact-inhibited cells were unable, under my conditions, to interact with BPV or AAV *oris*. Protein extracts prepared from HU-treated, unreleased BFL cells did not bind to an AAV terminus, while similarly treated HeLa cells contained a factor that bound non-specifically to an AAV or a BPV left terminus. HU specifically inhibits ribonucleotide reductase, depleting the cell's stock of nucleotides and arresting cells at the G1/S border (reviewed in Moore and Hurlbert, 1989). Cells, in general, will exhibit altered patterns of RNA and protein synthesis and activate DNA repair processes in response to genotoxic agents such as HU (Herrlich *et al.*, 1986, Moore and Hurlbert, 1989, Sarasin *et al.*, 1985). Severe DNA damage such as amplification or chromosome rearrangement has been noted for cells with abnormal chromosome complements. However, only stress responses are activated if HU is used alone and/or at low concentrations (reviewed in Moore and Hurlbert, 1989). The nonspecific binding seen with HU-treated, unreleased HeLa cells could be due to a stress-related protein that is not seen in BFL cells because of the differences in species origin or transformation state.

In agreement with the results of Im and Muzyczka (1989) and Ashktorab and Srivastava (1989), I did not detect any binding between nuclear extracts of asynchronous HeLa cells and an AAV terminus. However, DNA retardation assays revealed the formation of a specific complex when extracts were prepared from HU-synchronized HeLa cells, using conditions under which most cells are in the S-phase. The fact that binding to an AAV terminus was restricted to S-phase extracts correlates with the finding that helper-independent AAV replication cannot occur in asynchronous, untreated mammalian cells, but can occur after S-phase synchronization (Yakobson *et al.*, 1987), or other treatments (Schlehofer *et al.*, 1986, Yakobson *et al.* 1989, Yalkinoglu *et al.*, 1988, 1991). The precise function of adenovirus as helper during AAV infection is still unknown, although some adenoviral genes important for the AAV replication process have been mapped (reviewed in Carter, 1990). The results of Yakobson *et al.* (1987,

1989) and Yalkinoglu *et al.* (1988, 1991) suggest that the main function of the helper virus is to modulate or alter cellular gene expression, rendering the cells transiently permissive for AAV DNA replication. Adenovirus infection often leads to enhancement of the expression of a variety of cellular genes, some of which are expressed only during S-phase (Levine, 1990, Liu *et al.*, 1985, and reviewed in Boulanger and Blair, 1991, Flint and Shenk, 1989). The HeLa cell protein demonstrated in this study, which exhibited an S-phase-specific binding to the AAV *ori*, could be one of those permissive gene products.

Data obtained by Metcalf (1990), Braddon (submitted), and this study showed that proteins from nuclear extracts of BFL and HeLa cells interact specifically with terminal sequences of homologous and heterologous parvoviruses *in vitro*. Metcalf (1990) also reported that a BPV right terminus competed for binding of BFL cells to the BPV left terminus. Reciprocally, Braddon (submitted) showed that, the left *ori* of BPV could compete for the right *ori* in mobility shift assays. While these data suggest that, in BFL cells, one protein recognizes both *oris* of the BPV genome, my data suggest that another protein, or set of proteins, recognizes the AAV *ori* since neither BPV terminus was able to prevent complex formation between the AAV *ori* and the bovine cells. Even though AAV is not a natural pathogen of cattle, some serotypes can replicate in primary bovine kidney cells in the presence of bovine adenovirus (unpublished data cited in Cukor *et al.*, 1984). The bovine adenovirus may act similarly to the human adenovirus by activating cellular factors which possibly interact with the AAV *oris* to allow its replication in bovine cells.

The data presented here suggest that one protein in HeLa cells might recognize the BPV left and AAV *oris*, while another binds only the BPV right *ori*. A higher affinity for the AAV *ori* is postulated for the former protein since non-reciprocal competitions were observed between the AAV and the BPV left termini. I believe that a separate HeLa cell protein may bind to the BPV right *ori*, since the BPV right terminus neither competes for binding with the other parvoviral *oris*

nor is competed by these *oris*. The significance of the binding of a HeLa cell factor to the BPV termini is unclear, given the fact that BPV has a very restricted host range and has not been shown to replicate in HeLa cells (reviewed in Bates, 1990). Nevertheless, the capacity of these cellular proteins to bind to the *oris* of different parvoviruses suggest that they might have common characteristics.

The DNA-binding proteins studied may recognize primarily the nucleotide sequence of the viral genomes. The competition experiments with homologous termini revealed that the double-stranded linear forms of the three viral *oris* were efficient and sometimes more effective competitors than their hairpinned counterparts. Such findings were unexpected, considering that binding of some cellular (Metcalf *et al.*, 1990, Willwand and Kaaden, 1990) and viral proteins (Im and Muzyczka, 1989, Ashktorab and Srivastava, 1989, Willwand and Hirt, 1991, 1993) to other parvoviral termini were restricted to T-shaped DNA molecules and that the secondary structure of the *ori* is critical for the replication process (reviewed in Astell, 1990).

Interaction of cellular proteins, thought to be involved in the regulation rather than the synthesis of DNA, with origins of DNA replication has been described in several other viral (Dabrowski and Schaffer, 1991, Habiger *et al.*, 1992, Oh *et al.*, 1991, Traut and Fanning , 1988) and eukaryotic systems (Bergemann and Johnson, 1992, Hofmann and Gasser, 1991, Schmidt *et al.*, 1991). Among parvoviruses, the cellular proteins that were shown to interact specifically with viral terminal palindromic sequences have not yet been purified (Chow *et al.*, 1986, Metcalf *et al.*, 1990, Willwand and Kaaden, 1990, Wobbe and Mitra, 1985). Two cellular proteins capable of binding to MVM and KRV DNAs have been characterized as nucleolin and nuclear lamin, respectively (Barrijal *et al.*, 1992, Wobbe, 1984, and Wobbe and Mitra, 1985). However, the binding site of nucleolin does not appear confined to the MVM terminus (Barrijal *et al.*, 1992). The binding site of lamin on the KRV genome is yet undefined since binding of cellular proteins was investigated using the viral replicative form (RF) (Wobbe, 1984, Wobbe and Mitra, 1985).

Due to the fact that the parvoviral termini are critical for replication, a role for the cellular proteins studied here in the replication process would not be surprising.

It is also possible that these *ori*-binding cellular proteins have a role in regulation of gene expression, as noted for other cellular proteins (reviewed in DePamphilis, 1988). The three parvoviral *ori*s used in the DNA retardation assays contain consensus sequences for binding of transcription factors. Among them, the BPV left terminus has binding sites for CTF/NF-I and AP-1, while the AAV terminus has Sp1 consensus binding sequences. Although transcription factor binding sites are present in the right terminus of BPV, it is unlikely that they are used given their location. In B19, nucleotide sequences located within the left terminal palindrome, which contain Sp1 binding sequences (Blundell and Astell, 1989), positively regulate expression of the P6 promoter (Liu *et al.*, 1991). In MVMp, sequences upstream of the P4 promoter have been shown to interact with the transcription factors Sp1 (Pitluck and Ward, 1991), ATF/CREB (Perros *et al.*, 1993), NPY and USF (Gu *et al.*, 1993).

The dependent and autonomous parvoviruses share more common characteristics than previously thought, possibly including a common dependence on a protein produced or activated during the S-phase of the cell cycle. Although the requirement for an S-phase factor has been known since 1969 (Rhode, 1973, Siegl and Gautshi, 1973, Tennant *et al.*, 1969), its exact nature remains unknown. The cellular proteins studied here, whose concentration, conformation, or binding affinity are altered during the S-phase of the cell cycle might be one of the S-phase factor acting as positive regulator of parvoviral replication.

CHAPTER III

CHARACTERIZATION OF THE CELLULAR PROTEINS BOUND TO PARVOVIRAL *ORI*S

INTRODUCTION

Parvoviruses depend on structural and nonstructural viral proteins, as well as on host cell factors, for a productive life cycle (reviewed in Berns, 1990, Cotmore and Tattersall, 1987). Few of the required cellular factors have been identified to date. Some cellular replicative enzymes, such as topoisomerase and DNA polymerases, appear essential for parvoviral DNA replication (Gu and Rhode, 1991, Kolleck *et al.*, 1982, Pritchard *et al.*, 1981). Parvoviruses replicate in the nucleus of the infected cells, where viral DNA has been shown to be associated with or bound to the nuclear matrix (Bodnar *et al.*, 1989), to lamin (Wobbe, 1984, Wobbe and Mitra, 1985), or to nucleolin (Avalosse *et al.*, 1989, Barrijal *et al.*, 1992). Parvoviral DNAs have also been shown to bind to other, yet unidentified, cellular proteins (Chow *et al.*, 1986, Metcalf *et al.*, 1990, Willwand and Kaaden, 1990, Wobbe and Mitra, 1985). Initiation of DNA replication and gene expression of autonomous parvoviruses are dependent on an S-phase host factor (Cotmore and Tattersall, 1987, Wolter *et al.*, 1980). Replication of dependent parvoviruses may also possibly depend on similar cellular functions (Yakobson *et al.*, 1987, 1989). Investigation of the nature of

this S-phase factor has begun by detection of cellular protein(s), whose concentration, conformation or binding affinity is altered during the S-phase of the cell cycle, allowing binding to the BPV left (Metcalf *et al.*, 1990), BPV right (Braddon, submitted), and AAV (Chapter II) origins of replication (*oris*).

My objective was to characterize the cellular proteins bound to the BPV left and the AAV *oris*, detected by Metcalf (1990) and in Chapter II. The first approach was to determine the size of the bound proteins by SDS-polyacrylamide gel electrophoresis of the retarded complexes. The results of these experiments led me to investigate whether p53, an anti-oncoprotein, is present in the protein complexes bound to the parvoviral *oris*.

MATERIALS AND METHODS

Cells culture and labelling

Bovine fetal lung (BFL) cells and HeLa cells were cultured as detailed in Chapter II. BFL and HeLa cells were synchronized by hydroxyurea (HU) treatment, and harvested in the S-phase as detailed in Chapter II. BFL cells were *in vitro* labelled with L-[^{35}S]methionine (1000 Ci/mmol, Dupont NEN, Boston, MA) in Eagle's minimum essential medium with 5% dialyzed fetal bovine serum. Labelling was performed by addition of 3 µCi/ml of [^{35}S]methionine when the cells were seeded in HU, and by addition of 4.5 µCi/ml when the culture was replenished with complete medium after release from the HU block. HeLa cells were labelled with [^{32}P]orthophosphate (370 MBq/ml, Amersham, Arlington Heights, IL) in phosphate-free medium (Gibco BRL, Gaithersburg, MD) with 10% dialyzed fetal calf serum. The HeLa cells were labelled only during release from the HU block by addition of 10 µCi/ml of [^{32}P]orthophosphate.

Nuclear extracts

Radiolabelled nuclear extracts were prepared identically to the unlabelled extracts, as detailed in Chapter II. The specific activity of the nuclear extracts was determined by liquid scintillation, and was $1x10^{-5}$ μCi/μg for the ^{35}S-labelled BFL cells and $1.4x10^{-3}$ μCi/μg for the ^{32}P-labelled HeLa cells.

DNA retardation assays

DNA retardation assays of parvoviral termini were performed as described in Chapter II. Labelled nuclear extracts were used in the DNA retardation assay, at the same final protein concentration as unlabelled nuclear extracts. The preparative gels performed for protein analysis were fixed with 30% methanol, 10% acetic acid for 30 min before being dried on 3MM paper and exposed to Kodak XAR-5 film.

When competition experiments with antibodies were performed, the antibody solution was added in the incubation buffer with the nuclear extract and the non-specific competitor DNA. Incubation was carried out for 30 min at room temperature, and continued overnight at 4°C. The labeled viral DNA was then added to the binding reaction and incubated for 30 min at room temperature. The reactions were then electrophoresed as detailed in Chapter II. The anti-human p53 monoclonal antibody (Ig G_1)(pAb 1801, Banks *et al.*, 1986, a gift of C. Prives, Columbia University, NY) was obtained from mouse ascites fluid and was concentrated 10 fold with a Centricon 30 microconcentrator (Amicon, Beverly, MA) before use. A non-specific mouse monoclonal antibody (Ig G_1) directed against human Ig A, pAb H2K1E, which was also produced from ascites fluid (a gift of M. Nagarkatti, Virginia Polytechnic Institute and State University, VA), was used as a control. The protein concentration of the anti-p53 10X concentrate (6.9 μg/μl) and of the non-specific antibody (11.6 μg/μl) were determined by the BCA assay (Pierce, Rockford, IL). The two antibodies were used at the same final protein concentration (~ 25 μg/20 μl of binding reaction).

SDS polyacrylamide gels

The DNA samples or DNA-protein complexes to be analyzed were obtained, as described by Cleveland (1977), from the DNA retardation assays gels. The DNA or retarded complexes were excised from the dried gel and soaked for 30 min at room temperature in 0.125 M Tris-Cl [pH 6.8], 0.1% SDS, 1 mM EDTA, 0.8 U/ml aprotinin, a protease-inhibitor (Sigma, St Louis, MO). After removal of the 3MM paper, the gel was crushed into small pieces using a spatula. The pieces of gel were then pushed into the wells of the SDS-PAGE gel and electrophoresed (Laemmli, 1970) next to protein standards (High and low molecular weight standards, Sigma, St Louis, MO). The SDS gels were stained with 0.2% Coomassie blue in 50% methanol, 10% acetic acid for 15 min, and destained with 30% methanol, 10% acetic acid overnight. Gels containing samples labelled with [^{35}S]methionine were fluorographed with Amplify (Amersham), dried on 3MM paper, and then exposed to Kodak XAR-5 film. Gels containing only ^{32}P-labelled samples were dried and exposed directly.

RESULTS

A 55 kd protein, of bovine origin, is bound to the left end of the BPV virus

To determine the size of the protein(s) present in the complex formed with nuclear extract of BFL cells and the BPV left terminus (Metcalf *et al.*, 1990), BFL cells were S-phase synchronized by HU pretreatment and metabolically labelled with [^{35}S]methionine. A preparative DNA retardation assay was performed with either labelled or unlabelled BFL cells nuclear extract and a ^{32}P-labelled BPV left terminus. Gel samples containing either the unbound viral DNA or DNA-protein complexes were extracted and further electrophoresed on an SDS-polyacrylamide gel (SDS-PAGE). An autoradiograph of the SDS-PAGE is presented in Fig. 9. Lane 1 shows the unbound ^{32}P-BPV left terminus DNA. Lane 2 contains the DNA-protein complex obtained with unlabelled BFL cell extract and the labelled viral DNA. Lane 3 contains

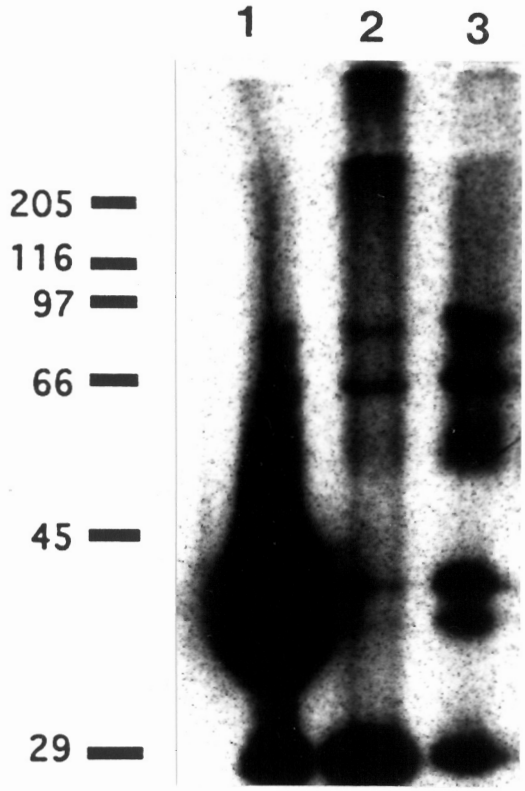


Figure 9. SDS-PAGE electrophoresis of the BFL cell protein bound to the BPV left terminus.
The unbound radiolabelled BPV left terminus was electrophoresed alone in lane 1. Retarded complexes formed between a labelled BPV left terminus and unlabelled BFL cell extract (lane 2), or [^{35}S]methionine-labelled BFL cell extract (lane 3) were electrophoresed after extraction from a preparative mobility shift assay.

the retarded complex formed with [^{35}S]methionine-labelled BFL cell extract and the ^{32}P-BPV left terminus DNA. In addition to the DNA species present in lanes 1, 2 and 3, an additional band was observed in lane 3, which corresponds to a 55 kd protein. The proteinaceous nature of this radiolabelled band was confirmed by its staining with Coomassie Blue (data not shown). The apparent size of this protein was estimated using the molecular weight marker.

Two phosphoproteins from HeLa cells are bound to the BPV left *ori*

A preparative DNA retardation assay using [^{32}P]orthophosphate S-phase labelled HeLa cells, was used to analyze the HeLa cell protein(s) bound to the BPV left terminus (Chapter II). An autoradiograph of the SDS-PAGE electrophoresis of the retarded complexes is shown in Fig. 10. The unbound ^{32}P-BPV left terminus DNA was electrophoresed in lane 1. Lane 2 contains the DNA-protein complex obtained with unlabelled HeLa cell extract and the labelled viral DNA. In lane 3, the nuclear extract used in the DNA retardation assay was prepared from ^{32}P-labelled HeLa cells; two additional bands, corresponding to two phosphoproteins of approximately 54 and 90 kd, can be observed (lane 3 compared to lanes 1 and 2). Lane 4 contains the retarded complex formed with ^{32}P-labelled HeLa cell extract and an unlabelled viral DNA. The radiolabelled nuclear extract, also extracted from preparative DNA retardation assay, was electrophoresed in lane 5, as a control.

Anti-p53 antibodies prevent retardation of the BPV left terminus

Figures 9 and 10 showed that a cellular protein of 54/55 kd, prepared from nuclear extract of two different cell types, was bound to the BPV left terminus. I decided to investigate if the proteins detected in these complexes were identical or related, since results from Chapter II suggest that (some of) the cellular proteins bound to different parvoviral *oris* have similar characteristics. Protein purification of HeLa and BFL cells nuclear extracts were difficult due to

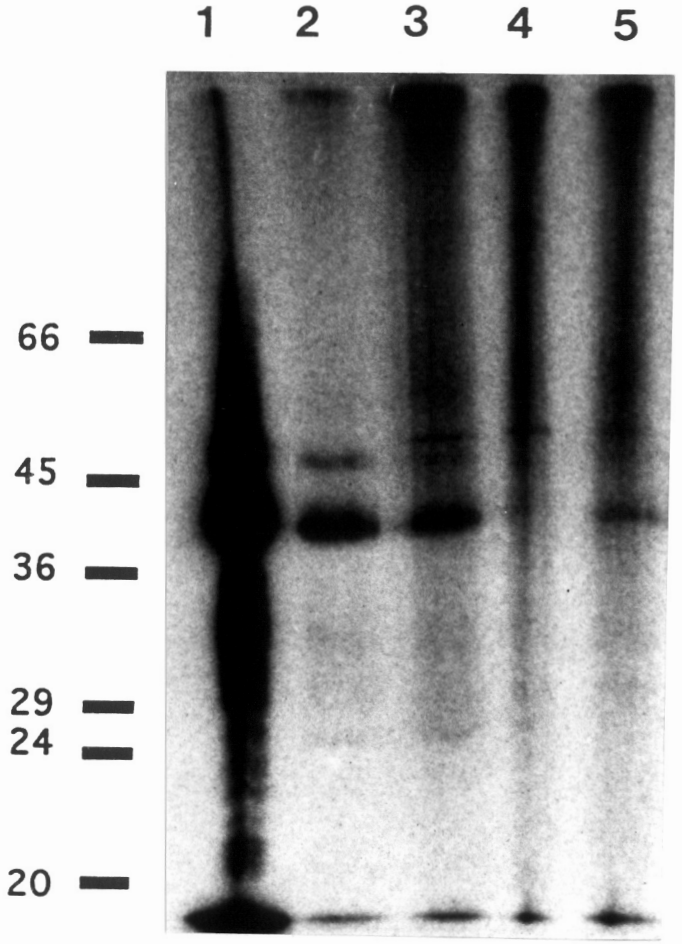


Figure 10. SDS-PAGE electrophoresis of the HeLa cell proteins bound to the BPV left terminus.
Lane 1 contains the unbound radiolabelled BPV left terminus. Lanes 2 to 4 contain the DNA-protein complexes formed with: radiolabelled terminus and unlabelled HeLa cell extract (lane 2), radiolabelled terminus and ^{32}P-labelled HeLa cell extract (lane 3), and unlabelled terminus and ^{32}P-labelled HeLa cell extract (lane 4). Lane 5 contains the ^{32}P radiolabelled HeLa cell extract.

the small quantities of protein recovered. Analysis of the characteristics shared by these proteins: i. e., size, nuclear location, cell-cycle regulation, and in the case of the HeLa cells protein, S-phase phosphorylation, led us to investigate the relationship of these proteins with the anti-oncoprotein p53.

Monoclonal antibodies to p53 are available and my approach was to use one of these antibodies as competitor in DNA retardation assay (Figs 11, 12). Fig. 11 shows that pAb 1801 (Banks *et al.*, 1986), a mouse monoclonal antibody directed against human p53, was able to prevent complex formation between a BPV left terminus and a BFL cell nuclear extract (lane 3, compared to lane 2). A non-specific monoclonal antibody of the same class, pAb H2K1E, used at the same concentration did not alter complex formation (lane 4). Fig. 12 revealed that pAb 1801 similarly prevented retardation of the BPV left terminus when the antibody was pre-incubated with the HeLa cell nuclear extract (lane 3 compared to lane 2). The non-specific monoclonal antibody, pAbH2K1E, had little effect on the complex formation (lane 4).

Anti-p53 antibodies reduce complex formation between an AAV terminus and HeLa cells nuclear extract

Nuclear extracts of S-phase HeLa cells were shown to interact specifically with an AAV terminus (Chapter II). Preincubation of HeLa cells nuclear extract with pAb 1801 reduced complex formation with an AAV terminus, as shown in Fig. 13 (lane 3 compared to lane 2). Preincubation of the protein extract with a non-specific antibody, pAb H2K1E, did not affect the binding capacity of the HeLa cell protein extract (lane 4).

DISCUSSION

Cellular proteins prepared from actively dividing or S-phase, hydroxyurea treated, synchronized BFL and HeLa cells were previously shown to bind to the BPV left and the AAV

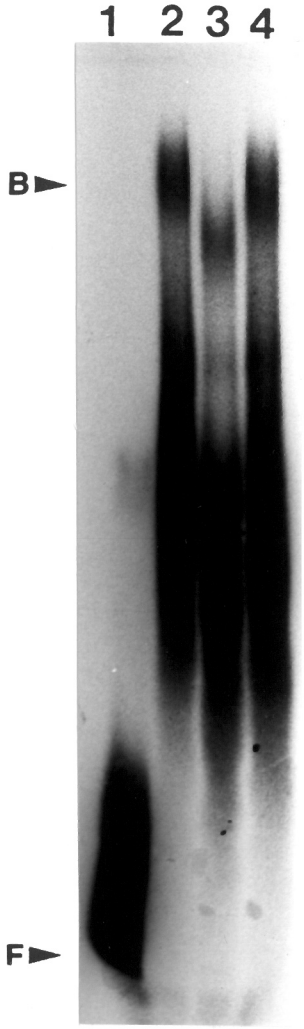


Figure 11. Specific competition of the DNA-protein complex formation between BFL cell protein and the BPV left *ori* , using anti-p53 antibodies.
The hairpinned BPV left terminus was electrophoresed alone (lane 1), or after incubation with nuclear extract of HU-treated S-phase synchronized BFL cells (lane 2 to 4). Monoclonal antibodies pAb 1801 (lane 3) or pAb H2K1E (lane 4) were added to the binding reaction as competitors. F: free probe, B: bound probe.

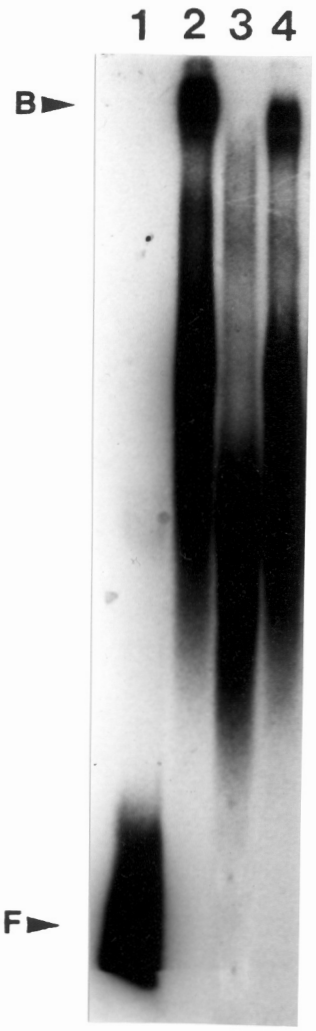


Figure 12. Specific competition of the DNA-protein complex formation between HeLa cell protein and the BPV left *ori* , using anti-p53 antibodies.
DNA retardation assays of the hairpinned BPV left terminus were carried out in the absence (lane 1), or presence (lanes 2 to 4) of nuclear extract from HU-treated HeLa cells. pAb 1801 and pAb H2K1E were added as competitor in lanes 3 and 4, respectively. F: free probe, B: bound probe.

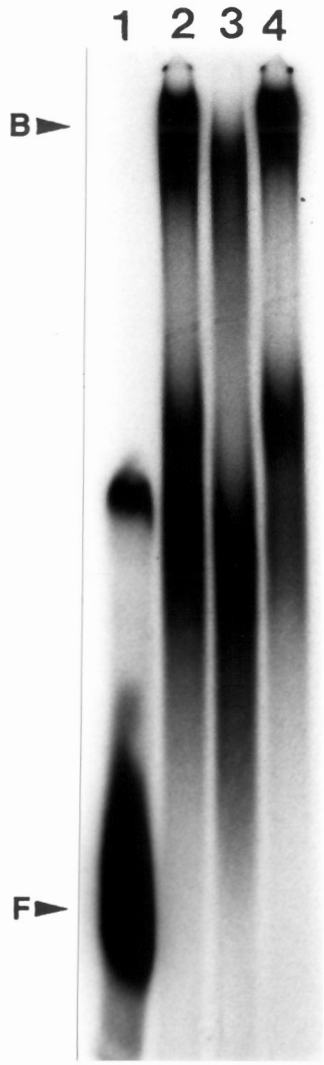


Figure 13. Specific competition of the formation of a DNA-protein complex between HeLa cell protein and the AAV terminus, using anti-p53 antibodies.
The hairpinned AAV terminus was electrophoresed alone (lane 1), or after incubation with nuclear extract of HU-treated S-phase synchronized HeLa cells (lane 2 to 4). Monoclonal antibodies pAb 1801 (lane 3) or pAb H2K1E (lane 4) were added to the binding reaction as competitors. F: free probe, B: bound probe.

oris (Metcalf *et al.*, 1990 and Chapter II). In order to begin to characterize the protein(s) involved in the complex formation, SDS-PAGE electrophoresis of the retarded complexes was performed. A 54 kd protein was contained in the complex formed with the BPV left terminus and BFL cell extract prepared from S-phase synchronized cells. Using a similar technique, I observed that two phosphoproteins of 55 and 90 kd were present in the retarded complex formed between a BPV left terminus and S-phase synchronized HeLa cell extract. An antibody directed against human p53, an anti-oncoprotein, was shown to compete binding of BFL cell extract and HeLa cell extract to the BPV left terminus. This antibody also competed the binding of HeLa cell extract to the AAV terminus.

I was able to detect one protein (54 kd) in the complex formed between BFL cells extract and the BPV left terminus, using [^{35}S]methionine labelling, and two proteins (55 and 90 kd) in the complex formed between extracts of HeLa cells and a BPV left terminus, using [^{32}P]-orthophosphate labelling. It is yet possible that other proteins were present in these complexes but were undetected due to a low concentration or/and to a poor labelling. Isotopic methionine was chosen for labelling since initiation of translation usually starts at a methionine codon. However, translation can also start at a valine, leucine, or threonine codon, and such proteins might not be labelled due to the low frequency of the methionine codon (Lewin, 1990). Similarly, protein phosphorylation occurs only at certain amino acid residues. The HeLa cell proteins used in the experiment were S-phase labelled, therefore, any protein that was un- or hypophosphorylated during the S-phase of the cell cycle probably remained undetected.

These results show that proteins immunoreactive with an anti-human p53 antibody were part of the DNA-protein complexes obtained with the BPV left and the AAV *oris*. The high specificity of the monoclonal antibody used (pAb 1801, Banks *et al.*, 1986) strongly suggests that the protein involved in these complex was p53. The cellular p53 protein is highly conserved across species (reviewed in Levine, 1990), however, the epitope(s) recognized by pAb 1801,

which are located close to the N-terminus, are human-specific and show a very low degree of homology with other p53 proteins, such as the murine p53 (Banks *et al.*, 1986). The presence of p53 in bovine cells has not been reported yet, but results from our laboratory (Lederman, unpublished) revealed that pAb 1801, although human-specific, can immunoprecipitate a protein of the appropriate size from extracts of BFL cells. The presence of p53 in nuclear extracts from S-phase synchronized BFL and HeLa cells that bind to the *ori* of a parvovirus, is in agreement with biological data gathered on p53. p53 is a nuclear phosphoprotein that can bind to DNA sequences (reviewed in Vogelstein and Kinzler, 1992). Its cellular concentration and phosphorylation are cell cycle regulated, its phosphorylation being maximal in S-phase (Bischoff *et al.*, 1990, and reviewed in Ullrich *et al.*, 1992). In addition, it has been shown recently that wild type p53 exists at a very low level in HeLa cells (Scheffner *et al.*, 1991), in spite of earlier evidence that the E6 oncoprotein of HPV binds to and degrades p53 in human papillomavirus (HPV)-positive cervical cancer lines, such as HeLa cells (Scheffner *et al* 1990, Wermess, *et al.*, 1990). I attempted to confirm the presence of p53 in the retarded complexes by immunoblotting the DNA retardation assays with anti-p53 antibody, but could only obtained background-level signals using a horseradish peroxidase-conjugated second antibody and a chemiluminescent detection assay (data not shown).

Results from Chapter II suggest that (some of) the proteins in BFL and HeLa cells that bind to parvoviral *oris* have common characteristics. A protein of 54/55 kd was detected bound to the BPV left terminus, in both BFL and HeLa cells extracts. An additional protein of 90 kd was also detected in the HeLa cell complex. Since different compounds were used to metabolically label these cells, and since the resolution of SDS-PAGE is low, the relatedness of these proteins cannot be assessed. However, the fact that an anti-p53 antibody was able to prevent or reduce complex formation between the BPV left terminus and both HeLa and BFL cells extract, and

between an AAV terminus and a HeLa cells extract suggest that a similar protein, most likely p53, might be indeed involved in the *ori*-binding complexes.

Competition experiments revealed that p53 interacted with two parvoviral *oris*, yet, it remains undetermined if the anti-oncoprotein was directly bound to the parvoviral DNAs or indirectly bound through other proteins. p53 has been shown to bind DNA at specific sequences (Kern *et al.*, 1991, Vogelstein and Kinzler, 1992) and also to interact with cellular or viral DNA-binding proteins (reviewed in Levine, 1990, Vogelstein and Kinzler, 1992, Pietenpol and Vogelstein, 1993). Sequences closely related to the p53-binding consensus sequence (PuPuPuC(A/T)(A/T)GPyPyPy) are present in the BPV and AAV termini. pAb 1801 recognizes epitopes located in the DNA transactivation domain of p53 (Banks *et al.*, 1986), thereby possibly preventing interaction with DNA. To determine if p53 could bind by itself to the parvoviral *oris*, we used purified human p53 in DNA retardation assays with the BPV left and the AAV termini. No complex formation was obtained (data not shown).

These data raise questions about the role, if any, of p53 in the parvoviral replication cycle. p53 acts as a tumor suppressor protein, and has a pleiotropic cellular role being involved in the control of the cell cycle, cell differentiation and programmed cell death (reviewed in Harris and Hollstein, 1993). The p53 protein has been shown to interfere with replication of oncogenic DNA viruses (Friedman *et al.*, 1990), but its effect on oncosuppressive viruses, such as parvoviruses, has not been widely studied yet. Telerman and colleagues (1993) used autonomous parvovirus H-1 as a tool for the selection of oncogenically suppressed cells. The author reports that infection of leukemic cells, in which a wild type p53 could not be detected, with H-1 parvovirus lead to selection of clones that are resistant to H-1 killing, but which released low level of infectious virus. These clones had a suppressed malignant phenotype and apparently expressed wild type p53. These experiments would suggest that wild type p53 confers resistance to the cytopathic effect of H-1 parvovirus. Such observations may be limited to the cell type used

in these experiments, since parvoviruses replicate in a wide range of cells with non-transformed phenotypes, which most likely express wild type p53.

Dependoparvoviruses generally require a helper virus, whose exact function is unknown, for a productive infection (reviewed in Berns, 1990, Carter, 1990). The adenovirus gene products required for helper function are not replicative proteins (reviewed in Berns, 1990, Carter, 1990) and include the oncoproteins E1b and E1a. E1a has been shown to induce p53 expression (Braithwaite *et al.*, 1990). In contrast, some of the herpes virus replication genes can provide the helper function needed for AAV replication (Weindler and Heilbronn, 1991). It has been shown that p53 co-localizes with the viral and the cellular replication gene products at the sites of viral replication in the nuclei of herpes infected cells (Wilcock and Lane, 1991). p53 might therefore play a direct or indirect role in the replication process of both autonomous and dependent parvoviruses, possibly as regulator of S-phase events.

Wild type p53 can act as positive regulator of transcription by binding to p53-binding consensus sequences (Farmer *et al.*, 1992, Zambetti *et al.*, 1992, Vogelstein and Kinzler, 1992), and also as a negative regulator of transcription from TATA boxes (Mack *et al.*, 1993, Seto *et al.*, 1992). In addition to interfering with replication of certain viral DNAs (Friedman *et al.*, 1990), p53 has been shown to inhibit DNA replication by inactivating the cellular replication factor RPA (Dutta *et al.*, 1993). In view of these properties, p53 would be likely to act on the parvovirus life cycle as a modulator of transcription or viral replication. Parvoviral gene expression and initiation of DNA replication are two S-phase dependent events (Cotmore and Tattersall, 1987, Wolter *et al.*, 1980); thus, p53 would probably act in a cell cycle-specific fashion. It has been proposed that changes in its conformational state and phosphorylation, which are all cell-cycle dependent events, regulate p53 function (Ullrich *et al.*, 1992). p53 might therefore repress transcription from the parvoviral TATA boxes during the G1 phase, or/and activate transcription of parvoviral genes in the early S-phase by binding to the consensus sequence variants found upstream of the early

promoter. Alternatively, p53 might negatively regulate parvoviral DNA replication during the G1 phase, as part of its G1-phase growth arrest properties.

CHAPTER IV

SPECIFIC SEQUENCES WITHIN THE MISMATCHED REGION OF THE BPV LEFT HAIRPIN ARE ESSENTIAL FOR VIRAL REPLICATION

INTRODUCTION

BPV, like all parvoviruses, contains self-complementary sequences that can form stable hairpins at both ends of its genome (Astell *et al.*, 1983, Bloom *et al.*, 1988, Chen *et al.*, 1986, Diffoot *et al.*, 1993, Kariatsumari *et al.*, 1991, Rhode and Paradiso, 1983, Shade *et al.*, 1986, Srivastava *et al.*, 1983). The BPV left terminus can fold into a T-shaped "stem-plus-arms" structure, while the right end can assume a U-shaped conformation. The secondary structure of the parvoviral ends is remarkably conserved across the autonomous genus. In addition, all autonomous parvoviruses except B19 contain unpaired/mismatched nucleotides in the duplex stem of their left hairpin, which forms a bubble (Astell *et al.*, 1979, Chen *et al.*, 1986, Deiss *et al.*, 1990, and reviewed in Astell, 1990). The right termini of rodent parvoviruses also have mismatched bases in their stem when maximally base-paired (Astell *et al.*, 1979, Astell, 1990). Most parvoviral termini have been found in two different sequences orientations, flip or flop, which are inverted complements of each others. The ratio for each of these conformations is

strand-specific for both termini of the viral genome (Astell *et al.*, 1979, Chen *et al.*, 1988, Deiss *et al.*, 1990, Lusby *et al.*, 1980, and reviewed in Astell, 1990). The nucleotide sequence of the bubble, in conjunction with the one of the hairpin arms, defines the orientation of a terminus.

The termini of parvoviruses were shown to be involved in replication (Rhode and Klassen, 1982, Salvino *et al.*, 1991, Shull *et al.*, 1988, Senepathy *et al.*, 1984, Snyder *et al.*, 1990 (b)), encapsidation (McLaughin *et al.*, 1988), excision of viral DNA (Samulski *et al.*, 1983, Rhode, 1989), and, in the case of AAV, integration (Samulski *et al.*, 1989). Several models have been proposed to explain the different encapsidation patterns and terminal sequences of the viral genomes (Astell *et al.*, 1985, Berns and Hauswirth, 1984, Chen *et al.*, 1989, Cotmore *et al.*, 1989, and reviewed in Berns, 1990, Cotmore and Tattersall, 1987). The unpaired region in the parvoviral termini might play a role in the replication process, either by acting as a potential signal sequence for specific nicking of the rodent parvovirus dimer replication intermediate (Astell *et al.*, 1985), or as part of the element that determines the terminal orientation, by influencing the hairpin transfer rate of the different parvoviral termini (Chen *et al.*, 1989). Mutagenesis experiments showed that the secondary structure of the arms of the AAV terminus is more important than its primary sequence for replication (Bohensky *et al.*, 1988, Lefebvre *et al.*, 1984, Snyder *et al*, 1993). The conservation of the hairpin secondary structure and of the bubble in the stem of the left hairpin throughout almost the entire autonomous parvovirus genus suggest that constraints exist during replication for their maintenance.

To analyze the possible role of these unpaired/mismatched nucleotides in the BPV life cycle, two mutant clones lacking the unpaired region were constructed by site-directed mutagenesis. These "bubbleless" BPV clones were obtained either by removal of the unpaired nucleotides, or by addition of the complementary bases. I report the replication properties of these mutants as analyzed after electroporation in permissive cells.

MATERIALS AND METHODS

Construction of the mutated BLOP clone

The two mutated "bubbleless" clones were constructed by site-directed mutagenesis using a two-step polymerase chain reaction (PCR). Restriction enzymes were purchased from New England Biolabs (Beverly, CA), or Boehringer Mannhein (Indianapolis, IN). Primers were synthesized on a Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Foster City, CA). PCR reactions were performed in a DNA thermal cycler (Perkin Elmer/Cetus, Norwalk, CT). All PCR reactions were performed in the following buffer: 10mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, using the reagents and *AmpliTaq* polymerase from the GeneAmp kit (Perkin Elmer/Cetus), and were overlaid with mineral oil to prevent evaporation during cycling.

Two genomic infectious clones of BPV (Shull *et al.*, 1988), pVT501 (flop orientation at the left end) and pVT502 (flip orientation at the left end) were digested with NheI and religated, to obtain BPV plasmids deleted from m.u. 6 to m.u. 92. These clones, pVT501ΔNheI and pVT502ΔNhe, were used as templates in the PCR reactions.

For the construction of the BLOP clone ("Bubbleless Left flOp Plus"), the first round of PCR amplification was performed on a pVT502ΔNheI template digested with HindIII, with a mutating primer (5'-AGCCGCTCGCATTGTAAGGCCTAAT-3'), which is complementary to nt 105 to 81 of the BPV minus strand in the flip orientation except for the underlined bases, and a second primer, P2' (5'-ACTGGAATTCTTGCTAGCCCCCCAGTAGCC-3'), which is complementary to nt 338 to 350/5058 to 5075 of the BPV plus strand (Fig. 16 A, step 1). This first PCR reaction yielded a 287 bp product. Due to the palindromic nature of the BPV left end, the mutating primer used in this first round of amplification could also anneal to the BPV plus strand (nucleotides 46 to 65) with 8 mismatches. To prevent extensive primer elongation from this location, the pVT502ΔNheI template used in the PCR reaction was digested with HindIII. The amplification

reaction was carried out in a 100 µl reaction, with 100 ng of template, 100 pmoles of each primer, 200 µM of dNTPS, and 2.5 Units of *AmpliTaq*. After an initial denaturation step of 2 min at 94°C, 35 cycles were performed as follows: 94°C, for 1 min, ramp time of 0.3°C/sec between 94°C, and 55°C to avoid hairpin formation, 55°C for 1 min, ramp time of 0.6°C/sec between 55 and 72°C, 72°C for 1 min, ramp time of 0.7°C/sec between 72°C and 94°C. The PCR product obtained was purified using the Magic PCR prep DNA purification system (Promega, Madison, WI), digested with 1 Unit of Nuclease S1 (Boehringer Mannhein) for 15 min at 37°C and digested with SmaI to select against the unmutated product (step 2). The 287 bp PCR product was purified in low-melting-point agarose (1.5% Nusieve:Seakem, 2:1, FMC, Rockland, ME), extracted from the gel using the Geneclean kit (Bio 101, La Jolla, CA)(step 2), and used in the second round of PCR amplification as one of the primers (step 3).

The second round of PCR amplification was performed on a pVT501ΔNheI template, in conjunction with the forward (-40) universal primer (5'- GTTTTCCCAGTCACGAC-3') and the 287 bp product (Fig. 16 A, step 3). Although only one strand (minus strand) of the 287 bp PCR product was used as primer for amplification with the universal primer, both strands were added to the reaction. To prevent elongation from the plus strand of the PCR product, the pVT501ΔNheI template used was digested with NheI. Conditions for this second PCR amplification were: an initial denaturation step at 94°C for 2 min, followed by 10 cycles of 94°C for 1 min, ramp time of 0.3°C/sec between 94°C and 55°C, 55°C for 10 min, ramp time of 0.6°C/sec between 55 and 72°C, 72°C for 1 min, ramp time of 0.7°C/sec between 72°C and 94°C. These cycles were performed in a 50 µl reaction, using 50 ng of template, 2 pmoles of the forward primer, about 1/10 (2 pmoles) of the 100 µl reaction producing the 287 bp PCR product, 200 µM of dNTPs, and 1.25 Unit of *AmpliTaq*. In order to obtain sufficient product, amplification was continued for 35 cycles after addition of 100 pmoles of both forward and P2' primers and 1.25 Unit of *AmpliTaq*, in a volume of 50 µl. The cycling parameters were identical to the one used during the first round

of amplification. This second round of amplification led to a 432 bp fragment (step 4). The PCR fragment was cleaned and digested again with SmaI to select against the unmutated product, and then digested with HindIII and NheI (step 4), in preparation for ligation. The restriction products were electrophoresed on a 1.5% agarose gel. The appropriate fragment was extracted using the GeneClean kit and ligated to a pVT502ΔHindIII–NheI vector. This ligation reaction produced a 3.5 kb plasmid, named BLOPΔNheI (step 5). The NheI fragment of BPV, m.u 6 to m.u. 92, was then inserted in BLOPΔNheI using the NheI fragment of the infectious genomic clone pVT501, to create a full length BLOP clone.

Construction of the mutated BLOM clone

For the BLOM (Bubbleless Left flOp Minus) construction, the first round of amplification was performed on a pVT502ΔNheI template digested with NheI, with a mutating primer 5'-TCGCGCGCGCATTATTGGCCCGGGCAGCCAAT-3', which is complementary to a BPV plus strand in the flip orientation nt 34 to 65, except the underlined bases, and with the universal primer (Fig. 16 B, step 1). This reaction lead to a 130 bp product. The PCR reaction was carried out in a 100 µl reaction, with 100 ng of template, 100 pmoles of each primer, 20 µM of dNTPS and 2.5 Units of *AmpliTaq*. After an initial denaturation step of 2 min at 94°C, 30 cycles were performed as follows: 94°C for 1 min, ramp time of 0.3°C/sec between 94°C and 55°C to prevent hairpin formation, 55°C for 3 min, ramp time of 1.3°C/sec between 55°C and 94°C to prevent extensive elongation by the *Amplitaq* at temperatures at which the error rate is increased (Innis and Gelfand, 1990). The PCR product obtained was separated by low-melting-point agarose electrophoresis, and extracted from the gel using the Qiaex gel extraction kit (Qiagen, Chatsworth, CA) (step 2).

The second round of PCR reaction was performed using a pVT501ΔNheI template digested with HindIII, in conjunction with the P2' primer and the 130 bp PCR product of the first

round of amplification as second primer (step 3). After an initial denaturation step of 2 min at 94°C, 15 cycles were performed as follow: 94°C 1 min, ramp time of 0.3°C/sec between 94°C and 55°C, 55°C for 15 min, ramp time of 1.3°C/sec between 55°C and 94°C. The 50 µl reaction was performed with 30 ng of template, 2 pmoles of P2', about 1/5 (2 pmoles) of the 100 µl reaction used to produce the first PCR product, 50 µM dNTPS and 1.25 Unit of *AmpliTaq*. Further amplification was performed after addition of 100 pmole of both P2' and universal primers and 1.25 unit of *AmpliTaq*, for 30 cycles. After an initial denaturation step of 2 min at 94°C, the cycling parameters were: 94°C for 1 min, ramp time of 0.3°C/sec between 94°C and 55°C, 55°C for 4 min, ramp time of 3.9°C/sec between 55°C and 94°C. The 432 bp product obtained was gel purified by low-melting point agarose gel electrophoresis, extracted with the Qiaex gel extraction kit (Qiagen). The PCR product was then digested with StuI to select against the unmutated product, digested with HindIII and NheI, and repurified by gel electrophoresis (step 4). The DNA fragment of the appropriate size was gel purified and ligated as a HindIII-NheI fragment, to a pVT501ΔHindIII-NheI vector (step 5). A BLOMΔNheI clone and a full-length BLOM clone were obtained.

Due to the high error rate of *Taq* polymerase (Innis and Gelfand, 1990) and to the nature of the mutation, all potential BLOP and BLOM clones were sequenced using the Sequenase 2.0 DNA sequencing kit (USB, Cleveland, OH). The HindIII-NheI fragments and the NheI junction sites of BLOP and BLOM were completely sequenced.

Propagation of plasmids

The mutated plasmids were propagated in *E. coli* SURE cells (Stratagene, La Jolla, CA) to minimize deletion and recombination events. Recombinant cells were grown in the presence of ampicillin (100 µg/ml), tetracyclin (10 µg/ml) and kanamycin (25 µg/ml) to maintain pressure on the transposons, as suggested by the manufacturer. PVT501, pVT502 (Shull *et al.*, 1988),

pVT501ΔKpnI (del m.u. 43-90), pVT501ΔXhoI (del m.u. 13-89) and pVT501ΔNheI (del m.u. 6-92) were grown in *E. coli.*, DH5α, as previously described, or in SURE cells. Large scale preparation of plasmid DNAs was performed using the Qiagen plasmid maxi kit (Qiagen, Chatsworth, CA). The volume of reagents was generally increased four-fold over the manufacturer's recommendations. Purity of the plasmid DNAs were verified by agarose gel electrophoresis and diagnostic restriction digests with HindIII, or NheI, or SmaI, or StuI, or BstXI.

Cell culture and electroporation

Bovine fetal lung (BFL) cells were cultured in monolayer in Eagle's minimum essential medium (MEM) in 10% fetal bovine calf serum (Hyclone, Logan, UT), as previously described (Parris and Bates, 1976). When grown in tissue culture plates, BFL cells were incubated at 37°C in an atmosphere containing 5% vol/vol of CO_2, and Fungizone (Flow, Mc Lean, VA) was added to the culture medium at the final concentration of 1.25 μg/ml..

BFL cells were electroporated with the various plasmid DNAs using a Biorad Gene Pulser (Hercules, CA), as described by Maxwell and Maxwell (1988). Electroporation was performed on 50% confluent cells. These were obtained by passing the cells 48 hours before electroporation. Approximately $1x10^6$ cells were electroporated with 10 μg of plasmid DNA and plated in a 100 x 20 mm plate (Primaria, Falcon, Lincoln Park, NJ).

Analysis of the infectivity of mutant clones

Detection of viral antigens by indirect immunofluorescence was performed as described previously (Shull *et al.*, 1988). Two primary antibodies were used for the assay: a rabbit antiserum directed against BPV capsids (rabbit 0118), and a serum obtained from an infected calf (calf 86) that react with both nonstructural and structural proteins. The secondary antibody was

either a fluorescein-conjugated goat anti-rabbit Ig (Cooper Biomedical Inc, West Chester, PA), or a fluorescein-conjugated goat anti-bovine Ig (Miles Scientific, Naperville, IL)

Extraction of low molecular weight DNA for assay of transient viral DNA replication was performed using a modified "Hirt" protocol (Hirt, 1967, Shull *et al.*, 1988). "Hirt DNA" samples were electrophoresed on a 0.8% to 1% agarose gel before or after digestion with DpnI. Agarose gels were depurinated 10 min in 0.25 N HCl, then denaturated in 0.4 N NaOH, and transferred to a nylon membrane (Pall Biodyne, East Hills, NY) in 0.4 N NaOH, by Southern transfer. Blots were probed with a non-radioactive digoxigenin-labelled-BPV DNA probe (Genius system, Boehringer Mannheim), following the manufacturer's recommendations. A series of washes, of 20 min each, was performed at room temperature as follows: 2xSSC and 0.1% SDS, 1xSSC and 0.1% SDS, 0.5xSSC and 0.1% SDS, 0.1xSSC and 0.1% SDS, and a last wash with 0.1xSSC and 1% SDS at 65°C. Detection of the digoxigenin-labelled DNAs was performed by chemiluminescence as directed (Genius system). Chemiluminescent signals, detected by exposure to Kodak XAR-5 films, were quantified by densitometry with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The efficiency of the DNA recovery during the extraction was monitored by adding a known amount (0.5 µg) of DNA of unrelated sequence to the harvested cells. An insertion element (IS 1141) contained in *Mycobacterium avium* plasmids (Via, 1993) was arbitrarily chosen as the internal standard. The *Mycobacterium* and BPV DNAs did not cross-hydridize, when blotting was performed using the normal assay conditions (data not shown). The pVT365 clone (Via, 1993) was used to obtain the *Mycobacterium* internal standard. pVT365 was digested with PvuII and the digestion products (vector (2.4 kb) and insert (2.2 kb)) were added to the harvested cells. The internal standard DNA was detected by dot-blotting the Hirt DNAs with a non-radioactive digoxigenin-labelled *Mycobacterium* DNA probe (Genius system). The amount of Hirt DNAs to be analyzed by blotting for transient BPV DNA replication

assay, was then adjusted for each sample before electrophoresis, depending on the amount of standard DNA recovered. All electroporation experiments were repeated.

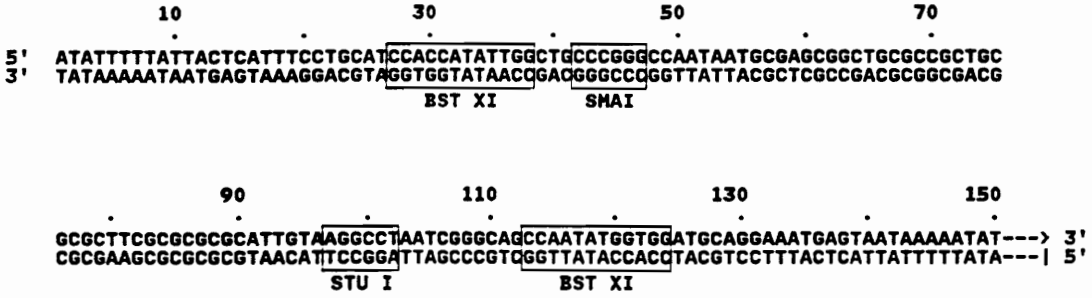
RESULTS

Construction of the mutated clones

The two "bubbleless" clones (Figs. 14 and 15) that lack the unpaired region found in the stem of the BPV wild type left terminus were constructed by site-directed mutagenesis that either added or removed nucleotides. One clone referred as "BLOP" (for "Bubbleless Left flOp Plus") contains 5 "extra" nucleotides that are complementary to the unpaired nucleotides of the BPV wild type sequence and has two mutated bases, so as to have a perfectly matched sequence (Fig. 15). The other clone, referred as "BLOM" (for "Bubbleless Left flOp Minus") lacks 5 of the unpaired nucleotides found in the BPV wild type sequence and has two mutated bases (Fig. 15). The nucleotide sequences (position 99 to 105 of a wild type flop BPV) that have been mutated to create the BLOP clone are now identical to wild type BPV flip sequences (nucleotides 94 to 105). The SmaI site normally present at position 106 in a wild type flop sequence (shown on Figs. 14 and 15) is lost in the BLOP clone, and replaced by a StuI site. Similarly, the nucleotides sequences (position 46 to 57 of a wild type flop BPV) that have been mutated to create the BLOM clone are now identical to wild type BPV flip sequences (nucleotides 46 to 52). As a result, BLOM has lost the StuI site at position 51 and gained a SmaI site.

Our first attempt was to synthesize a 110 bp fragment (nucleotides 21 to 130 of a BPV flop sequence), which would have contained the desired mutation, by polymerase chain reaction (PCR) using mutating primers. The resulting PCR fragment would then have been cloned after restriction digestion as a BstXI fragment into a BPV clone. The use of only one primer was needed for the construction of either clone, since each primer would perfectly anneal to one DNA

(A) FLIP



(B) FLOP

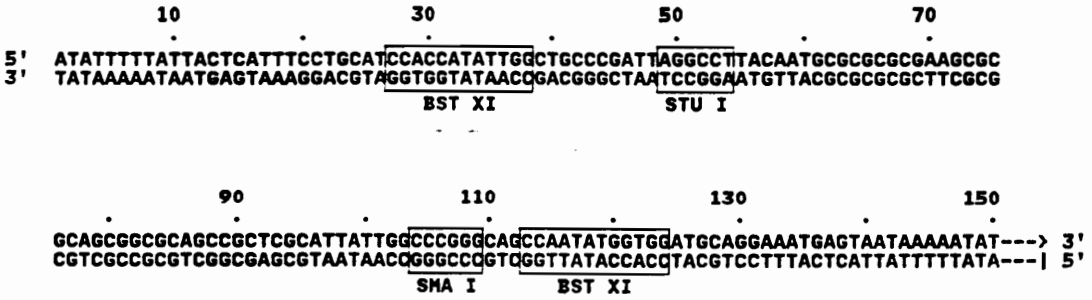


Figure 14. DNA sequence of the BPV left terminus.

(A), flip orientation, (B), flop orientation. Restrictions sites of interest are indicated by boxes.

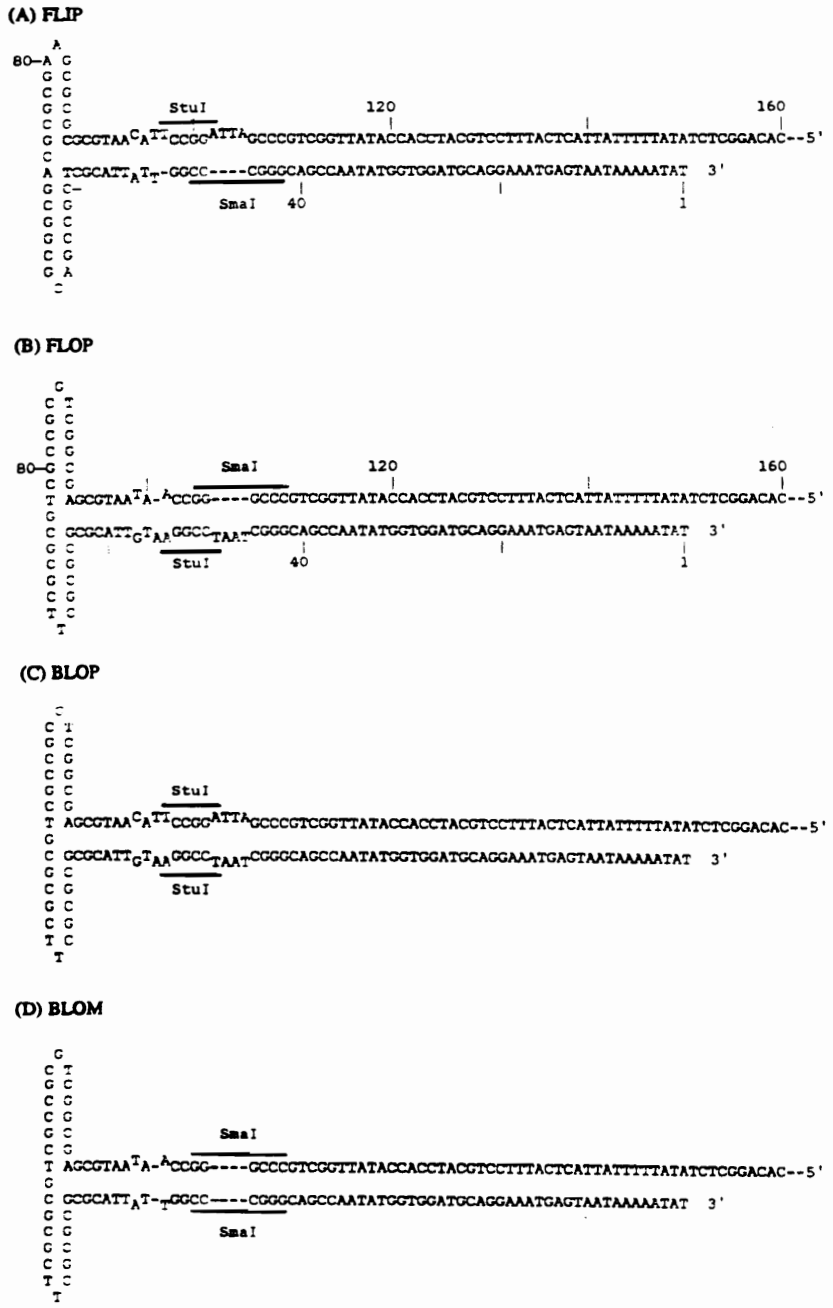


Figure 15. Hairpin conformations of the wild-type and mutated BPV left termini.
(A) Flip conformation of the left terminus of the BPV minus strand, (B) Flop conformation of the left terminus of the BPV minus strand, (C) left terminus of the mutated BLOP ("Bubbleless Left flOp Plus") minus strand, and (D) left terminus of the mutated BLOM ("Bubbleless Left flOp Minus") minus strand.

strand and also anneal to the other DNA strand, with mismatches, thereby creating the desired mutation. For example, for the construction of the BLOP, a primer which anneals to nucleotides 63 to 21 of the BPV minus strand, would also anneal, with 7 mismatches, to nucleotides 93 to 130 of the BPV plus strand. Very little PCR product was detected using this technique. On the other hand, a normal amount of PCR product was detected when amplification was carried out with two primers of wild type sequence, indicating that the absence of amplification probably resulted from poor annealing of the primers to the template.

A second approach was tried to obtain this mutant 110 bp fragment by PCR, in which primer elongation would take place in the absence of template by using overlapping primers. I successfully obtained the two "primary" PCR products of 60 bp (nucleotides 71 to 130 of a BPV flop sequence) and of 66 bp (nucleotides 21 to 86). Unfortunately, I could not obtain the desired 110 bp product when these two PCR fragments where used together for further amplification, probably as a result of their secondary structure.

The third, successful, strategy was to synthesize using a two-step polymerase chain reaction a mutant fragment of 432 bp (BPV nucleotides 1 to 350), that would then be cloned as a HindIII-NheI fragment into a BPV clone (Figs. 16 A and B). The two-step polymerase chain reaction, also called megaprimer amplification (reviewed in Barik, 1993), uses the product of the first PCR reaction as one of the primers during the second round of amplification. The mutating primers that were used to construct either BLOP or BLOM, contained sequences complementary to both flip and flop sequences (see Fig. 15 and Materials and Methods). For example, the mutating primer for the construction of the BLOP is complementary at its 5' end (primer bases 1 through 13) to BPV flop sequences nt 86 to 98 and at its 3' part (primer bases 8 through 25) to nt 94 to 105 of a BPV flip sequence. Our strategy was therefore to use different templates, alternatively of flip or flop conformations, in order to have sufficient annealing between the template and the mutating primer for both rounds of amplification (Figs. 16 A and B). Due to the

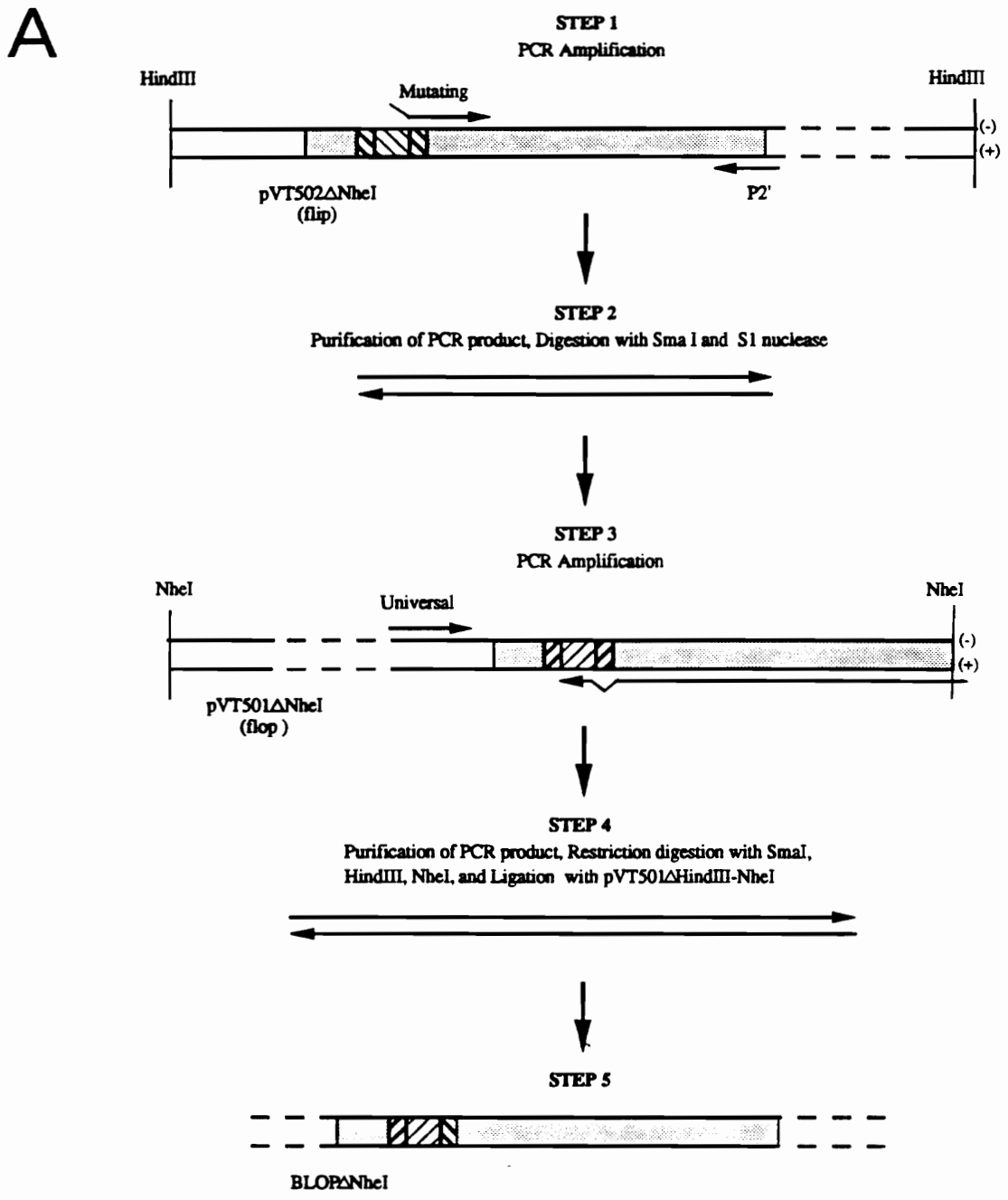


Figure 16. Construction of BLOP and BLOM clones.
(A), construction of the BLOP clone; (B), construction of the BLOM clone.
- vector sequences,
- BPV sequences common to both the flip and the flop orientations (nt 1-45 and 106-350),
- BPV sequences of the hairpin "stem", flip orientation (nt 46-52, and 94-105),
- BPV sequences of the hairpin "stem", flop orientation (nt 46-57, and 99-105),
- BPV sequence of the hairpin "arms", flip orientation (nt 53-93),
- BPV sequence of the hairpin "arms", flop orientation (nt 58-98).

B

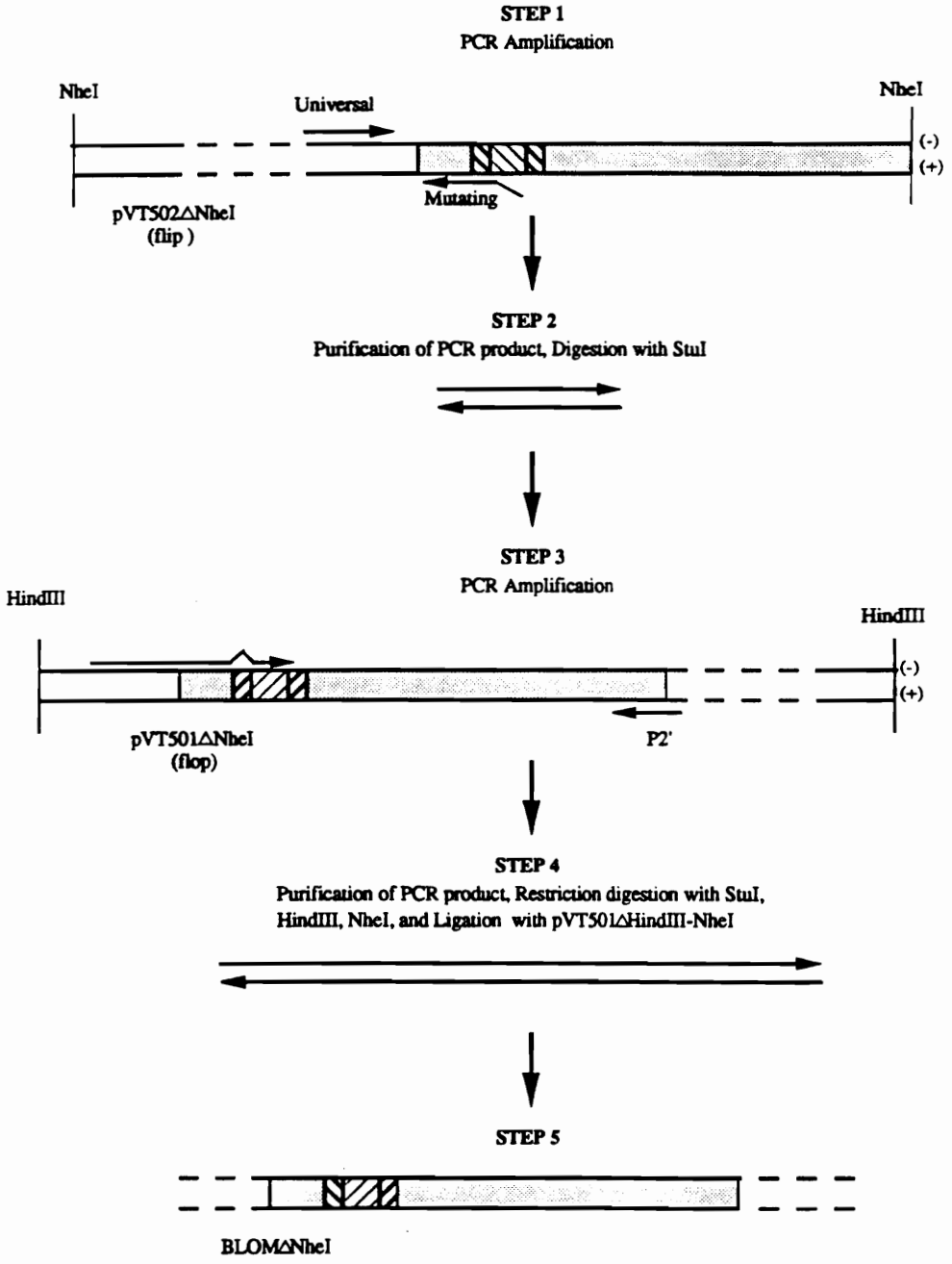


Figure 16. Construction of BLOP and BLOM clones. Continued
(A), construction of the BLOP clone; (B), construction of the BLOM clone.

- vector sequences,
- BPV sequences common to both the flip and the flop orientations (nt 1-45 and 106-350),
- BPV sequences of the hairpin "stem", flip orientation (nt 46-52, and 94-105),
- BPV sequences of the hairpin "stem", flop orientation (nt 46-57, and 99-105),
- BPV sequence of the hairpin "arms", flip orientation (nt 53-93),
- BPV sequence of the hairpin "arms", flop orientation (nt 58-98).

high secondary structure of the BPV left terminus, the first PCR reaction was designed to amplify sequences located outside (upstream or downstream) of the hairpin (Figs. 16 A and B, steps 1). Amplification of the DNA containing the hairpin sequences was achieved in the second round of amplification (Figs. 16 A and B, steps 3), during which the length of the megaprimer probably stabilizes the replication fork.

The BLOP clone was constructed as described in Fig. 16A. Sequencing of the first set of mutated clones revealed that the PCR fragment obtained during the first round of amplification (step 1) contained an extra adenosine at its minus strand 3' OH end. This misincorporation was most likely due to the intrinsic property of the *Taq* polymerase to add non-template nucleotides at the 3' end of its synthesis products (Clark, 1988). Therefore an additional step was used to remove this "extra" adenosine before further amplification, by S1 nuclease treatment (Fig. 16 A, step 2). The mutation efficiency using this technique was quite low; approximately 40% of the recombinant clones were resistant to SmaI digestion. In addition, due to the high error rate of *Taq* polymerase (Innis and Gelfand, 1990), only one BLOP clone that contained the desired mutation and no added misincorporation was found.

The BLOM clone was constructed with a similar technique (Fig. 16 B), but the extension temperature and dNTPS concentration were lowered to increase the fidelity of the *Taq* polymerase (Innis and Gelfand, 1990). In addition, the start of the mutating primer was positioned 5' upstream of an A on the template strand, so that addition of an A by *Taq* polymerase at the 3' end of the PCR products would not alter the original viral sequence at this location. Only 33% of the recombinant clones contained the desired mutation, and similarly, only one clone with no added misincorporation was found. One or two other BLOM clones might have been of correct sequence, but these were not sequenced entirely.

Primary sequence but not secondary structure of the bubble is required for viral replication

The ability of BLOM and BLOP to replicate was examined first by observation of cytopathic effect (CPE), after electroporation of the mutant plasmids in bovine fetal lung (BFL) cells (data not shown). CPE after BPV infection is usually characterized by cells rounding up at the point of infection. No CPE was observed for the BLOP clone, even by 12 days post-electroporation. Only little CPE was observed for the BLOM clone 6-7 days after the assay and the cell layer was completely lysed after 12-13 days. In comparison, BFL cells electroporated with wild-type pVT501, an infectious clone of BPV (Shull *et al.*, 1988), showed CPE at 3-4 days, and were completely lysed by 5-6 days. Excision of the BPV DNA sequences from the mutant clones before electroporation, by digestion with SalI, gave similar results. The infectivity of pVT501 and BLOM was however increased when the clones were electroporated as linear viral sequences. Replication of the BLOP clone was not affected. To detect if any infectious virus was released by BLOM and BLOP, part of the culture medium that was harvested 12 days after electroporation with excised clones, was used to infect cell layers. Blind passage revealed that the BLOM clone produced infectious viruses since the cell layer showed CPE after a few days, while no CPE could be observed for BLOP even 7 days after infection.

An indirect immunofluorescence assay was used to detect the presence of virally-coded proteins in the electroporated cells. A rabbit serum directed against BPV capsids (rabbit 0118) or a calf serum directed against the structural and non-structural proteins (calf 86) was used. The BLOP clone gave negative immunofluorescence results with either antibody (Fig. 17, panels A and B), while BLOM (panels D and E), and pVT501 (panels G and H) gave positive results, seen by the presence of fluorescent nuclei. The number of positive cells was much lower in the cell layer electroporated with BLOM (Fig. 17, panels D and E) compared to pVT501 (panels G and H). Control assays were performed omitting the primary antibody (panels C, F, and I).

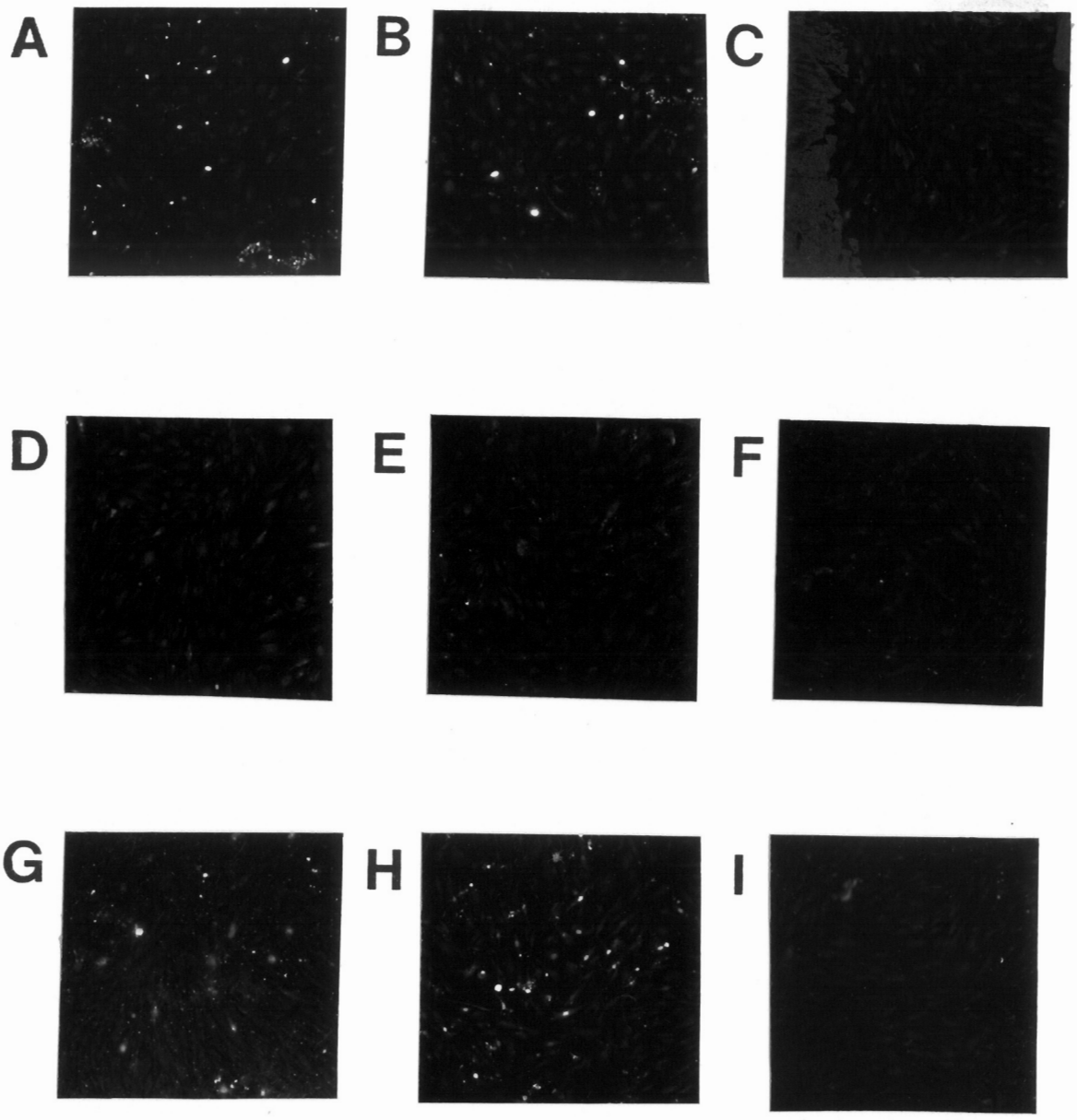


Figure 17. Analysis of viral proteins production by indirect immunofluorescence assay. Panels A, B, and C are cell layers electroporated with the BLOP clone and assayed with (A) calf 86 antibody, (B) rabbit 0118 antibody, or (C) no primary antibody. Panels D, E, and F are cell layers electroporated with the BLOM clone and assayed with (D) calf 86 antibody, (E) rabbit 0118 antibody, or (F) no primary antibody. Panels G, H, and I are cell layers electroporated with the wild-type pVT501 clone and assayed with (G) calf 86 antibody, (H) rabbit 0118 antibody, or (I) no primary antibody.

The replication ability of the mutant BPV clones was further studied by isolation of low molecular-weight DNA from the cells 5 to 6 days after electroporation. The Hirt DNAs were electrophoresed on agarose gels, blotted and analyzed by hybridization with a BPV-specific probe. pVT501 was replicated as indicated by the presence of monomer and dimer replication intermediates (Fig 18, lane 3). Digestion of the Hirt DNA with DpnI, which digests the input methylated plasmid DNA, showed a similar gel pattern (lane 4). Very little monomer-length DNA was obtained from the BFL cells electroporated with the BLOM clone (lanes 5, 6), confirming the time required for the appearance of CPE on the cell layers. Replication of BLOM occured only at about 8% of the level of pVT501 replication, as assessed by densitometric comparison of the intensity of the 5 kb bands. Doubling of the amount of DNA used in the electroporation did not yield an increase in DNA replication, but rather resulted in a decrease in the amount of DNA produced (lanes 7, 8). The BLOP plasmid was not replicated since no genome-length DNA was detected after DpnI digestion of the Hirt DNA (lanes 9, 10). Electroporation with twice the amount of plasmid DNA did not lead to detectable replication (lanes 11, 12).

A second set of electroporations was performed using excised viral DNA sequences rather than circular plasmids. Fig. 19 showed that replication of BLOM was increased when the BLOM construct was electroporated after excision form the plasmid sequence. The intensity of the 5 kb band produced by the excised BLOM was about 20% of the intensity of the band produced by wild-type pVT501 (lanes 5, 6 compared to 3, 4). Lanes 7 and 8 revealed that BLOP DNA was unreplicated. Replication of the BLOM clone was best when the cells were electroporated at a rather low density, since its replication is slower than that of pVT501 and requires actively dividing cells for more than 5 days.

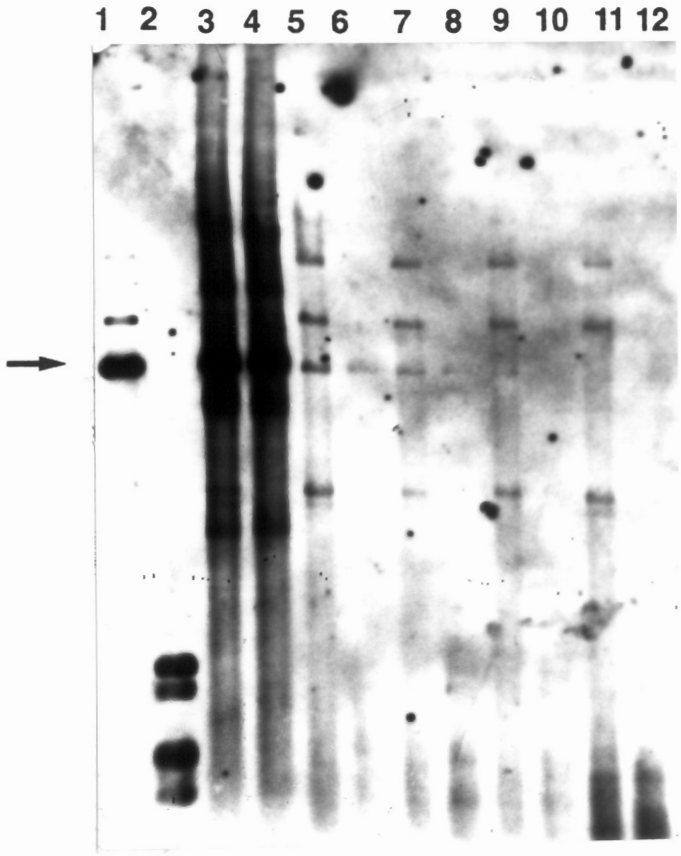


Figure 18. Analysis of low molecular weight DNA after electroporation of BFL cells with the bubbleless mutant clones.
The marker in lane 1 is the SalI fragment of pVT501. Lane 2 shows the DpnI product of a pVT501 plasmid. Lanes 3 to 12 contains the Hirt DNAs extracted from cells electroporated with the following circular plasmids: lanes 3 and 4, pVT501 10 µg; lanes 5 and 6, BLOM 10 µg; lanes 7 and 8, BLOM 20 µg; lanes 9 and 10, BLOP 10 µg; lanes 11 and 12, BLOP 20 µg. In lanes 4, 6, 8, 10 and 12 the DNAs were electrophoresed after DpnI restriction digestion. The arrow indicates the genome-length replicated DNA.

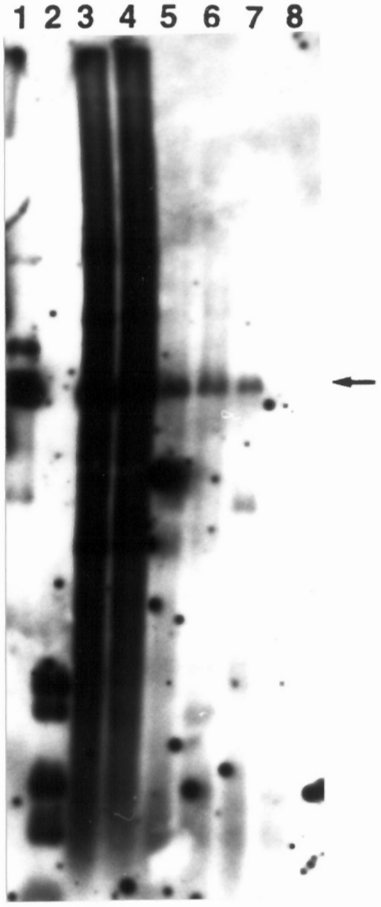


Figure 19. Analysis of low molecular weight DNA after electroporation of BFL cells with the excised bubbleless mutant clones.
The marker in lane 1 is the SalI fragment of pVT501. Lane 2 shows the DpnI digestion product of a pVT501 plasmid. Lanes 3 to 8 contains the Hirt DNAs extracted from cells electroporated with the following plasmids: lanes 3 and 4, pVT501 10 µg; lanes 5 and 6, BLOM 10 µg; lanes 7 and 8, BLOP 10 µg. In lanes 4, 6, and 8 the DNAs were electrophoresed after DpnI restriction digestion. The viral sequences were excised from the plasmid DNAs by digestion with SalI, before electroporation. The arrow indicates the genome-length replicated DNA.

Complementation between replication-deficient clones

pVT501, BLOP and BLOM clones were co-electroporated with a replication-deficient minigenome (pVT501ΔKpnI) at a 1:1 molar ratio, to answer whether full length clones could provide the factors needed in *trans* for minigenome replication. The viral sequences of all clones were excised before electroporation. Replication of pVT501 was greatly reduced, to about 21% of the level of pVT501 alone, when the clone was co-electroporated with pVT501ΔKpnI (Fig. 20, lane 4 compared to lane 3). Amplification of the BPV sequences contained in pVT501ΔKpnI should yield a 2.9 kb fragment. A band of the appropriate size was observed when this minigenome was co-electroporated with pVT501 (lane 4). Replication of BLOM was reduced by the addition of pVT501ΔKpnI, to a level of about 5% of the level of pVT501 alone (lane 5). In comparison, when BLOM was electroporated alone (Fig. 19), it replicated at about 20% of the level of pVT501. However, if the level of replication of BLOM is compared to the level of replication of pVT501, when both clones are co-electroporated with pVT501ΔKpnI, this figure is of 22%. Only a very faint band corresponding to the pVT501ΔKpnI DNA was observed in lane 5. Co-electroporation of BLOP with pVT501ΔKpnI resulted in the production of a 5 kb length molecule (lane 6). Densitometric comparison revealed that BLOP, in the presence of pVT501ΔKpnI, replicated at about 9% of the intensity of pVT501 alone. pVT501ΔKpnI was unable to replicate by itself as shown in lane 7. Two similar sets of co-electroporation experiments were performed with other smaller minigenomes (pVT501ΔXhoI or pVT501ΔNheI) and gave similar results (data not shown).

Indirect immunofluorescence assays were performed on cell layers electroporated with both BLOP and pVT501ΔKpnI (Fig. 21). Positive results were obtained with either the rabbit or the calf serum at day 4 (panels A and B), while no fluorescence was seen when BLOP was electroporated alone (as shown in Fig. 17 panels A and B).

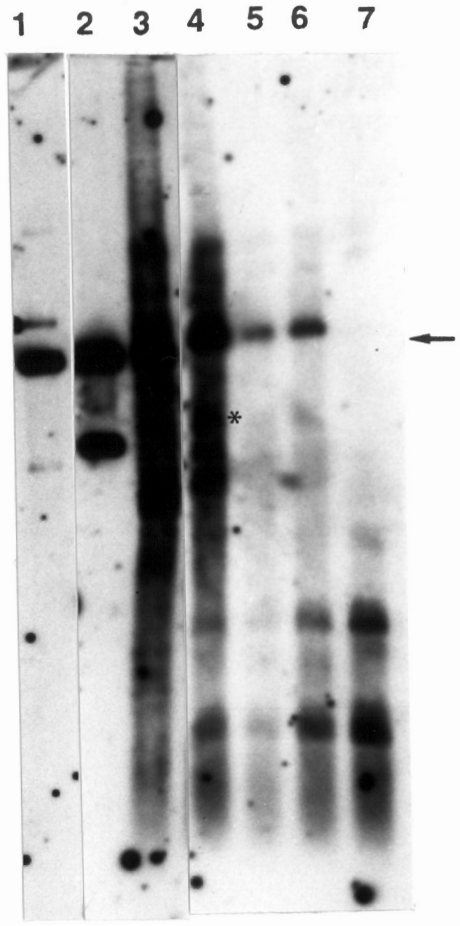


Figure 20. Analysis of transient DNA replication by complementation between replication-deficient clones.
The markers in lanes 1 and 2 are the SalI fragments of pVT501 and pVT501ΔKpnI, respectively. Lanes 3 to 7 contains the DpnI digested DNAs extracted from cells electroporated with the following plasmids: lane 3, pVT501 10 μg; lane 4, pVT501 10 μg and pVT501ΔKpnI 6.8 μg; lane 5, BLOM 10 μg and pVT501ΔKpnI 6.8 μg; lane 6, BLOP 10 μg and pVT501ΔKpnI 6.8 μg; lane 7, pVT501ΔKpnI 6.8 μg. The viral sequences were excised from the plasmid DNAs by digestion with SalI, before electroporation. The arrow indicates the 5kb length replicated DNA, and the asterisk indicates the 2.9 kb replicated DNA.

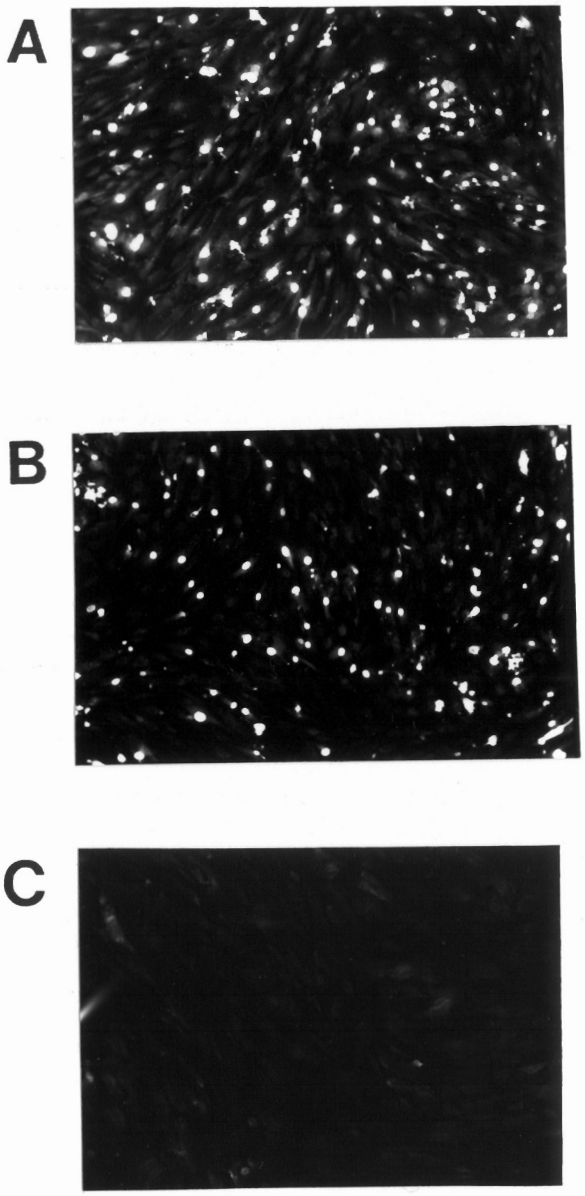


Figure 21. Indirect immunofluorescence assay on cell layers electroporated with BLOP and pVT501ΔKpnI.
Panel A was assayed with calf 86 antibody, panel B with rabbit 0118 antibody, and panel C with no primary antibody.

DISCUSSION

The secondary structure of the viral ends is remarkably conserved among parvoviruses. Of particular interest is the presence of mismatched/unpaired nucleotides, forming a bubble, in the stem of the left hairpin of almost all autonomous parvoviruses. To analyze the possible role of these unpaired/mismatched nucleotides in the BPV life cycle, two mutants clones lacking the bubble region were constructed and their replicative properties were analyzed after electroporation in permissive cells. Infectivity of the mutant clones was determined by three techniques: observation of cytopathic effect, detection of virally-coded proteins by indirect immunofluorescence, and transient DNA replication assays. The mutant clone containing duplicate sequences of the (mismatched) nucleotides numbered 46 to 57 (BLOP) was defective for replication. The other bubbleless clone (BLOM), that contains duplicate sequences of the (mismatched) nucleotides 99 to 105, was able to replicate. The BLOM clone produced monomer-length viral DNA at about 20% of the level of the infectious genomic clone of BPV, when electroporated as a linear excised sequence. This clone was infectious since it could be propagated by subsequent passage. Expression of viral structural proteins was seen by an indirect immunofluorescence assay using anti-capsid antibodies. The BLOM construct, like a wild-type genome, is sensitive to a defective interfering particle; its replication is decreased in the presence of a minigenome containing intact termini. These results suggest that the bubble in the left hairpin of BPV is not required for the viral life cycle, but that specific sequences within the mismatched/unpaired region are necessary to the viral replication process.

These results confirm that the terminal repeats are important to the parvoviral life cycle and that deletion or mutation within these sequences might interfere with the replicative properties of these viruses (Bohensky *et al.*, 1988, Lefebvre *et al.*, 1984, Rhode and Klassen, 1982, Salvino *et al.*, 1991, Samulski *et al.*, 1983, Senepathy *et al.*, 1984, Shull *et al.*, 1988, Snyder *et al.*, 1993). Extensive studies on AAV plasmids showed that the secondary structure and

symmetry of the hairpin arms are important for replication (Bohensky *et al.*, 1988, Lefebvre *et al.*, 1984, Samulski *et al.*, 1983, Snyder *et al.*, 1993) and that deletion in the stem of the hairpin could be repaired to wild type when an intact terminus was present. The mutations introduced in the bubbleless clones altered both the primary and the secondary sequence of the stem of the BPV hairpin. The use of two different types of mutants showed that the secondary structure of the bubble, although needed to obtain wild type level of DNA replication, is not an absolute requirement for the viral life cycle. These experiments also suggest that the bubble-coding DNA sequences that are located furthest from the 3' end of a BPV flop genome, nucleotides 99-105, are essential for the viral replication process. These data are in agreement with Shull's observations (1988) that deletion of the first 52 bases at the 3' end of a BPV clone, in the flip orientation, is lethal. A 52 base deletion in a BPV flip clone removes the information for the bubble, by deleting its potential complementary bases. Smaller deletions of the BPV left end, up to 34 bases, were shown not to affect replication and were repaired to wild type sequences (Shull *et al.*, 1988). Deletion experiments of the right end of MVM revealed that an 82 base deletion, which similarly prevented formation of a bubble by deleting its potential complementary bases, reduced, but did not abolish the infectivity of MVM (Salvino *et al.*, 1991). This MVM clone apparently regenerated a bubble at the same position (Costello *et al.*, 1993).

The mutant clones used in the electroporation experiments were both in the flop orientation, although the orientation could now only be defined through the sequences of the hairpin arms. This conformation was chosen because the dominant orientation at the BPV left end, regardless of the strand polarity, is flip (Chen *et al.*, 1988). Therefore replication from a flop template, which generates a flip progeny, is proposed to proceed or initiate faster (Chen *et al.*, 1989). It has been shown that BPV genomic clones with a flip or a flop left end were equally infectious (Shull *et al.*, 1988). It is thus expected that identical results would have been obtained if bubbleless clones in the flip orientation had been used.

The termini of parvoviruses act in *cis* for viral DNA replication (Rhode and Klassen, 1982, Salvino *et al.*, 1991, Shull *et al.*, 1988, Senepathy *et al.*, 1984, Snyder *et al.*, 1990 (b)), and are involved in encapsidation and excision of viral DNA (McLaughin *et al.*, 1988, Samulski *et al.*, 1983). In addition, they are located upstream of the parvoviral TATA promoters, and contain binding sites for several transcription factors (Blundell and Astell, 1989, Gu *et al.*, 1993, Perros *et al.*, 1993, Pitluck and Ward, 1991), thereby probably affecting transcription. While replication of the BLOM clone shows that the secondary structure of the bubble is not required for any of these viral processes, our experiments cannot yet answer which of these steps is retarded or blocked when nucleotides 99 through 105 are deleted.

Removal of the bubble probably affects excision of the viral sequences from the transfecting plasmid, a function of the NS1 nonstructural protein (Rhode, 1989), since replication was increased two-fold when the mutant BLOM genome was electroporated as a linear excised sequence compared to a circular sequence. The mutations introduced might have affected viral DNA replication by interfering with initiation of replication at the 3' *ori*, or by affecting recognition of the DNA sequences by viral proteins, or alternatively, by interfering with the hairpin resolution step. The non-structural proteins are required in *trans* for AAV and autonomous parvovirus replication (Hermonat *et al.*, 1984, Rhode, 1989, Senepathy *et al.*, 1984, Tratschin *et al.*, 1984). DNaseI protection assays revealed that protein extracts containing Rep 68 and 78 interact with sequences in the stem and the arms of the AAV hairpin (Im and Muzyczka, 1989, Ashktorab and Srivastava, 1989). Resolution of the replicative intermediate at the terminal resolution site (*trs*) by Reps 68 and 78 also requires the intact secondary structure of the hairpin and possibly other sequence elements within the stem of the hairpin. (Snyder *et al.*, 1993). Snyder (1993) reported that addition of 8 bases in the AAV hairpin stem, which moved the *trs* further away from the end of the genome, reduced nicking by 50 fold. Both of the mutations introduced in these bubbleless clones altered the length of the stem of the BPV left hairpin. The BLOM

mutation reduced by 5 bases the spacing between the 3' end of the genome and the arms of the hairpin, while the BLOP mutation added 5 bases downstream of the arms of the hairpin. The location of the predicted *trs* of BPV remains, however, unchanged. Conformation of the termini has been proposed to affect the kinetics of hairpin transfer during replication (Chen *et al.*, 1989). Thus, removal of the orientation-coding sequences in the BPV hairpin stem might modify the hairpin transfer rates.

NS1 acts as a transcriptional regulator for both autonomous and dependoparvoviruses (Beaton *et al.*, 1989, Doerig *et al.*, 1990, Hanson and Rhode, 1991). Stimulation of p4 expression in LuIII occurs by interaction of NS1 with the terminal repeats (Hanson and Rhode, 1991). In addition, several transcription factors were shown to bind to the terminal sequences of autonomous parvoviruses (Blundell and Astell, 1989, Gu *et al.*, 1993, Perros *et al.*, 1993, Pitluck and Ward, 1991). Thus, transcription might also be affected in these bubbleless clones. Expression of structural or nonstructural proteins could not be detected from BLOP by an indirect immunofluorescence assay, while virally coded proteins were expressed from BLOM. Complementation experiments with BPV *trans*-activating clones, or quantification of the gene expression levels of these clones compared to the wild type clone would reveal if gene expression was modified in these clones. Although encapsidation might not have been affected in the BLOM clone, it is difficult to determine if this process was altered in the BLOP clone.

Hermonat (1984) reported that recombination occurred, at a low level, between mutant AAV genomes during lytic growth. I similarly suspect that recombination occurred between the replication-deficient BLOP clone and the replication-deficient minigenomes that contained wild type *oris*. Recombination between these clones could recreate a full length wild-type genome capable of replication. In fact a low level of replication and production of viral proteins by indirect immunofluorescence were observed, when these clone were co-electroporated.

The unpaired/mismatched nucleotides of the BPV left hairpin appear therefore as positive effectors of parvovirus replication, in a sequence-specific manner. The specific step(s) at which these sequences are required needs to be determined and might help elucidate what type of constraint exists for the conservation of the bubble almost throughout the entire autonomous parvovirus genus.

CONCLUSIONS

The first part of my project was to detect specific binding of S-phase proteins to the *oris* of both homologous and heterologous parvoviruses. A DNA-protein complex was detected between the AAV terminus and an extract of a permissive human cell line. We also observed binding between nuclear proteins and *oris* of heterologous parvoviruses: proteins from bovine cells were able to bind to an AAV terminus, and protein extracts from human cells interacted with both *oris* of the BPV genome. These results suggest that similar factors, present in these two different cell types, can recognize different parvoviral *oris*. Although binding of these proteins was not identical for each viral terminus tested, these data suggest a general role for these proteins. Binding to these termini seemed independent of the viral DNA secondary structure, which could suggest that these proteins might interact with different forms of the parvoviral DNA, such as intermediates with different secondary structure produced during infection. The binding site of these proteins on the viral termini could be determined by DNAse protection assays and competition experiments with short oligonucleotide sequences.

In order to begin to characterize these proteins, we performed SDS-PAGE electrophoresis of some retarded complexes. These assays showed that a 54 kd protein of bovine origin was bound to the BPV left terminus and that two phosphoproteins of 55 and 90 kd were present in the complex formed between a BPV left terminus and HeLa cell extract. Different approaches, such

as Southwestern blotting, could also have been used for the size determination of these proteins. Conventional purification methods, such as affinity chromatography, would allow identification of the proteins involved in these complexes. Unfortunately, to achieve this long-term goal, technical difficulties, such as the small amount of nuclear proteins recovered after extraction, must be overcome.

Helper-independent replication of AAV suggests a closer link than previously thought between the two vertebrate parvovirus groups and possibly a common dependence on a protein produced or activated during the S-phase of the cell cycle. Specific binding to the parvoviral *oris* was only observed when the protein extracts were prepared from S-phase synchronized cells. This raises the question as to whether these *ori*-binding proteins might be one of the constituents of the S-phase factor that is required for autonomous parvovirus replication. Since little information is yet available on the nature of this S-phase factor, specific experiments designed to answer this question might not be easy to design.

In view of the results of the SDS-PAGE, an anti-p53 antibody was used as competitor in the DNA retardation assays. This antibody competed with the binding of both cell extracts to the BPV left terminus and with the HeLa cell extract to the AAV terminus. Competition was observed for the formation of all three DNA-protein complexes tested, suggesting that a similar protein, most likely p53, might be involved in the *ori*-binding complexes. More experiments are needed to confirm the presence of p53 in these complexes, to determine if p53 was directly bound to the DNA, and to determine the involvement of p53, if any, in the parvoviral life cycle. p53-deficient cells are available and could be used, for example, to investigate the requirement of p53 for parvoviral replication. If p53 is indeed needed for viral replication, the stage at which this anti-oncoprotein acts will need to be determined. Either the replication or transcription of parvoviruses could be directly affected. Alternatively, p53 could act as a general factor, either as a

modulator of cellular transcription, repair mechanisms, cell death pathways, or cellular phenotype.

In order to determine the possible role of the unpaired/mismatched nucleotides of BPV in its life cycle, two mutant BPV clones, differing from the wild type genome solely by the lack of the bubble, were constructed. The replication of these clones was analyzed by indirect immunofluorescence for the detection of viral proteins, and by extraction of low molecular weight replicative DNA. We report that one clone (BLOP, in which bases were added to remove the bubble) did not replicate, while the other clone (BLOM, in which bases were deleted to base pair this region) replicated at a low level but was able to produce infectious viruses. This suggests that the sequence of one of the strands found in the bubble, that which is missing in the BLOP clone, is required for the viral life cycle, while the sequence of the other strand, missing in BLOM, is not essential for replication. Others experiments would be needed to distinguish between requirement for the specific sequence and the requirement for a bubble to obtain wild-type level of replication. Using site-directed mutagenesis, the wild type sequence could be maintained on one strand while introducing a mutation on the other strand by inserting a bubble of unrelated sequence. It would also be interesting to insert or remove bases in the stem of the hairpin to move the bubble further upstream or downstream from its original location. The assays performed in this study did not allow us to determine which viral pathway(s) was affected in the replication of these two clones. Experiments aimed at determining the transcription level of these clones, and complementation experiments with clones providing the *trans* factors required for replication could be performed. Determination of the binding site of the major BPV nonstructural protein to the viral left terminus should provide more insight on the precise sequences required for replication. Finally, to see if these mutations affected the hairpin transfer rate or the hairpin transfer process, complete analysis of the flip to flop and plus to minus distributions of the virion

produced by one of the clone could be undertaken. If such data can be obtained, they should be compared to the distributions observed for the AAV and B19 genomes both of which lack a bubble in their hairpins.

LITERATURE CITED

Alexandersen, S., M. E. Bloom, and S. Perryman. 1988. Detailed transcription map of Aleutian mink disease parvovirus. J. Virol. 62:3684-3694.

Antoni, B. A., A. B. Rabson, I. J. Miller, J. P. Trempe, N. Chejanovsky, and B. J. Carter. 1991. Adeno-associated virus Rep protein inhibits human immunodeficiency virus type 1 production in human cells. J. Virol. 65:396-404.

Arella, M., S. Garzon, J. Bergeron, and P. Tijssen. 1990. Physicochemical properties, production, and purification of parvoviruses, p. 11-30, vol. I. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL.

Armentrout, R., R. Bates, K. Berns, B. Carter, M. Chow, D. Dressler, K. Fife, W. Hauswirth, G. Hayward, G. Lavelle, S. Rhode, S. Straus, P. Tattersall, and D. Ward. 1978. A standardized nomenclature for restriction endonuclease fragments, p. 523-526. *In* D. C. Ward and P. Tattersall (eds.), Replication of mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor, N. Y.

Ashktorab, H., and A. Srivastava. 1989. Identification of nuclear proteins that specifically interact with adeno-associated virus type 2 inverted terminal repeat hairpin DNA. J. Virol. 63:3034-3039.

Astell, C. R. 1990. Terminal hairpins of parvovirus genomes and their role in DNA replication, p. 59-80, vol. I. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL.

Astell, C. R., M. B. Chow, and D. C. Ward. 1985. Sequence analysis of the termini of virion and replicative forms of minute virus of mice suggests a modified rolling hairpin model for autonomous parvovirus DNA replication. J. Virol. 54:171-177.

Astell, C. R., E. M. Gardiner, and P. Tattersall. 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM (i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. 57:656-669.

Astell, C. R., M. Smith, M. B. Chow, and D. C. Ward. 1979. Structure of the 3' hairpin termini of four rodent parvovirus genomes: nucleotide sequence homology at origins of DNA replication. Cell 17:691-703.

Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward. 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. 11:999-1018.

Atchison, R. W., B. C. Casto, and W. McD Hammond. 1965. Adeno-associated defective virus particles. Science. 149:754-755.

Attardi, B., and G. Attardi. 1967. A membane-associated RNA of cytoplasmic origin in HeLa cells. Proc. Natl. Acad. Sci. 58:1051-1058.

Avalosse, B. L., S. Barrijal, Y. Q. Chen, J.-J. Cassiman, and J. Rommelaere. 1989. Identification of a transformation-sensitive nuclear protein from normal human fibroblasts that specifically interacts with minute virus of mice DNA and correlates with cell resistance to the parvovirus. Mol. Carcinogenesis 2:245-251.

Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Wilson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249:912-915.

Ball-Goodrich L. J., and P. Tattersall. 1992. Two amino acid substitutions within the capsid are coordinately required for acquisition of fibrotropism by the lymphotropic strain of minute virus of mice. J. Virol. 66:3415-3423.

Banks, L., G. Matlashewski, and L. Crawford. 1986. Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. Eur. J. Biochem. 159, 529-534.

Barbis, D. P., S.-F. Chang, and C. R. Parrish. 1992. Mutations adjacent to the dimple of the canine parvovirus capsid structure affect sialic acid binding. Virology 191:301-308.

Barik, S. 1993. Site-directed mutagenesis by double polymerase chain reaction, p. 277-286. *In* B. A. White (ed.), Methods in molecular biology, vol 15: PCR protocols: Current methods and applications. Humana Press Inc., Totowa, N.Y.

Barrijal, S., M. Perros, Z. Gu, B. L. Avalosse, P. Belenguer, F. Amalric, and J. Rommelaere. 1992. Nucleolin forms a specific complex with a fragment of the viral (minus) strand of minute virus of mice DNA. Nucleic Acids Res. 19:5053-5060.

Basak, S., and H. Turner. 1992. Infectious entry pathway for canine parvovirus. Virology 186:368-376.

Bates, R. C. 1990. Clinical and pathological features: diagnosis of bovine parvovirus infections, p. 181-192, vol. II. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL.

Beaton, A., P. Palumbo, and K. I. Berns. 1989. Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated in *trans* by the Rep protein. J. Virol. 63:4450-4454.

Bergemann, A. D., and E. M. Johnson. 1992. The HeLa Pur factor binds single-stranded DNA at a specific element conserved in gene flanking regions and origins of DNA replication. Mol. Cell. Biol. 12:1257-1265.

Berns, K. I. 1990. Parvoviridae and their replication, p. 1743-1763, vol. II. *In* B. N. Fields and D. M. Knipe (eds.), Virology, 2nd ed. Raven Press, Ltd., New York, N.Y.

Berns, K. I., and W. W. Hauswirth. 1984. Adeno-associated virus DNA structure and replication, pp. 1-31. *In* K. I. Berns (ed.), The Parvoviruses. Plenum Press, New-York. N.Y.

Bischoff, J. R., P. N. Friedman, D. R. Marshak, C. Prives, and D. Beach. 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc. Natl. Acad. Sci. USA 87:4766-4770.

Blacklow, N. R. 1988. Adeno-associated viruses of humans, p. 165-174. *In* J.R. Pattison (ed.), Parvoviruses and human disease, CRC Press, Inc. Boca Raton, FL.

Bloom, M. E., S. Alexandersen, S. Perryman, D. Lechner, and J. B. Wolfinbarger. 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV. J. Virol. 62:2903-2915.

Bloom, M. E., R. E. Race, and J. B. Wolfinbarger. 1982. Identification of a nonvirion protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonvirion proteins. J. Virol. 43:608-616

Blundell, M. C., and C. R. Astell. 1989. A GC-box motif upstream of the B19 parvovirus unique promoter is important for *in vitro* transcription. J. Virol. 63: 4814-4823.

Bodnar, J. W., P. I. Hanson, M. Polvino-Bodnar, W. Zempsky, and D. C. Ward. 1989. The terminal regions of adenovirus and minute virus of mice DNAs are preferentially associated with the nuclear matrix in infected cells. J. Virol. 63:4344-4353.

Bohenzky, R. A., R. B. Lefebvre, and K. I. Berns. 1988. Sequence and symmetry requirements within the internal palindromic sequences of the adeno-associated virus terminal repeat. Virology. 166:316-327.

Boulanger, P. A., and G. E. Blair. 1991. Expression and interactions of human adenovirus oncoproteins. Biochem. J. 275:281-299.

Braithwaite, A., C. Nelson, A. Skulimowsli, J. McGovern, D. Pigott, and J. Jenkins. 1990. Transactivation of the p53 oncogene by E1a gene products. Virology 177:595-605.

Buller, R. M., J. E. Janik, E. D. Sebring, and J. A. Roses. 1981. Herpes simplex virus types 1 and 2 completely help adeno-associated virus replication. J. Virol. 40:241-247.

Carter, B. J. 1990. Adeno-associated virus helper functions, p. 255-282, vol. I. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL.

Cater, J. E., and D. J. Pintel. 1992. The small non-structural protein NS2 of the autonomous parvovirus minute virus of mice is required for virus growth in murine cells. J. Gen. Virol. 73:1839-1843.

Cavalier-Smith, T. 1974. Palindromic base sequences and replication of eukaryote chromosome ends. Nature (London) 250:467-470.

Chen, K. C., B. C. Shull, M. Lederman, E. R. Stout, and R. C. Bates. 1988. Analysis of the termini of the DNA of bovine parvovirus: demonstration of a sequence inversion at the left terminus and its implication for the replication model. J. Virol. 62:3807-3813.

Chen, K. C., B. C. Shull, E. A. Moses, M. Lederman, E. R. Stout, and R. C. Bates. 1986. Complete nucleotide sequence and genome organization of bovine parvovirus. J. Virol. 60:1085-1097.

Chen, K. C., J. J. Tyson, M. Lederman, E. R. Stout, and R. C. Bates. 1989. A kinetic hairpin transfer model for parvoviral DNA replication. J. Mol. Biol. 208:283-296.

Cheung, A. K.-M., M. D. Hoggan, W. W. Hauswirth, and K. I. Berns. 1980. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J. Virol. 33: 739-748 .

Chejanovsky, N., and B. J. Carter. 1989. Mutagenesis of an AUG codon in the adeno-associated virus *rep* gene: effects on viral DNA replication. Virology 173:120-128.

Chow, M., J. W. Bodnar, M. Polvino-Bodnar, and D. C. Ward. 1986. Identification and characterization of a protein covalently bound to DNA of minute virus of mice. J. Virol. 57:1094-1104.

Clark, J. M. 1988. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. Nucl. Acid Res. 16:9677-9686.

Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102-1106.

Cooper, G. M. Oncogenes. 1990. Jones and Barlett Publishers, Boston, MA.

Costello, E., R. Sahli, P. Beard, and B. Hirt. 1993. Analysis of the mismatched nucleotides/bubble in the 5' terminal hairpin of MVM(p). Program Abstr. 5th International Parvovirus Workshop, abstr. P1-11.

Cotmore, S. F., M. Gunther, and P. Tattersall. 1989. Evidence for a ligation step in the DNA replication of the autonomous parvovirus minute virus of mice. J. Virol. 63:1002-1006.

Cotmore, S. F., J. P. F. Nuesch, and P. Tattersall. 1992. *In vitro* excision and replication of 5' telomeres of minute virus of mice DNA from cloned palindromic concatemer junctions. Virology 190:365-377.

Cotmore, S. F., L. J. Sturzenbecker, and P. Tattersall. 1983. The autonomous parvovirus MVM encodes two nonstructural proteins in addition to its capsid polypeptides. Virology 129:333-343.

Cotmore, S. F, and P. Tattersall. 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. 58:724-732.

Cotmore, S. F, and P. Tattersall. 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. 33:91-174.

Cotmore, S. F., and P. Tattersall. 1988. The NS-1 polypeptide of minute virus of mice is covalently attached to the 5' termini of duplex replicative-form DNA and progeny single strands. J. Virol. 62:851-860.

Cotmore, S. F., and P. Tattersall. 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. J. Virol. 63:3902-3911.

Cukor, G., N. R. Blacklow, M. D. Hoggan, and K. I. Berns. 1984. Biology of adeno-associated virus, p. 33-66, *In* K. I. Berns (ed.), The parvoviruses. Plenum Press, New-York, N.Y.

Dabrowski, C. E., and P. A. Schaffer. 1991. Herpes simplex virus type 1 origin-specific binding protein: *ori*S-binding properties and effects of cellular proteins. J. Virol. 65:3140-3150.

Deiss, V., J.-D. Tratschin, M. Weitz, and G. Siegl. 1990. Cloning of the human parvovirus B19 genome and structural analysis of its palindromic termini. Virology. 175:247-254.

DePamphilis, M. L. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell 52:635-638.

Diffoot, N. 1992. Ph.D. thesis. Virginia Polytechnic Institute and State University, VA.

Diffoot, N., K. C. Chen, R. C. Bates, and M. Lederman. 1993. The complete nucleotide sequence of parvovirus LuIII and localization of a unique sequence possibly responsible for its encapsidation pattern. Virology. 192;339-345.

Diffoot, N., B. C. Shull, K. C. Chen, E. R. Stout, M. Lederman, and R. C. Bates. 1989. Identical ends are not required for the equal encapsidation of plus- and minus-strand parvovirus LuIII DNA. J. Virol. 63:3180-3184.

Doerig, C., B. Hirt, J.-P. Antonietti, and P. Beard. 1990. Nonstructural protein of parvoviruses B19 and minute virus of mice controls transcription. J. Virol. 64:387-396.

Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery Jr, J. S. Butel and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature (London) 356:215-221.

Dutta, A., J. M. Ruppert, J. C. Aster, and E. Winchester. 1993. Inhibition of DNA replication factor RPA by p53. Nature (London) 365:79-82.

Farmer, G., J. Bargonetti, H. Zhu, P. Friedman, R. Prywes, and C. Prives. 1992. Wild-type p53 activates transcription in vitro. Nature (London) 358:83-86.

Finlay, C. A., P. W. Hinds, and A. J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell 57:1083-1093.

Flint, J., and T. Shenk. 1989. Adenovirus E1A protein paradigm viral transactivator. Annu. Rev. Genet. 23:141-161.

Friedman, P. N., S. E. Kern. B. Vogelstein, and C. Prives. 1990. Wild-type, but not mutant human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA. 87:9275-9279.

Gottlieb, J., and N. Muzycka. 1988. *In vitro* excision of adeno-associated virus DNA from recombinant plasmids: isolation of an enzyme fraction from HeLa cells that cleaves DNA at poly(G) sequences. Mol. Cell. Biol. 6:2513-2522.

Green, M. R., and R. G. Roeder. 1980. Transcripts of the adeno-associated virus genome: mapping of the major RNAs. J. Virol. 36:79-92.

Gu, M.-L., F.-X. Chen, and S. L. Rhode. 1992. Parvovirus H-1 P38 promoter requires the *trans*-activating region (tar), an Sp1 site, and a TATA box for full activity. Virology 187:10-17.

Gu, Z., S. Plaza, C. Dinsart, J. J. Cornelis and J. Rommelaere. 1993. Mutually exclusive binding of NF-Y and USF transcription factors to an upstream element of the MVM(p) P4 promoter. Program Abstr. 5th International Parvovirus Workshop, abstr. 3-1.

Gu, M.-L., and S. L. Rhode. 1991. Autonomous parvovirus DNA replication requires topoisomerse I and its activity is increased during infection. J. Virol. 65:1662-1665.

Habiger, C., G. Stelzer, U. Schwarz, and E.-L. Winnacker. 1992. Two cellular single-strand-specific DNA-binding proteins interact with two regions of the bovine papillomavirus type 1 genome, including the origin of DNA replication. J. Virol. 66:5988-5998.

Hanson, N. D., and S. L. Rhode III. 1991. Parvovirus NS1 stimulates P4 expression by interaction with the terminal repeats and through DNA amplification. J. Virol. 65:4325-4333.

Harris, C. C., and M. Hollstein. 1993. Clinical implications of the p53 tumor-suppressor gene. N. Engl. J. Med. 329:1318-1327.

Hermonat, P. L. 1989. The adeno-associated virus rep 78 gene inhibits cellular transformation by bovine papillomavirus. Virology 172:253-261.

Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka. 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. 51:329-339.

Herrlich, P., P. Angel, H. J. Rahmsdorf, U. Mallick, A. Poting, L. Hieber, C. Lucke-Huhle, and M. Schorpp. 1986. The mammalian genetic stress response. Adv. Enz. Regul. 25:485-504.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. 26:365-369.

Hofmann, J. F.-X., and S. M. Gasser. 1991. Identification and purification of a protein that binds the yeast ARS consensus sequence. Cell 64:951-960.

Hong, G., P. Ward, and K. I. Berns. 1992. *In vitro* replication of adeno-associated virus DNA. Proc. Natl. Acad. Sci. USA 89:4673-4677.

Hong, G., P. Ward, and K. I. Berns. 1994. Intermediates of adeno-associated virus DNA replication *in vitro*. J. Virol. 68:2011-2015.

Horiuchi, M., N. Ishiguro, H. Goto, and M. Shinagawa. 1992. Characterization of the stage(s) in the virus replication cycle at which the host-cell specificity of the feline parvovirus subgroup is regulated in canine cells. Virology 189:600-608.

Hunter, L. A., and R. J. Samulski. 1992. Colocalization of adeno-associated virus Rep and capsid proteins in the nuclei of infected cells. J. Virol. 66:317-324.

Im, D. S., and N. Muzyczka. 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. 63:3095-3104.

Im, D. S., and N. Muzyczka. 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell 61: 447-457.

Im, D. S., and N. Muzyczka. 1992. Partial purification of adeno-associated virus Rep78, Rep52, and Rep40 and their biochemical characterization. J. Virol. 66:1119-1128.

Innis, M. A., and D. H. Gelfand. 1990. Optimization of PCRs, p. 3-11. *In* M. A. Innis, D. H. Gelfand, J. J. Snisky, and T. J. White (eds.), PCR protocols: a guide to methods and applications. Academic Press Inc., San Diego, CA.

Iversen, P., and S. L. Rhode. Parvovirus proteins, p 81-90, vol. I. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL.

Kajigaya, S., H. Fujii, A. Field, S. Anderson, S. Rosenfeld, L. Anderson, T. Shimada, and N. S. Young. 1991. Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. Proc. Natl. Acad. Sci. USA 88:4646-4650.

Kariatsumari, T., M. Horiuchi, E. Hama, K. Yaguchi, N. Ishigurio, N. Goto, and M. Shinagawa. 1991. Construction and nucleotide sequence analysis of an infectious DNA clone of the autonomous parvovirus, mink enteritis virus. J. Gen. Virol. 72:867-875.

Kern, S. E., K. W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein. 1991. Identification of p53 as a sequence-specific DNA-binding protein. Science 252:1708-1711.

Kollek, R., B. Y. Tseng, and M. Goulian. 1982. DNA polymerase requirements for parvovirus H-1 DNA replication *in vitro*. J. Virol. 41:982-989.

Konchuba, A. M., P. F. Schellhammer, J. P. Alexander, and G. L. Wright. 1989. Flow cytometric study comparing paired bladder washing and voided urine for bladder cancer detection. Urology 33:89-96.

Kotin, R. M., M. Siniscalco, R. J. Samulski, X. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns. 1990. Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. USA 87:2211-2215.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Lane, D. P., and L. V. Crawford. 1979. T antigen is bound to a host protein in SV-40 transformed cells. Nature (London) 278:201-203.

Langeveld, J. P. M., J. I. Casal, C. Vela, K. Dalsgaard, S. H. Smale, W. C. Puijk, and R. H. Meloen. 1993. B-cell epitopes of canine parvovirus: distribution on the primary structure and exposure on the viral surface. J. Virol. 67:765-772.

Lederman, M., R. C. Bates and E. R. Stout. 1983. *In vitro* and *in vivo* studies of the bovine parvovirus proteins. J. Virol. 48:10-17.

Lederman, M., S. F. Cotmore, E. R. Stout, and R. C. Bates. 1987 (a). Detection of bovine parvovirus proteins homologous to the nonstructural NS-1 proteins of other autonomous parvoviruses. J. Virol. 61:3612-3616.

Lederman, M., J. T. Patton, E. R. Stout, and R. C. Bates. 1984. Virally coded noncapsid protein associated with bovine parvovirus infection. J. Virol. 49:315-318.

Lederman, M., B. C. Shull, E. R. Stout, and R. C. Bates. 1987 (b). Bovine parvovirus DNA-binding proteins: identification by a combined DNA hydridization and immunodetection assay. J. Gen. Virol. 68:147-157.

Lefebvre, R. B., S. Riva, and K. I. Berns. 1984. Conformation takes precedence over sequence in adeno-associated virus DNA replication. Mol. Cell. Biol. 4:1416-1419.

Levine, A. J. 1990. The p53 protein and its interaction with the oncogene products of the small DNA tumor viruses. Virology. 177:419-426.

Lewin, B. 1990. Genes IV. Cell Press, Cambridge, MA

Li, X., and S. L. Rhode III. 1991. Nonstructural protein NS2 of parvovirus H-1 is required for efficient viral protein synthesis and virus production in rat cells *in vivo* and *in vitro*. Virology 184:117-130.

Liu., H. T., R. Baserga, and W. E. Mercer. 1985. Adenovirus type 2 activates cell cycle-dependent genes that are a subset of those activated by serum. Mol. Cell. Biol. 5:2936-2942.

Liu, J. M., S. W. Green, T. Shimada, and N. S. Young. 1992. A block in full-length transcript maturation in cells nonpermissive for B19 parvovirus. J. Virol. 66:4686-4692.

Liu, J. M., S. W. Green, H. Yu-Shu, K. T. McDonagh, N. S. Young, and T. Shimada. 1991. Upstream sequences within the terminal hairpin positively regulate the P6 promoter of B19 parvovirus. Virology. 185:39-47.

Luo, W., and C. R. Astell. 1993. A novel protein encoded by small RNAs of parvovirus B19. Virology 165:448-455.

Lusby, E., K. H. Fife, and K. I. Berns. 1980. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. J. Virol. 34:402-409.

Mack, D. H., J. Vartikar, J. M. Pipas, and L. A. Laimins. 1993. Specific repression of TATA-mediated but not initiator-mediated transcription by wild type p53. Nature (London) 363:281-283.

Matsunaga, Y., and S. Matsuno. 1983. Structural and nonstructural proteins of a rabbit parvovirus. J. Virol. 45:627-633.

McLaughin, S.K., P. Collis, P.L. Hermonat, and N. Muzyczka. 1988. Adeno-associated virus general transduction vectors: analysis of proviral structures. J. Virol. 62:1963-1973.

McPherson, R. A., and J. A. Rose. 1983. Structural proteins of adeno-associated virus: subspecies and their relatedness. J. Virol. 46:523-529.

Maxwell, I. H., and F. Maxwell. 1988. Electroporation of mammalian cells with a firefly luciferase expression plasmid: kinetics of transient expression differ markedly among cell types. DNA. 7:557-562

Mendelson, E., J. P. Trempe, and B. J. Carter. 1986. Identification of the *trans* acting rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. J. Virol. 60:823-832.

Merchlinsky, M. J., P. J. Tattershall, J. L. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward. 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. 47:227-232.

Metcalf, J. B., R. C. Bates, and M. Lederman. 1990. Interaction of virally coded protein and a cell cycle-regulated cellular protein with the bovine parvovirus left terminus *ori*. J. Virol. 64: 5485-5490.

Momand, J., G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69:1237-1245.

Moore, E. C., and R. B. Hurlbert. 1989. The inhibition of ribonucleoside diphosphate reductase by hydroxyurea, guanazole and pyrazoloimidazole (IMPY), p. 165-201. *In* J. G. Cory and A. H. Cory (eds.), Inhibitors of ribonucleoside diphosphate reductase activity. Pergamon Press Inc., New York, N. Y.

Oh, S.-J., T. Chittenden, and A. J. Levine. 1991. Identification of cellular factors that bind specifically to the Epstein-Barr virus origin of DNA replication. J. Virol. 65:514-519.

Ozawa, K., J. Ayub, H. Yu-Shu, G. Kurtzman, T. Shimada, and N. Young. 1987. Novel transcription map for the B19 (human) pathogenic parvovirus. J.Virol. 61:2395-2406.

Ozawa K., and N. Young. 1987. Characterization of capsid and non-capsid proteins of B19 parvovirus propagated in human erythroid bone marrow cell culture. J. Virol. 61:2627-

Parris, D. S. 1975. Ph.D. thesis. Virginia Polytechnic Institute and State University, VA.

Parris, D. S., and R. C. Bates. 1976. Effect of bovine parvovirus replication on DNA, RNA, and protein synthesis in S phase cells. Virology. 73:72-78.

Parris, D. S., R. C. Bates, and E. R. Stout. 1975. Hydroxyurea synchronization of bovine fetal spleen cells. Explt. Cell. Res. 96:422-425.

Pattison, J. R. 1990. Parvoviruses. Medical and biological aspects. p. 1765-1784, vol. II. *In* B. N. Fields and D. M. Knipe (eds.), Virology, 2nd ed. Raven Press, Ltd., New York, N.Y.

Perros, M., S. Faisst, N. Spruyt, J.-M. Vanacker, and J. Rommelaere. 1993. Interaction of the parvovirus MVM(p) P4 promoter with the ATF family of transcription factors. Program Abstr. 5th International Parvovirus Workshop, abstr. P1-13.

Pfeiffer, S. E., and L. J. Tolmach. 1967. Inhibition of DNA synthesis in HeLa cells by hydroxyurea. Cancer Res. 27:124-129.

Pietenpol, J. A., and B. Vogelstein. 1993. No room at the p53 inn. Nature (London) 365:17-18.

Pintel, D., D. Dadachanji, C. R. Astell, and D. C. Ward. 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res. 11:1019-1038.

Pitluck, Z. W., and D. C. Ward. 1991. Unusual Sp1-GC box interaction in a parvovirus promoter. J. Virol. 65:6661-6670.

Pritchard, C., E. R. Stout, and R. C. Bates. 1981. Replication of parvoviral DNA. Characterization of a nuclear lysate system. J. Virol. 37:352-362.

Rhode, S. L. 1973. Replication process of the parvovirus H-1. Kinetics in a parasynchronous cell system. J. Virol. 11:856-861.

Rhode, S. L. 1985. *Trans*-activation of parvovirus p38 promoter by the 76K noncapsid protein. J. Virol. 55:886-889.

Rhode, S. L. 1989. Both excision and replication of cloned autonomous parvovirus DNA require the NS1 (*rep*) protein. J. Virol. 63:4249-4256.

Rhode, S. L., and P. Iversen. 1990. Parvoviruses genomes: DNA sequences, p. 31-57, vol. I. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL.

Rhode, S.L., and B. Klassen. 1982. DNA sequence of the 5' terminus containing the replication origin of parvovirus replicative form DNA. J. Virol. 41:990-999.

Rhode, S. L., and P. R. Paradiso. 1983. Parvovirus genome: nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J. Virol. 45:173-184.

Rommelaere, J. and P. Tattersall. 1990. Oncosuppression by parvoviruses , p. 41-57, vol. II. *In* P. Tijssen (ed.), Handbook of parvoviruses. CRC Press Inc., Boca Raton, FL..

Salvino, R., M. Skiadopoulos, E. A. Faust, P. Tam, R. O. Shade, and C. R. Astell. 1991. Two spacially distinct elements constitute a bipartite DNA replication origin in the minute virus of mice genome. J. Virol. 65:1352-1363.

Samulski, R. J., L.-S. Chang, and T. Shenk. 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised *in vitro* and its use to study viral replication. J. Virol. 61:3096-3101.

Samulski, R. J., L.-S. Chang, and T. Shenk. 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J. Virol. 63:3822-3828.

Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka. 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell 33:135-143.

Sarasin, A. 1985. SOS response in mammalian cells. Cancer Invest. 3:163-174.

Scheffner, M., K. Münger, J. C. Byrne, and P. Howley. 1991. The state of p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA 88:5523-5527.

Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129-1136.

Schlehofer, J. R., M. Ehrbar, and H. Zur Hausen. 1986. Vaccinia virus, herpes simplex virus, and carcinogens induce DNA amplification in a human cell line and support replication of a helpervirus dependent parvovirus. Virology. 152:110-117.

Schmidt, M. A., S. U. Herterich and B. Krauss. 1991. A single-stranded DNA binding protein from *S. cerevisiae* specifically recognizes the T-rich strand of the core sequence of ARS elements and discriminates against mutant sequences. EMBO. 10:981-985.

Schoborg, R. V., and D. Pintel. 1991. Accumulation of MVM gene products is differentially regulated by transcription initiation, RNA processing and protein stability. Virology 181:22-34.

Senepathy, P., J. D. Tratschin, and B. J. Carter. 1984. Replication of adeno-associated virus DNA: complementation of naturally occuring rep- mutants by a wild type genome or an *ori-* mutant and correction of terminal palindrome deletions. J. Mol. Biol. 179:1-20.

Seto, E., A. Usheva, G. P. Zambetti, J. Momand, N. Horikoshi, R. Weinmann, A. J. Levine, and T. Shenk. 1992. Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc. Natl. Acad. Sci. USA 89:12028-12032.

Shade, R. O., M. C. Blundell, S. F. Cotmore, P. Tattersall, and C. R. Astell. 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J. Virol. 58:921-936.

Shahrabadi, M. S., H. J. Cho, and R. G. Marusyk. 1977. Characterization of the protein and nucleic acid of Aleutian disease virus. J. virol. 23: 353-362.

Shull, B. C., K. C. Chen, M. Lederman, E. R. Stout, and R. C. Bates. 1988. Genomic clones of bovine parvovirus: construction and effect of deletion and terminal sequence inversions on infectivity. J. Virol. 62:417-426.

Sidransky, D., T. Mikkelsen, K. Schwechheimer, M. Rosenblum, W. Cavanee, and B. Vogelstein. 1992. Clonal expansion of p53 mutant cells is associated with brain tumor progression. Nature (London) 355:846-847.

Siegl, G., R. C. Bates, K. I. Berns, B. J. Carter, D. C. Kelly, E. Kurstak, and P. Tattersall. 1985. Characteristics and taxonomy of Parvoviridae. Intervirology. 23:61-73.

Siegl, G., and M. Gautschi. 1973. The multiplication of parvovirus LuIII in a synchronized culture system. Biochemical characteristics of virus replication. Arch. Gesamte Viruforsch. 40:119-127.

Snyder, R. O., D.-S. Im, and N. Muzyczka. 1990 (a). Evidence for covalent attachment of the adeno-associated virus (AAV) Rep protein to the ends of the AAV genome. J. Virol. 64:6204-6213.

Snyder, R. O., D.-S. Im, T. Ni, X. Xia, R. J. Samulsky, and N. Muzyczka. 1993. Features of the adeno-associated virus origin involved in substrate recognition by the viral Rep protein. J. Virol. 67:6096-6104.

Snyder, R. O., R. J. Samulsky, and N. Muzyczka. 1990 (b). *In vitro* resolution of covalently joined AAV chromosome ends. Cell 60:105-113.

Spalholz, B. A., and P. Tattersall. 1983. Interaction of minute virus of mice with differentiated cells: strain-dependent target cell specificity is mediated by intracellular factors. J. Virol. 46:937-943.

Srivastava, A. 1987. Replication of the adeno-associated virus DNA termini *in vitro*. Intervirology 27:138-147.

Srivastava, A., E. W. Lusby, and K. I. Berns. 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. 45:555-564.

Sraus, S. E., E. D. Sebring, and J. A. Rose. 1976. Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. Proc. Natl. Acad. Sci. USA. 73:742-746.

Symonds, H., J. Chen, and T. Van Dyke. 1991. Complex formation between the lymphotropic papovavirus large tumor antigen and the tumor suppressor protein p53. J. Virol. 65:5417-5424.

Tam, P., and C. R. Astell. 1993. Replication of minute virus of mice minigenomes: novel replication elements required for MVM DNA replication. Virology 193:812-824.

Tattersall, P. 1972. Replication of the parvovirus MVM. Dependence of virus multiplication and plaque formation on cell growth. J. Virol. 10:586-590.

Tattersall, P, and J. Bratton. 1983. Reciprocal productive and restrictive virus-cell interactions of immunosuppressive and prototype strains of minute virus of mice. J. Virol. 46:944-955.

Tattersall, P., A. J. Shatkin, and D. C. Ward. 1977. Sequence homology between the structural polypeptides of minute virus of mice. J. Mol. Biol. 111:375-394

Telerman, A., M. Tuynder, T. Dupressoir, B. Robaye, F. Sigaux, E. Shaulian, M. Oren, J. Rommelaere, and R. Amson. 1993. A model for tumor suppression using H-1 parvovirus. Proc. Natl. Acad. Sci. USA. 90:8702-8706.

Tennant, R. W., K. R. Layman, and R. E. Hand, Jr. 1969. Effect of cell physiological state on infection by rat virus. J. Virol. 4:872-878.

Tratschin, J. D., I. L. Miller, and B. J. Carter. 1984. Genetic analysis of adeno-associated virus: properties of deletion mutants constructed *in vitro* and evidence for an adeno-associated virus replication function. J. Virol. 51:611-619.

Tratschin, J. D., J. Tal, and B. J. Carter. 1986. Negative and positive regulation in *trans* of gene expression from adeno-associated virus vector in mammalian cells by a viral rep gene product. Mol. Cell. Biol. 6:2884-2894.

Traut, W., and E. Fanning. 1988. Sequence-specific interactions between a cellular DNA-binding protein and the simian virus 40 origin of DNA replication. Mol. Cell. Biol. 8:903-911.

Tsao, J., M. S. Chapman. M. Agbandje, W. Keller, K. Smith, H. Wu, M. Luo, T. J. Smith, M. G. Rossman, R. W. Compans, and C. R. Parrish. 1991. The three-dimensional structure of canine parvovirus and its functional implications. Science 251:1456-1464.

Tseng, B. Y., R. H. Grafstrom, D. Revie, W. Oertel, and M. Goulian. 1978. Studies on early intermediates in the synthesis of DNA in animal cells. Cold Spring Harbor Symp. Quant. Biol. 43:263-270.

Tullis, G. E., L. R. Burger, and D. Pintel. 1993. The minor capsid protein VP1 of the autonomous parvovirus minute virus of mice is dispensable for encapsidation of progeny single-stranded DNA but is required for infectivity. J. Virol. 67:131-141.

Ullrich, S. J., C. W. Anderson, W. E. Mercer, and E. Appella. 1992. The p53 tumor suppressor protein, a modulator of cell proliferation. J. Biol. Chem. 267:15259-15262.

Vasudevacharya, J., and R. W. Compans. 1992. The NS and capsid genes determine the host range of porcine parvovirus. Virology 187:515-524.

Via, L. E. 1993. Ph.D. thesis. Virginia Polytechnic Institute and State University, VA.

Vogelstein, B., and K. W. Kinzler. 1992. p53 function and dysfunction. Cell 70:523-526.

Weinberg, R. A. 1991. Tumor suppressor genes. Science 254:1138-1146.

Weindler, F. W., and R. Heilbronn. 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. J. Virol. 65:2476-2483.

Wermess, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76-79.

Wilcock, D. M., and D. P. Lane. 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature (London) 349:429-431.

Willwand, K., and B. Hirt. 1991. The minute virus of mice capsid specifically recognizes the 3' hairpin structure of the viral replicative-form DNA: mapping of the binding site by hydroxyl radical footprinting. J. Virol. 65:4629-4635.

Willwand, K., and B. Hirt. 1993. The major capsid protein VP2 of minute virus of mice (MVM) can form particles which bind to the 3'-terminal hairpin of MVM replicative-form DNA and package single-stranded viral progeny DNA. J. Virol. 67:5660-5663.

Willwand, K., and O.-R. Kaaden. 1990. Proteins of viral and cellular origin bind to the Aleutian disease virus (ADV) DNA 3'-terminal hairpin: presentation of a scheme for encapsidation of ADV DNA. J. Virol. 64:1598-1605.

Wilson, G. M., H. K. Jindal, D. E. Yeung, W. Chen, and C. R. Astell. 1991. Expression of minute virus of mice major nonstructural proteins in insect cells: purification and identification of ATPase and helicase activities. Virology 185:90-98.

Winocour, E. L., M. F. Callaham, and E. Huberman. 1988. Perturbation of the cell cycle by adeno-asociated virus. Virology 167:393-399.

Winocour, E. L. Puzis, S. Etkin, T. Koch, B. Danovitch, E. Mendelson, E. Shaulian, S. Karby, and S. Lavi. 1992. Modulation of the cellular phenotype by integrated adeno-asociated virus. Virology 190:316-329.

Wobbe, C. R. 1984. Ph. D. thesis. University of Tennessee, TN.

Wobbe, C. R., and S. Mitra. 1985. Proteins tightly associated with the termini of replicative form DNA of Kilham rat virus, an autonomous parvovirus. Proc. Natl. Acad. Sci. USA. 82:8335-8339.

Wolter, S., R. Richards, and R. W. Armentrout. 1980. Cell cycle-dependent replication of the DNA of minute virus of mice, a parvovirus. Biochem. Biophys. Acta. 607:420-431.

Yakobson, B., T. A. Hrynko, M. J. Peak, and E. Winocour. 1989. Replication of adeno-associated virus in cells irradiated with UV light at 254 nm. J. Virol. 63:1023-1030.

Yakobson, B., T. Koch, and E. Winocour. 1987. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. J. Virol. 61:972-981.

Yalkinoglu, A. O., R. Heilbronn, A. Burkle, J. R. Schlehofer, and H. Zur Hausen. 1988. DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. Cancer. Res. 48:3123-3129.

Yalkinoglu, A. O., H. Zentgraf, and U. Hubscher. 1991. Origin of adeno-associated virus DNA replication is a target of carcinogen-inducible DNA amplification. J. Virol. 65:3175-3184.

Yang, Q., and J. P. Trempe. 1993. Analysis of the terminal repeat binding abilities of mutant adeno-associated virus replication proteins. J. Virol. 67:4442-4447.

Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Strong, and G. M. Wahl. 1992. Wild type p53 restores cell cycle control and inhibits gene amplificaition in cells with mutant p53 alleles. Cell 70:937-9348.

Yoshimoto K., S. Rosenfeld, N. Frickhofen, D. Kennedy, R. Hills, S. Kajigaya, and N. S. Young. 1991. A second neutralizing epitope of B19 parvovirus implicates the spike region in the immune response. J. Virol. 65:7056-7060.

Zambetti, G. P., J. Bargonetti, K. Walker, C. Prives, and A. J. Levine. 1992. Wildtype p53 mediates positive regulation of gene expression through a specific DNA sequence element. Genes and Dev. 6:1143-1152.

LIST OF ABBREVIATIONS

AAV: adeno-associated virus

ADV: Aleutian disease virus

BFL: bovine fetal lung cells, a tissue culture host for bovine parvovirus

BLOM: mutant clone of bovine parvovirus bubbleless at its left end, Bubbleless Left flOp Minus

BLOP: mutant clone of bovine parvovirus bubbleless at its left end, Bubbleless Left flOp Plus

BPV: bovine parvovirus

Cap: structural proteins of adeno-associated virus

CPV: canine parvovirus

HeLa: epitheloid carcinoma human cells, a tissue culture host for adeno-associated virus

HU: hydroxyurea

KRV: Kilham rat virus

MEV: mink enteritis virus

MVM: minute virus of mice

NS: viral nonstructural proteins

Rep: nonstructural proteins of adeno-associated virus involved in replication

RV: rat parvovirus

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel

VP: viral structural proteins

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PUBLISHED ABSTRACT

Deville, C.M., and M. Lederman. Protein interaction with parvovirus termini. II. Cellular proteins of different cell types interact with different parvovirus termini. The Virginia Journal of Sciences, Vol. 42, No. 2, Summer 1991.

MANUSCRIPTS SUBMITTED OR IN PREPARATION

Deville, C. M., V. R. Braddon, and M. Lederman. Binding of cellular proteins of different cell types to three parvoviral *oris*. submitted to the Journal of Virology, January 1994.

Deville, C. M., K. C. Chen, and M. Lederman. Specific sequences within the mismatched region of the BPV left hairpin are essential for viral replication, in preparation.

PRESENTATIONS

Deville, C. M.*, V. R. Braddon, and M. Lederman. Characterization of cellular proteins binding to parvoviral origins of replication. Presented at the 11th Annual Meeting of the American Society for Virology, Cornell University, Ithaca, N. Y., July 1992.

Braddon, V. R.*, C. M. Deville, and M. Lederman. Study of the binding sites of cellular proteins to parvoviral termini by competition assays. Presented at the 11th Annual Meeting of the American Society for Virology, Cornell University, Ithaca, N. Y., July 1992.

Deville, C. M.*, and M. Lederman. Is there a common protein in different cells responsible for parvovirus permissivity? Presented at the annual meeting of the Virginia Branch of the American Society for Microbiology, Blacksburg, VA, November 1991.

Deville, C. M., V. R. Braddon, and M. Lederman*. Interaction of parvoviral origins of replication with cellular proteins of different cell types. Presented at the 4th International Parvovirus Workshop, Elsinore, Denmark, August 1991.

Braddon, V.R., C.M. Deville and M. Lederman*. Interaction of cellular proteins with parvoviral origins of replication. Presented at the 11th Annual Seminar of Cancer Researchers in Virginia, sponsored by the Virginia Branch of the American Cancer Society, Charlottesville, VA, April, 1991.

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