C. Maisonpierre *et al.*, *Science* **247**, 1446 (1990); J. Leibrock *et al.*, *Nature* **341**, 149 (1989).
14. K. Kadomatsu, M. Tomomura, T. Muramatsu, *Biochem. Biophys. Res. Commun.* **151**, 1312 (1988).
15. Peptide (GKKEKPEKKVKKSDCGEWQ) was synthesized, conjugated to thyroglobulin, and emulsified (1:2) with complete Freund's adjuvant. New Zealand white rabbits were injected subcutaneously (100 μg per rabbit) in multiple dorsal sites. Boosters were given at 2- to 3-week intervals and contained 50 μg of conjugated peptide in incomplete Freund's adjuvant. Rabbit antiserum was passed over protein A Sepharose column (Pharmacia), and bound antibodies were eluted as described by Pharmacia protocol.
16. Y.-S. Li, P. G. Milner, A. K. Chauhan, M. A. Watson, R. M. Hoffman, C. M. Kodner, J. Milbrandt, T. F. Deuel, unpublished data.
17. R. S. Bradley and A. M. C. Brown, *EMBO J.* **9**, 1569 (1990).
18. P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987).
19. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
20. R. D. Ye, T. C. Wun, J. E. Sadler, *J. Biol. Chem.* **262**, 3718 (1987).
21. J. Milbrandt, *Science* **238**, 797 (1987).
22. For immunoprecipitation, the labeled cells were washed three times with cold PBS and lysed in 0.5 ml of lysis buffer containing 50 mM tris-HCl, pH 7.5, 150 mM NaCl, 0.1 percent SDS, 0.5 percent sodium deoxycholate, 1 percent NP-40, 2 mM PMSF and aprotinin at 2 μg/ml for 30 minutes on ice. The lysate was centrifuged, and the supernatants were diluted with 50 mM tris-HCl, pH 7.5, 150 mM NaCl to 1.0 ml. The immunoprecipitation was done as described by J. Sambrook, E. F. Fritsch, and T. Maniatis [*Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989), pp. 18 to 26].
23. The bovine and human PTN sequence data will appear in EMBL nucleotide sequence data bases under the accession numbers X52945 and X52946, respectively. We thank E. Sadler for providing the human placenta cDNA library, R. Kawahara for stimulating discussion, and M. Chu for technical assistance during cloning. Supported by NIH grants HL31102, HL14147, and CA49712; a grant from the Monsanto Company; and a Merck fellowship from the American College of Cardiology (to P.G.M.)

6 July 1990; accepted 15 November 1990

Targeting the E1 Replication Protein to the Papillomavirus Origin of Replication by Complex Formation with the E2 Transactivator

IAN J. MOHR, ROBIN CLARK, SHAW SUN, ELLIOT J. ANDROPHY, PAUL MACPHERSON, MICHAEL R. BOTCHAN

The mechanism by which transcription factors stimulate DNA replication in eukaryotes is unknown. Bovine papillomavirus DNA synthesis requires the products of the viral E1 gene and the transcriptional activator protein encoded by the E2 gene. Experimental data showed that the 68-kilodalton (kD) E1 protein formed a complex with the 48-kD E2 transcription factor. This complex bound specifically to the viral origin of replication, which contains multiple binding sites for E2. Repressor proteins encoded by the E2 open reading frame failed to complex with E1 suggesting that the 162–amino acid region of E2 that participates in transactivation contained critical determinants for interaction with E1. The physical association between a replication protein and a transcription factor suggests that transcriptional activator proteins may function in targeting replication initiator proteins to their respective origins of replication.

THE INITIATION OF DNA SYNTHESIS IS A PRECISELY ORchestrated event performed at defined genetic loci termed origins of replication. Such elements have been isolated from bacteria, bacteriophage, yeasts, and viruses that infect eukaryotic cells (*1*). They are cis-acting elements that direct the assembly of specialized nucleoprotein complexes that function in duplicating the genome (*2*). Replication origins frequently contain multiple DNA sequence motifs that are recognized by proteins directly involved in unwinding the origin (*3*). However, several proteins that bind specifically to origins of replication also function in the control of transcription (*4*). The function of transcription factors in the initiation of DNA synthesis is ambiguous.

Much of the information regarding the function of transcription factors in DNA replication stems from analyses of DNA sequences that are required for origin function. In these studies, in vivo replication is measured after the deletion of DNA sequences that bind a specific transcription factor in vitro or stimulate transcription from heterologous promoters in vivo. Thus, the replication of polyoma virus displays a requirement for a transcriptional enhancer sequence (*5*). In addition, a core origin of replication for SV40 is stimulated by auxiliary elements that function in transcriptional control (*6*). A transcriptional enhancer is also a component of the Epstein-Barr virus origin of replication (oriP), and its activity is dependent on the Epstein-Barr nuclear antigen (EBNA) replication protein. The replication and transcription functions of EBNA, which binds to both core origin and enhancer sequences, may involve similar segments of the polypeptide (*7*). Biochemical studies with the cellular proteins CCAAT transcription factor–nuclear factor I (CTF-NFI) and octamer transcription factor 1 (OTF1)–NFIII revealed that they stimulate adenovirus DNA replication in vitro by enhancing the formation of the preterminal protein (deoxycytosine monophosphate) initiation complex (*8*). While NFI DNA binding sites are required for replication in vivo, effects of NFIII binding sites have not been reported (*9*).

The mechanism by which transcription factors function to augment replication is equally obscure. They may participate directly in the initiation of DNA synthesis by bringing replication factors to the template or indirectly by causing localized alterations in chro-

I. J. Mohr, S. Sun, P. MacPherson, and M. R. Botchan are in the Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkely, CA 94720. R. Clark is at the Cetus Corporation, Emeryville, CA 94608. E. J. Androphy is in the Department of Dermatology, New England Medical Center, Tufts University School of Medicine, Boston, MA 02111.

matin structure (*10*). Much of the work that addresses these issues in eukaryotes was performed with viruses which, with the exception of EBV, undergo multiple rounds of DNA synthesis in a given cell cycle, followed by cell lysis. In the case of adenovirus, a mode of initiation is used that is different from that which operates in the replication of cellular chromosomes. Furthermore, the soluble in vitro replication systems developed for SV40 and polyoma do not exhibit a requirement for transcriptional control sequences or the cognate factors that bind to them (*11*).

Bovine papilloma virus type 1 (BPV-1) provides a system in which to study regulated DNA synthesis in eukaryotic cells, as the virus replicates in synchrony with the host chromosome (*12*). BPV causes fibropapillomas in cattle and transforms rodent culture cells with single hit kinetics. In these transformed cells, the virus maintains itself as a multicopy nuclear plasmid (*13*). The establishment and maintenance of this state requires both the E1 and E2 open reading frames (ORF's). Immunochemical studies in BPV-transformed cells have identified 23-kD and 68-kD phosphoproteins as the products of the E1 ORF (*14*). However, all of the E1 functions necessary for extrachromosomal replication are provided by the 68-kD protein. This protein binds adenosine triphosphate (ATP); furthermore, mutant genomes that contain lesions in the ATP binding region of the E1 protein are integrated into the host cell chromosome and thus do not maintain themselves as plasmids (*14*). E1 mutants also display an overall increase in the amount of viral transcription (*15*).

Mutations in the E2 ORF display pleiotropic effects, some of which extend to viral DNA synthesis. Three related site-specific DNA binding proteins are encoded by the E2 ORF: a 48-kD transactivator, a 31-kD transcriptional repressor (E2C), which lacks the amino-terminal 162–amino acid activation region, and a 28-kD repressor derived from a fusion of the E8 ORF (11 amino acids) with the carboxyl-terminal 202 amino acids of E2 (E8-E2) (*16*). The relative concentrations of these proteins in transformed cells determine an exquisitely complex program of transcriptional control and may directly affect DNA replication as well. Mutant genomes that fail to produce the E2C repressor protein display an increase in overall plasmid copy number (*17*). Furthermore, viral genomes that harbor temperature sensitive mutations that affect the full-length transactivator integrate into the host cell chromosome at the nonpermissive temperature (*18*). Frameshift mutations and premature termination codons distributed throughout the E2 ORF lead to weakly transforming viruses that also integrate into the host chromosome (*18, 19*). A direct function for E2 in BPV DNA replication was previously discounted, as a deletion which removed almost all of the E2 ORF (Bal 15) maintained itself as a plasmid in a manner identical to wild-type BPV (*20*). This result was possibly due to either the persistence or repair synthesis of the mutant genomes or the presence of a critical cell factor that could functionally replace E2 (*21*). The genetic data are now entirely consistent with an absolute requirement for the E2 ORF in the maintenance of BPV genomes as extrachromosomal elements (*21*).

The BPV genome contains 17 E2 binding sites, 11 of which lie in the noncoding upstream regulatory region (*22*). Two clusters of E2 binding sites in this region (E2RE1 and E2RE2) function as E2-dependent transcriptional enhancer elements (*23*). Moreover, the origin of DNA replication has recently been mapped to a 200–base pair (bp) region that contains E2RE1, further suggesting a function for E2 in viral DNA synthesis (*24*). Here, we document a physical complex between the 68-kD E1 replication protein and the 48-kD E2 transcriptional activator, and demonstrate that this protein complex binds specifically to DNA fragments that contain the viral origin of replication.

Association of the E1 replication protein with DNA by the E2 transactivator. The 68-kD E1 protein has limited sequence similarity to SV40 large T antigen in regions involved in nucleotide binding (*25*). Because E1 is an adenosine triphosphate (ATP) binding protein, it may execute a subset of its replication functions in a manner analogous to SV40 large T. A salient feature of this mode of initiation involves direct recognition of the origin of replication by the initiator protein. Detection of papillomavirus proteins in vivo under the natural conditions of viral infection is extremely difficult. To address the DNA binding potential of E1 and to obtain sufficient quantities of material for biochemical studies, the 68-kD protein was produced in a baculovirus expression system (*26*). Approximately 70 to 80 percent of the protein produced was insoluble under nondenaturing conditions, while the remainder was salt-extracted (NaCl, 300 mM) from nuclei. The soluble fraction, which was used in the experiments described below, was chromatographed on DEAE-Sepharose, phosphocellulose, and mono Q columns. Some fractions of the partially purified E1 protein were not efficiently retained by resins that contained immobilized calf thymus DNA. Because most site-specific DNA binding proteins have affinity for nonspecific DNA, the question arose as to how this initiator protein recognizes the BPV origin of replication. In that the origin contained the E2RE1 enhancer element (*24*), we tested the ability of purified E2 to modify the origin binding activity of E1. Partially purified E1, E2 purified to homogeneity, or a mixture of these proteins was incubated with ^{32}P-labeled DNA fragments. Protein-DNA complexes were then analyzed by immunoprecipitation with either antibodies to E1 (anti-E1) to examine E1 containing complexes, or antibodies to E2 (anti-E2) to isolate complexes that harbored E2. The DNA fragments bound in the immune complexes were separated by electrophoresis and visualized on autoradiograms. Partially purified E1 displayed a weak affinity for the 1.4-kilobase (kb) fragment, which contained E2 binding sites 5 to 13 (Fig. 1). This relatively weak binding was increased in the presence of the 48-kD E2 transactivator. In the presence of a fixed amount of E1, increasing amounts of E2 resulted in increasing amounts of the specific fragment bound. In the absence of E1,

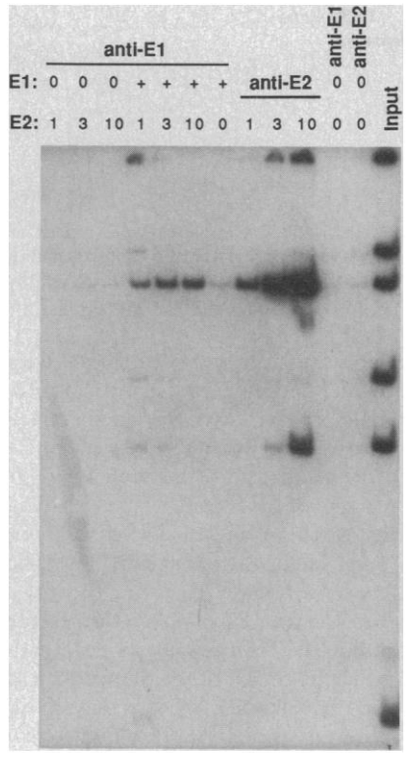

Fig. 1. Stimulation of the specific DNA binding activity of E1 by E2. A fixed amount of partially purified E1 and various amounts of purified E2 were incubated with a mixture of ^{32}P end labeled DNA fragments. Protein-DNA complexes were then immunoprecipitated with either anti-E1 or anti-E2 as indicated. Labeled DNA bound in the immune complexes was fractionated on native polyacrylamide gels (5 percent) and visualized by autoradiography. The lane marked Input contains a sample of the starting mixture. The numbers above each lane refer to microliters of either partially purified E1 or pure E2 (2.5 μg/ml). The E1 and E2 protein preparations were obtained as described (*45*). Immunoprecipitations were performed as in (*46*).

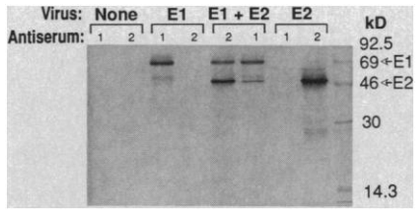

Fig. 2. Formation of a specific complex between E1 and E2. [^{35}S]Methionine-labeled extracts from Sf9 cells infected with E1, E2, or E1+E2 recombinant baculoviruses (*47*) were immunoprecipitated with antibodies to E1 (anti-E1) or E2 (anti-E2). Proteins bound in immune complexes were subject to electrophoresis on an SDS-polyacrylamide gel and visualized on autoradiograms. Molecular size markers are shown on the right. The numbers in the track labeled antiserum refer to either anti-E1 (1) or anti-E2 (2).

binding of the E2 protein to this fragment was not detected with anti-E1, but was visualized by immunoprecipitation with anti-E2. At higher concentrations of E2, a smaller fragment that contained the weaker E2 binding sites was also observed with anti-E2. The E2 transactivator thus facilitated the association of E1 with DNA. It may be that E2 altered the structure of the fragment, thus enabling the E1 protein to recognize either an altered structure or an E2:DNA complex. On the other hand, there could be a specific protein-protein interaction between E1 and E2.

Formation of a complex between the E2 transcription factor and the E1 replication protein. We infected insect cells with baculoviruses that expressed either E1 or E2, or we coinfected cells with both recombinant viruses to determine whether an E1-E2 complex could be detected. Cells were labeled with [^{35}S]methionine and extracts were passed through DEAE-Sepharose to remove endogenous nucleic acids. Immunoprecipitates obtained with either anti-E1 or anti-E2 were resolved by electrophoresis and visualized on autoradiograms. Anti-E1 precipitated a 68-kD protein from cells infected with the E1-expressing baculovirus, while anti-E2 precipitated a 48-kD protein from cells infected with an E2-expressing baculovirus (Fig. 2). Both anti-E1 and anti-E2 immunoprecipitated the 68-kD E1 protein and the 48-kD E2 protein from cells infected with both recombinant baculoviruses. This demonstrated that a physical complex existed between E1 and E2 and formed in the absence of exogenous nucleic acids. Moreover, this complex can be formed in vitro by mixing individual infected cell extracts that contain either E1 or E2 (see Fig. 5).

Binding of the E2-E1 complex to a DNA fragment that contains the E2 responsive transcription element. The specific DNA fragment bound by the E1-E2 complex in the previous experiment spanned nucleotides 7480 to 945. To further delineate the cis elements recognized by the E1-E2 complex, the viral DNA was digested with a different set of restriction enzymes. Protein-DNA complexes were then immunoprecipitated with either anti-E1 or anti-E2 and processed as in Fig. 1. Partially purified E1 (immunoprecipitated with anti-E1) displayed a weak specific DNA binding activity in the absence of E2. Addition of increasing amounts of purified E2 increased binding to a 596-bp Dde I–Cla I (Fig. 3A) fragment or a 319-bp Taq I fragment (Fig. 3B). Purified E2 (immunoprecipitated with anti-E2) bound to a 596-bp fragment in the Dde I–Cla I mixture (Fig. 3C) and to a 319-bp fragment in the Dde I–Taq I mixture (Fig. 3D). This 319-bp fragment spanned nucleotides 7477 to 7796 and contained E2 binding sites 5 to 10. DNA sequences involved in the control of transcription (*23*) as well as the origin of replication are present on this fragment (*24*). These results demonstrate that the E2-E1 complex specifically binds to sequences that contain the E2RE1 transcriptional enhancer element.

To obtain preparative amounts of the E1-E2 complex for biochemical analysis, we developed an immunoaffinity purification scheme. Nuclear extracts from insect cells infected with both E1 and E2 recombinant baculoviruses were fractionated through DEAE-Sepharose and phosphocellulose. Because purified E2 flowed through the phosphocellulose resin (*27*) and a fraction of E1 bound to phosphocellulose, E2 must have been retained on this resin by virtue of its association with E1. The protein fraction was eluted from phosphocellulose with 400 mM NaCl and was applied to the immunoaffinity matrix that contained the E2 monoclonal antibody, B202, immobilized on protein A–Sepharose. The resin was washed with a buffer that contained 1M LiCl and eluted with a high pH buffer, and the fractions were immediately neutralized. The 68-kD E1 protein and the 48-kD E2 protein were the major protein species eluted from this column (Fig. 4A) and were present in stoichiometric amounts. Their identity was confirmed by immunoblot analysis. The retention and subsequent coelution of both the 68-kD E1 protein and the 48-kD E2 protein from the immunoaffinity matrix, after extensive washing with a stringent high salt buffer further suggests that these proteins are complexed and that formation of the complex is driven by hydrophobic interactions. The E1-E2 complex can also be purified in a single step from coinfected cell extracts by this immunoaffinity procedure. This shortened procedure, however, does not remove any uncomplexed E2 present in the extract. Elution of this material from Q-Sepharose with a linear salt gradient yields a distinct fraction of E2, which co-chromatographs with E1. Furthermore, the 68-kD E1 and 48-kD components of this Q-Sepharose fraction cosediment as a distinct peak on a glycerol gradient (*28*). Thus, by immunological and physical criteria, the 68-kD E1 protein forms a stable complex with E2.

The stable complex eluted from this immunoaffinity matrix retained its specific DNA binding activity. Increasing amounts of the E1-E2 complex bound increasing amounts of the 596-bp Dde I–Cla I fragment when immunoprecipitated with anti-E1 (Fig. 4B). At higher protein concentrations, a 140-bp fragment that contained the weaker E2 binding sites 2, 3, and 4 was also bound by the E1-E2 complex. This upstream regulatory region contains a cis-acting element termed plasmid maintenance sequence (PMS)–1. This region (6945 to 7476) is involved in maintaining recombinant vectors in a plasmid state (*20*). The BPV origin of replication may thus be a multicomponent genetic element that requires the interaction of multiple E2 binding sites, as the replication origin maps to the strong E2 binding sites in a region distal to PMS-1 (*24*).

The E2 ORF encodes proteins that function both as transcriptional activators and repressors (*16*). Furthermore, mutations that

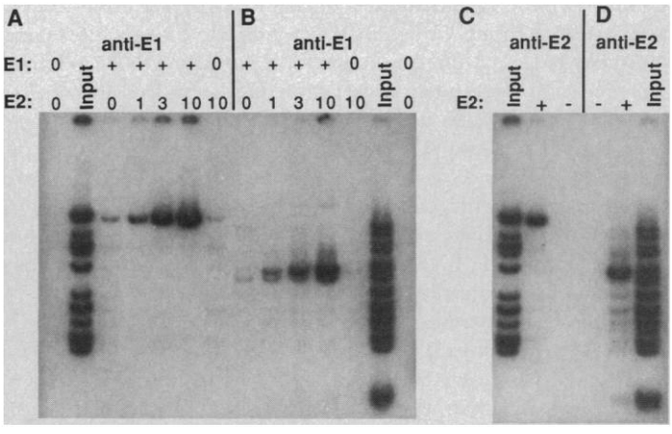

Fig. 3. Binding of the E2-E1 complex to the BPV-1 origin of replication, which contains E2RE-1. A fixed amount of partially purified E1 and various amounts of purified E2 were incubated with different mixtures of ^{32}P-labeled DNA. Protein-DNA complexes were immunoprecipitated with anti-E1 or anti-E2. Labeled DNA bound in the immune complexes was fractionated on native polyacrylamide gels and visualized by autoradiography. (**A** and **C**) PUC237 was digested with Dde I and Cla I. (**B** and **D**) PUC237 was digested with Dde I and Taq I. DNA binding and subsequent immunoprecipitations were performed as described (*46*).

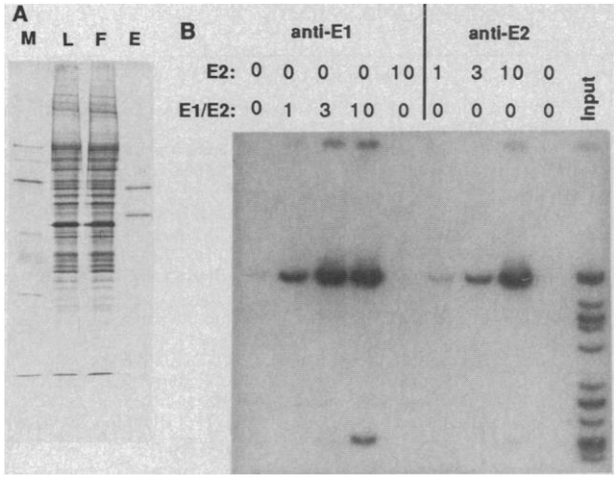

Fig. 4. Characterization of the E2-E1 complex. (**A**) Immunoaffinity purification of the E2-E1 complex. Protein fractions eluted from an immunoaffinity matrix that contain immobilized B202, a monoclonal antibody to E2 (*48*), were subjected to electrophoresis on SDS polyacrylamide gels. L, load; F, flowthrough; E, material eluted from the column. The gel was silver-stained; the molecular size markers (lane M) are as follows: 200 kD, 116 kD, 97 kD, 68 kD, 45 kD, and 30 kD. (**B**) Specific DNA binding activity of the purified E1/E2 complex. Purified E1-E2 complex or purified E2 was incubated with a ^{32}P end-labeled mixture of DNA fragments (PUC237 digested with Dde I and Cla I). Protein-DNA complexes were immunoprecipitated and processed (*46*). DNA bound in the immune complex was fractionated by electrophoresis on native polyacrylamide gels (5 percent) and visualized on autoradiograms.

eliminate the repressors display an increase in plasmid copy number (*17*). To address the potential regulatory function of the E2C repressor form, we tested its ability to form a complex with E1 by mixing infected cell extracts in vitro. In immunoprecipitation experiments, anti-E1 failed to coimmunoprecipitate E2C, and anti-E2 did not coprecipitate E1 (Fig. 5A). This was confirmed by the DNA binding assay (Fig. 5B). Addition of purified E2C failed to stimulate the weak specific binding of partially purified E1, as evidenced by immunoprecipitation with anti-E1. The E2C preparation used in these experiments bound efficiently to E2 binding sites when immunoprecipitated with anti-E2. The 48-kD E2 transactivator, however, formed a complex with the E1 protein and efficiently bound to the 596-bp fragment. The region of E2 that promotes stable complex formation with E1 thus maps to the amino-terminal 162 amino acids previously shown to participate in the activation of transcription. This region may directly form the surface contacted by E1 or it may facilitate a conformational change that unmasks the E1 binding determinants.

The function of the E2-E1 complex in DNA replication. Genetic analyses have demonstrated that both the E1 and E2 ORF's are important for viral DNA replication. This raises the possibility that transcription factors directly participate in DNA replication in addition to modulating gene expression. This idea is consistent with studies showing that the expression of both E1 and E2 ORF's from constitutive promoters is necessary and sufficient to support the transient replication of plasmids that bear BPV origin sequences (*29*).

The E2-E1 complex may serve to load the E1 protein onto the DNA or may have an enzymatic activity that requires both subunits. In addition to its site-specific DNA binding activity, the E2 protein binds to nonspecific single-stranded DNA (*30*). This single-stranded DNA binding function may combine with the E1 replication protein to generate a multisubunit DNA helicase that functions in the initial unwinding of the DNA template. Immune complexes that contain the E1 protein exhibit an adenosine triphosphatase activity, and this hydrolysis could provide energy for the unwinding process (*31*). This mode of action would most closely resemble the initiation functions executed by SV40 large T antigen (*32*). Another possibility is that the ATP binding function of E1 is similar to that of bacterial dnaA replication protein, where only the ATP bound form of the protein is active in promoting initiation (*33*).

Our DNA binding experiments reveal that partially purified E1 interacts weakly with specific DNA fragments. Further studies are necessary to determine whether this DNA binding activity is an intrinsic property of the 68-kD E1 polypeptide. Such activity could also be due to an association with cellular proteins present in the preparation. In support of this possibility, the DNA binding activity of E1 decreases substantially as purification increases. This does not preclude the possibility that E1 itself may have a weak affinity for origin DNA, and this affinity could be stabilized by E2 or by cellular transcription factors. However, the complex eluted from the immunoaffinity column is stable to washing with a stringent high-salt solution and subsequent chromatography. Moreover, this preparation of E1-E2 binds specifically to BPV origin fragments, an indication that the preformed E1-E2 complex may indeed be a specific DNA binding entity.

Transcription factors and the initiation of DNA synthesis. The region of E2 that binds E1 may be identical to the region that potentiates transcriptional activation. Mutant BPV genomes can be complemented in trans with E2 fusion genes that retain the DNA binding specificity of E2, but carry the activation domain of the herpesvirus transactivator, viral protein 16 (VP16). However, the transformed lines do not maintain the mutant genomes as plasmids. The transformation efficiency of these genomes under complementation conditions is identical when either wild-type E2 or the VP16-E2 fusion protein are provided in trans (*34*). This is consistent with our biochemical data as it suggests a peculiar characteristic of the E2 transactivation domain that is important for DNA

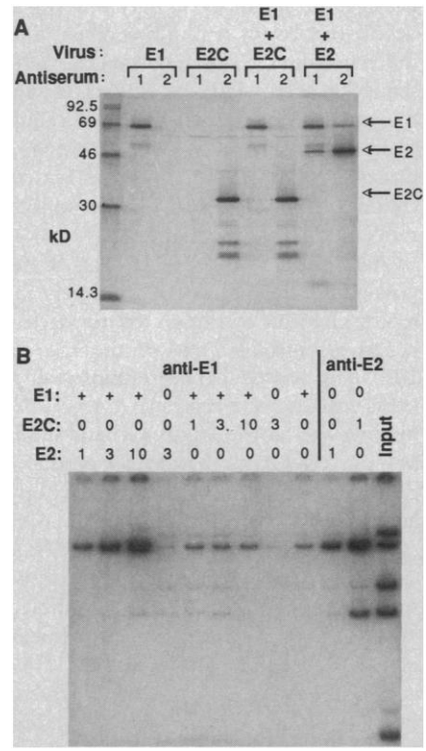

Fig. 5. Failure of the E2C repressor to complex with E1 and stimulate DNA binding. (**A**) Sf9 cells were infected with recombinant baculoviruses that produced either E1, E2, or E2C and starved prior to labeling as described (*47*). ^{35}S Trans-label (ICN) (200 μCi) and [^{35}S] L-cysteine (250 μCi) were used to label each 10-cm dish. Extracts were prepared as described (*47*). Complexes were formed in vitro by mixing extract (20 μl) with a buffer that contained 50 mM Hepes, pH 7.6, 100 mM NaCl, 1 mM EDTA, and 10 percent glycerol (20 μl). Immunoprecipitations were performed and processed as described (*47*). (**B**) Partially purified E1 and various amounts of purified E2 or E2C were incubated with an end-labeled mixture of DNA fragments (PUC237 digested with Hind III, Cla I, and Eco RI). Protein-DNA complexes were immunoprecipitated and processed as described (*46*). DNA bound in immune complexes was fractionated by electrophoresis on native polyacrylamide gels and visualized on autoradiograms.

replication.

Many models have been proposed to explain the function of transcription factors in DNA replication. It is possible, however, that transcription factors may exert effects on replication via multiple, distinct pathways. Transcription itself may be important in some cases (*35*), while in other cases, chromatin structure could be operative (*10*). Identification of the E1-E2 complex shows that a eukaryotic transcriptional activator can physically associate with a replication protein and is consistent with a direct function of E2 in viral DNA synthesis. Transcription factors may function by forming complexes with replication proteins and targeting them to their respective origins. In accord with such a model, the E1-E2 complex purified in a single chromatographic step by immunoaffinity chromatography directs the in vitro initiation of DNA synthesis on plasmids that harbor the BPV upstream regulatory region (*36*). A parallel situation exists in prokaryotes where replication proteins stimulate transcription from bacteriophage T4 late promoters (*37*).

Electron microscopy (*38*) and two-dimensional gel mapping techniques have localized the BPV origin of replication to regions that overlap with the transcriptional element, E2RE1 (*24*). Analysis of the upstream regulatory region (nucleotides 6959 to 7947) reveals the existence of several polypurine-polypyrimidine tracts interspersed among E2 binding sites. These sequences are unstable duplex elements that are predisposed to unwinding (*39*). Similar tracts are found at the SV40 origin of replication and at the bacterial origin, oriC (*40*). Furthermore, these sequences are the first to be unwound upon binding of the respective initiator proteins (*40, 41*). Although replication initiates at defined regions in eukaryotic chromosomes, the precise nature of these origins of replication and the protein determinants that recognize these regions as initiation sites are unclear. While specific cis-acting replication control elements have been identified in viruses and yeasts, none have been isolated from higher eukaryotic cells. In fact, it appears that precise sequence requirements do not exist for DNA to replicate in amphibian eggs (*42*). The possibility remains that specific sequences that define origins of replication exist, but they are fundamentally different from their bacterial counterparts. Inherently unstable DNA sequences or unwinding elements may function in conjunction with transcription factors to compose cellular origins. Such modular structures eliminate the need for a single canonical initiator protein that acts at its own binding site. Unwinding of origins may then commence through the interaction of cellular helicases with various stable transcription complexes.

The association of E1 with the 48-kD E2 protein raises several possibilities for the regulation of viral DNA synthesis. The ratio of repressor forms to the 48-kD transactivator may be an important negative control of DNA synthesis, as E2C failed to complex with E1. These relative concentrations may change throughout the cell cycle enabling the repressor forms to effectively compete for E2 binding sites at the origin. Genetic studies support this hypothesis, as mutant genomes that do not produce the E2C repressor protein exhibit an increase in BPV plasmid copy number (*17*). Post translational modification of E2 and E1 may also oscillate with the cell cycle and adds another level of complexity to the regulatory hierarchy. In the absence of E1, E2 is phosphorylated primarily at two serine residues (*43*). Mutations in these sequences do not alter the protein's ability to function as a transactivator, but result in an increase in BPV copy number (*44*). This suggests that phosphorylation may attenuate the E2-E1 interaction. Detection of the complex in BPV-transformed cells may be hampered if the complex exists only at a discrete time in the cell cycle. Soluble in vitro systems that faithfully replicate BPV DNA will allow an examination of the function of this complex in DNA synthesis and should permit exploration into cell cycle regulatory circuits that may control viral, and perhaps host cell, DNA synthesis.

REFERENCES AND NOTES

1. T. Kelley and B. Stillman, *Eukaryotic DNA Replication* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
2. A. Kornberg, *DNA Replication* (Freeman, San Francisco, 1980); B. M. Alberts, *Cold Spring Harbor Symp. Quant. Biol.* **49**, 1 (1984); H. Echols, *Science* **233**, 1050 (1986).
3. D. Bramhill and A. Kornberg, *Cell* **52**, 743 (1988); F. B. Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 16 (1987); M. Schnos, K. Zahn, R. B. Inman, F. R. Blattner, *Cell* **52**, 385 (1988).
4. M. L. DePamphilis, *Cell* **52**, 635 (1988); A. H. Brand, G. Micklem, K. Nasmyth, *ibid.* **51**, 709 (1987).
5. J. DeVilliers, W. Schaffner, C. Tyndall, S. Lupton, R. Kamen, *Nature* **312**, 242 (1984).
6. A. DeLucia, S. Deb, K. Partin, P. Tegtmeyer, *J. Virol.* **57**, 138 (1986).
7. D. Reisman and B. Sugden, *Mol. Cell. Biol.* **6**, 3838 (1986); J. L. Yates and S. M. Camiolo, in *Eukaryotic DNA Replication*, T. Kelly and B. Stillman, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 197–205.
8. N. Mermod, E. A. O'Neill, T. J. Kelly, R. Tjian, *Cell* **58**, 741 (1989); E. A. O'Neil *et al.*, *Science* **241**, 1210 (1988).
9. M. D. Challberg and T. J. Kelly, *Annu. Rev. Biochem.* **58**, 671 (1989).
10. L. Cheng and T. J. Kelly, *Cell* **59**, 541 (1989).
11. B. Stillman, R. D. Gerard, R. A. Guggenheimer, Y. Gluzman, *EMBO J.* **4**, 2933 (1985); J. J. Li, K. W. C. Peden, R. A. F. Dixon, T. Kelly, *Mol. Cell. Biol.* **6**, 1117 (1986); C. Prives *et al.*, *Mol. Cell. Biol.* **7**, 3694 (1987).
12. M. Botchan, L. Berg, J. Reynolds, M. Lusky, in *Papillomaviruses* (Wiley, New York, 1986), pp. 53–67; D. Gilbert and S. Cohen, *Cell* **50**, 59 (1987).
13. P. M. Howley, in *Virology*, B. N. Fields and D. M. Knipe, Eds. (Raven Press, New York, 1990), pp 1625–1650.
14. L. Thorner, N. Bucay, J. Choe, M. Botchan, *J. Virol.* **62**, 2474 (1988); S. Sun, L. Thorner, M. Lentz, P. MacPherson, M. Botchan, *ibid.* **64**, 5093 (1990).
15. P. F. Lambert and P. M. Howley, *ibid.* **62**, 4009 (1988); J. T. Schiller, E. Kleiner, E. J. Androphy, D. R. Lowy, H. Pfister, *ibid.* **63**, 1775 (1989).
16. N. L. Hubbert, J. T. Schiller, D. R. Lowy, E. J. Androphy, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5864 (1988); B. A. Spalholz, Y. C. Yang, P. M. Howley, *Cell* **42**, 183 (1985); P. F. Lambert, B. A. Spalholz, P. M. Howley, *ibid.* **50**, 68 (1987); J. Choe, P. Vaillancourt, A. Stenlund, M. Botchan, *J. Virol.* **63**, 1743 (1989); P. F. Lambert, N. L. Hubbert, P. M. Howley, J. T. Schiller, *ibid.*, p. 3151.
17. P. L. Lambert, B. C. Monk, P. M. Howley, *J. Virol.* **64**, 950 (1990); D. J. Riese II, J. Settleman, K. Neary, D. DiMaio, *ibid.*, p. 944.
18. D. DiMaio and J. Settleman, *EMBO J.* **7**, 1197 (1988).
19. M. Sarver, M. S. Rabson, Y. C. Yang, J. C. Byrne, P. M. Howley, *J. Virol.* **52**, 377 (1984); D. E. Groff, W. D. Lancaster, *Virology* **150**, 221 (1986); E. Kleiner, W. Dietrich, H. Pfister, *EMBO J.* **5**, 1945 (1986); M. S. Rabson, Y. C. Yang, P. M. Howley, in *DNA Tumor Viruses: Control of Gene Expression and Replication*, M. Botchan and T. Grodzicker, P. A. Sharp, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), pp. 235–243; D. DiMaio, *J. Virol.* **57**, 475 (1986); P. L. Hermonat and P. M. Howley, *ibid.* **61**, 3889 (1987).
20. M. Lusky and M. R. Botchan, *Cell* **36**, 391 (1984); *J. Virol.* **53**, 955 (1985).
21. M. Lusky, L. Thorner, M. Botchan, unpublished data.
22. R. Li, J. Knight, G. Bream, A. Stenlund, M. Botchan, *Genes Dev.* **3**, 510 (1989).
23. B. A. Spalholz, Y. C. Yang, P. M. Howley, *Cell* **42**, 183 (1985); B. A. Spalholz, P. F. Lambert, C. Yee, P. Howley, *J. Virol.* **61**, 2128 (1987).
24. L. Yang and M. Botchan, *J. Virol.* **64**, 5903 (1990).
25. P. Clertant and I. Seif, *Nature* **311**, 276 (1984).
26. V. A. Luckow and M. D. Summers, *Bio/technology* **6**, 47 (1988); G. E. Smith, M. D. Summers, M. J. Fraser, *Mol. Cell. Biol.* **3**, 2156 (1983).
27. J. Knight and M. Botchan, unpublished data.
28. After the isolation of the E1-E2 complex via a single chromatographic step on the immunoaffinity column, the E1-E2 complex (300 μg) was loaded onto a Q Sepharose column equilibrated in 25 mM tris-HCl, pH 8.0, 1 mM EDTA, 5 percent glycerol, 0.1 percent Triton X-100. The column was developed with a linear gradient from 0.2 to 1.0 M NaCl in the same buffer. Fractions that contained the peak amounts of E2 and E1 were pooled, dialyzed against 20 mM potassium-phosphate, pH 7.4, 20 mM potassium-glutamate, 0.1 mM EDTA, 10 percent glycerol, 1 mM DTT (dithiothreitol), concentrated by dialysis against solid Ficoll (type 400), and redialyzed against the potassium-phosphate buffer. Approximately 1.5 μg of protein was loaded on a 20 to 40 percent glycerol gradient prepared in 20 mM tris-HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT. Gradients were centrifuged for 8 hours at 4°C in a TLS55 rotor using the TL100 ultracentrifuge. Fractions (five drops) were collected from the bottom of each tube and a portion of each fraction was subject to electrophoresis on an SDS-polyacrylamide gel. Greater than 95 percent of the 68-kD E1 protein and 48-kD E2 protein bands were detected in a single peak.
29. M. Ustav and A. Stenlund, personal communication.
30. E. J. Androphy, D. R. Lowy, J. T. Schiller, *Nature* **325**, 70 (1987).
31. P. MacPherson and M. Botchan, unpublished data.
32. H. Stahl, P. Dröge, R. Knippers, *EMBO J.* **5**, 1939 (1986).
33. K. Sekimizu, D. Bramhill, A. Kornberg, *Cell* **50**, 259 (1987).
34. R. Li, T. Nottoli, M. Botchan, unpublished data.
35. T. A. Baker and A. Kornberg, *Cell* **55**, 113 (1988).
36. L. Yang, I. Mohr, M. Botchan, unpublished data.
37. D. R. Herendeen, G. A. Kassavetis, J. Barry, B. M. Alberts, E. P. Geiduschek, *Science* **245**, 952 (1989); D. R. Herendeen, K. P. Williams, G. A. Kassavetis, E. P. Geiduschek, *ibid.* **248**, 573 (1990).

38. W. Waldeck, F. Rosl, H. Zentgraf, *EMBO J.* **3**, 2173 (1984).
39. R. D. Wells, *J. Biol. Chem.* **263**, 1095 (1988); D. Kowalski and M. J. Eddy, *EMBO J.* **8**, 4335 (1989).
40. J. A. Borowiec and J. Hurwitz, *EMBO J.* **7**, 3149 (1988); J. W. Zyskind and D. W. Smith, *Cell* **46**, 489 (1986).
41. D. Bramhill and A. Kornberg, *Cell* **52**, 743 (1988).
42. R. M. Harland and R. A. Laskey, *ibid.* **21**, 761 (1980).
43. A. A. McBride, J. B. Bolen, P. M. Howley, *J. Virol.* **63**, 5076 (1989).
44. A. A. McBride and P. M. Howley, personal communication.
45. The BPV E1 coding sequence was recombined into the *Autographa californica* baculovirus (AcNPV) via the transfer vector, pAcE1. Plasmid pMTE1DM was used as the source for the BPV E1 ORF. This construct contains a G to A substitution at nucleotide 1236, which prevents production of the spliced M protein. The 68-kD E1 protein produced by genomes that have this lesion maintain BPV DNA as an extrachromosomal element. pAcE1 was constructed by insertion of the E1-encoding Xba I fragment from pMTE1DM into the Xba I site of pAcC13 (*49*). This places the E1 open reading frame downstream of the baculovirus polyhedrin promoter. To generate recombinant virus, transfer vector (2 μg) was cotransfected with wild-type DNA (1 μg) into Sf9 cells as described (*50*). Recombinant virus (occlusion-negative) was isolated from the transfution supernatant by plaque purification (*26*). Protein production was monitored by immunoblot analysis. Baculoviruses that expressed E2 or E2C were prepared in a similar fashion. The E2 expression vector constructs have been described (*51*). The E2 protein was purified by specific oligonucleotide affinity chromatography as described (*51*). In order to produce recombinant E1, Sf9 cells were infected with 5 to 10 plaque-forming units of recombinant virus per cell. Suspension cultures were grown in a 500-ml stirred flask that contained protein-free medium (*52*). The cells were infected at 1 to 1.5×10^6 cells per milliliter and were harvested 48 hours after infection. Infected cell pellets were frozen in liquid nitrogen and stored at −70°C. Cells were lysed by thawing and immediately suspended in five-pellet volumes of hypo buffer [20 mM tris-HCl, pH 8.0, 5 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, leupeptin at 10 μg/ml, and 1 mM phenylmethylsulfonyl chloride (PMSF)]. A nuclear fraction was prepared with 18 strokes of a dounce homogenizer (B pestle), and the mixture was clarified by centrifugation at 5000 rpm for 10 min (HB4 rotor). The pellet was washed twice with nuclei wash buffer (20 mM tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin at 10 μg/ml, and 10 percent sucrose), and suspended in five-pellet volumes of hypo buffer. Salt (NaCl) was slowly added to a final concentration of 0.3 M and the mixture was slowly agitated at 4°C for 30 min. After centrifugation at 10,000 rpm for 10 min in an HB4 rotor, the supernatant was applied to a DEAE-Sepharose fast flow column (Pharmacia) equilibrated in buffer A (20 mM tris-HCl, and pH 8.0, 1 mM EDTA, 5 percent glycerol, and 0.05 percent Triton X-100) that contained 0.3 M NaCl (A-300). The column was rinsed with 3 volumes of A-300 and this washing along with the DEAE flow-through fraction were adjusted to 0.2 M NaCl with buffer A. The individual fractions were then sequentially applied to a Phosphocellulose P-11 matrix (Whatman) equilibrated in A-200. The column was washed with A-200 and eluted stepwise with buffer A that contained 0.4 M NaCl followed by 1 M NaCl. These individual fractions were then dialyzed against A-200. Each pool was individually applied to a Mono Q 5/5 column (Pharmacia) equilibrated in 25 mM tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 5 percent glycerol, and 0.1 percent Triton X-100. The column was rinsed with this buffer and developed with a linear gradient from 0.2 to 0.5 M NaCl in this buffer. The linear gradient was followed by a step elution with 1 M NaCl in the same buffer. Fractions that contained E1 were identified by immunoblotting, pooled, and the indicated amounts used in experiments.
46. The PUC237 (a gift from G. Bream) vector contains the Xba I–Sma I fragment (nucleotides 6132 to 945) of BPV-1 cloned into PUC18. The plasmid was digested with Hind III, Cla I, and Eco RI, treated with calf intestinal alkaline phosphatase and labeled with [γ-^{32}P]ATP. The labeled DNA was extracted with phenol-chloroform and precipitated with ethanol. Partially purified E1 (4 μl) was incubated with labeled PUC237 (25 ng) in the presence or absence of various amounts of purified E2 in a final volume of 50 μl. Binding buffer consisted of 10 mM Hepes, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$, 5 percent glycerol, 1 mM DTT, and salmon sperm DNA at 20 μg/ml. The final ionic strength of the reaction was between 160 and 200 mM. Reactions were incubated at 37°C for 40 to 50 min, after which they were returned to room temperature. Polyclonal antiserum to E1 (1.5 μl) was then added, and the incubation was continued for 20 min. A 10 percent slurry of protein A–Sepharose (Pharmacia) (100 μl) equilibrated in 50 mM Hepes, pH 7.4, 200 mM NaCl, 5 mM EDTA, 0.05 percent Triton X-100, and salmon sperm DNA at 20 μg/ml was added, and the reactions were rocked for 50 min at room temperature. The beads were centrifuged and washed three times with 50 mM Hepes, pH 7.4, 200 mM NaCl, 5 mM EDTA, and 0.05 percent Triton X-100. In order to avoid additional dilution of reactions with anti-E2 tissue culture supernatants, the B202 was first bound to protein A–Sepharose beads (50 μl of B202 per 100 μl of 10 percent protein A–Sepharose) prior to its addition to the reactions. Alternatively, pure B202 (10 μg) was added, and the immunoprecipitations were performed as described for anti-E1. The final pellets were resuspended in 1 percent SDS, 25 mM EDTA, and incubated at 65°C for 15 min. Reactions were then adjusted to 20 mM tris-HCl, pH 8.0 and proteinase K was added to 50 μl/ml. After 60 min at 37°C, carrier transfer RNA (5 μg) was added, and the reactions were extracted with phenol-chloroform and precipitated with ethanol. DNA fragments purified from immune complexes were subjected to electrophoresis on native polyacrylamide gels (5 percent). The gels were dried onto DE-81 paper (Whatman) and exposed to Kodak XAR film.
47. The Sf9 cells (3×10^6) were transferred from suspension culture to 6-cm plates and infected with either E1, E2, or both recombinant baculoviruses (the multiplicity of infection was approximately 10 for the single infections, and 5 for each virus of the coinfection). At 48 hours after infection, the infected cells were starved for 1 hour in Grace's medium that lacked methionine and contained 10 percent dialyzed fetal calf serum. Cells were incubated for 5 hours in media (0.75 ml) that contained ^{35}S-translabel (100 μCi) (ICN). Extracts were prepared by lysing cells in 50 mM Hepes, pH 7.6, 300 mM NaCl, 1 mM EDTA, 10 percent glycerol, 0.5 percent NP40, leupeptin at 10 μg/ml, and 1 mM PMSF (0.5 ml). The plates were incubated at 0°C for 30 min after which the contents were scraped into a microfuge tube and centrifuged for 2 min. The supernatant was transferred to a vessel that contained DEAE-Sepharose fast flow (0.2 ml settled bed volume) (Pharmacia) equilibrated in lysis buffer. The mixture was stirred by rotation at 4°C for 5 min, centrifuged in a microfuge, and the resin was washed with lysis buffer (0.4 ml). Pooled supernatants (0.2 ml) were diluted with 50 mM Hepes, pH 7.6, 200 mM NaCl, 1 mM EDTA, and 10 percent glycerol (0.3 ml). Protein A–Sepharose (in lysis buffer that contained 100 mM NaCl) bound to either anti-E1 or anti-E2 was added and the reactions were mixed by rotation at 4°C for 1 hour. The beads were pelleted and washed four times with 50 mM Hepes, pH 7.6, 200 mM NaCl, 1 mM EDTA, and 0.05 percent NP40. The pellets were subjected to electrophoresis on SDS-polyacrylamide gels. The gels were fixed, dried, and exposed to Kodak XAR film.
48. The 400 mM phosphocellulose fraction prepared from Sf9 cells coinfected with recombinant E1 and E2 viruses was adjusted 200 mM NaCl with buffer A and applied to a column that contained immobilized B202. The resin was washed with 10 volumes of A-200, 50 volumes of buffer A with 1 M LiCl, and reequilibrated with an additional 10 volumes of A-200. Proteins were eluted with 20 mM triethylamine, and fractions were immediately neutralized with one-twentieth volume of 1 M Hepes, pH 7.4. Peak fractions were pooled, frozen in a dry ice–ethanol bath, and stored at −70°C. The immunoaffinity matrix was constructed by incubating pure B202 with protein A–Sepharose in 100 mM tris-HCl, pH 8.0 (6 A_{280} units of antibody per milliliter of resin) overnight on a rotator at 4°C. The mixture was washed with 100 mM borate buffer, pH 9.0, suspended in 20 mM dimethyl pimelimidate hydrochloride (Pierce), and mixed by rotation for 1 hour at room temperature. Unreacted crosslinker was blocked by suspending the beads in 40 mM ethanolamine-HCl, pH 8.0, at room temperature. The beads were stored in 100 mM borate buffer, pH 8.0 (*53*).
49. S. Munemitsu *et al.*, *Mol. Cell. Biol.*, in press.
50. M. D. Summers and G. E. Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures* (Texas Agricultural Experiment Station Bulletin No. 1555, 1987).
51. J. Knight and M. Botchan, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
52. B. Maiorella, D. Inlow, A. Shauger, D. Harano, *Bio/technology*, **6**, 1406 (1988).
53. C. Schneider, R. A. Newman, D. R. Sutherland, U. Asser, M. F. Greaves, *J. Biol Chem.* **257**, 10766 (1982); V. Simanis and D. P. Lane, *Virology* **144**, 88 (1985).
54. We thank R. Kanaar for assistance with the FPLC system. G. Bream and R. Kanaar for numerous discussions. L. Brindley for technical assistance in preparing the recombinant baculoviruses, A. Stenlund and M. Ustav for sharing their unpublished results, Y. Gluzman for critically reading the manuscript, and T. Monk for inspiration. Supported by NIH grants CA-30490 and CA-42414, and a postdoctoral fellowship from the American Cancer Society (I.J.M.).

14 September 1990; accepted 19 November 1990