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An Element of the BK Virus Enhancer Required for DNA Replication

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The human papovavirus BK virus contains three 68-base-pair (bp) repeats that act as transcriptional enhancers. An analysis of plasmids containing the BK virus origin revealed that sequences within the 68-bp enhancer are required for DNA replication as well as transcription of the early promoter in COS-1 cells. Origins with a single 68-bp repeat replicated as efficiently as did those with three repeats when transfected into COS-1 cells. Replication did not occur in the absence of enhancer sequences and could not be restored by distal placement of enhancers to enhancerless origins. However, as with simian virus 40, replication in vitro was not dependent on the presence of any enhancer sequences. Deletion analysis showed that replication of BK virus origins was dependent on the presence of the first 21 bp of the enhancer contiguous with the A-T-rich stretch of the origin. This 21-bp element is referred to as the *rep* element. Although in combination with *rep* the remaining 47 bp of the enhancer appear to increase replication by two- to fivefold, they alone are not sufficient to support replication. Deletions or insertions in the enhancer which did not alter the *rep* element had no major effect on replication. Site-directed mutagenesis of the Sp1-like site within the *rep* element, the NF1 site present in the enhancer, or the NF1 site in adjacent late-side sequences each reduced transcription by two- to fivefold, but had no effect on replication, suggesting that replication and transcription can be uncoupled.

The human papovavirus BK (BKV) was first isolated in 1971 by Gardner et al. from the urine of a renal transplant recipient on immunosuppressive therapy (18). Approximately 80% of the population of the world has antibodies to BKV (17, 50). This virus appears to be acquired during early childhood and may persist throughout life (6, 39). Reactivation of the virus by immunosuppression has been associated with hemorrhagic cystitis (1). Although BKV can transform human cells (reviewed in reference 24) and BKV DNA has been found in a variety of human tumors (13), its relationship to human cancers has not been clearly established.

The size and morphology of BKV are similar to those of other papovaviruses such as simian virus 40 (SV40), polyomavirus (Py), and JC virus. BKV has a double-stranded, circular DNA genome (5, 196 base pairs [bp] in the Gardner strain) which is remarkably similar to that of SV40. Nucleotide sequence homology between the two viruses is greater than 80% (49, 57). Both viruses have complex regulatory regions at their origins of replication from which early mRNAs encoding large and small tumor antigens and late mRNAs encoding viral capsid proteins (VP1, VP2, VP3, and Agno) are synthesized by host cell RNA polymerase II in opposite directions on opposite strands. Replication of BKV and SV40 DNA requires host cell DNA polymerases and viral T antigen. Replication of BKV and SV40 can be supported by the heterologous T antigen (5, 36, 38), and in fact, SV40 T antigen has been shown to contact specific nucleotides within the three BKV T-antigen-binding sites (46).

Although the overall arrangement of elements within the regulatory regions of SV40 and BKV is similar, there are critical differences. The regulatory region of both viruses from the early to late side begins with start sites for early transcription, three T-antigen-binding sites at the origin of replication, an A-T-rich stretch, a set of transcriptional enhancers, and late transcription start sites. BKV and SV40 diverge greatly with respect to their enhancer elements. SV40 contains three 21-bp tandem repeats situated between the A-T-rich stretch and the two 72-bp transcriptional enhancers (52, 54, 58). Each 21-bp repeat contains two copies of a G-C "box" sequence which forms the recognition sequence for the transcription factor Sp1 (14). One function of the 21-bp repeats is to augment transcription from both early and late promoters (2, 28). The 72-bp enhancers bind a number of different factors (i.e., AP-1, AP-2, and AP-3) involved in transcription (27, 55). In contrast to SV40, BKV has a unique set of three 68-bp tandem repeats: the middle repeat has a deletion of 18 nucleotides (nt) (16, 49). Similar to those of SV40, the BKV enhancers stimulate transcription from the early promoter (11, 45) and can stimulate transcription from heterologous promoters as well (11). It is likely that the enhancer elements play a critical role in determining the host range of these viruses (29, 43).

Papovaviral enhancers can augment replication as well as transcription. Replication in vivo from the SV40 origin still occurs in the absence of either the 72-bp enhancers or the 21-bp repeats but is dramatically reduced when both of these *cis*-acting elements are eliminated (7, 22, 25, 34). Stimulation of replication by the 21-bp repeats or the 72-bp enhancers or both occurs independently of their orientation, but is lost when these elements are placed distal to the A-T-rich stretch (22, 32). However, neither the 21-bp nor the 72-bp repeats are required to support SV40 DNA replication in vitro (34). Replication of Py is dependent on its enhancer element (8, 40, 53). A combination of two of the four nonhomologous subdomains within the Py enhancer can support replication, suggesting that they are functionally redundant (53). Moreover, either the SV40 enhancer or the immunoglobulin enhancer can substitute for the Py enhancer in replication (4, 8). Although Muller et al. (40) have shown that Py enhancer sequences lose their ability to support replication if positioned further than 400 bp from the origin, others have suggested that stimulation of Py replication by SV40 enhancers can take place over much greater distances (8).

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In this study we reveal that the BKV enhancer is required for the replication of plasmids containing a BKV origin in COS-1 cells. A BKV origin plasmid with a single 68-bp enhancer replicates as well as a plasmid containing three enhancers. Deletion analysis showed that replication requires that the first 21 bp of the enhancer be contiguous with the A-T-rich stretch. Distal placement of the BKV enhancers failed to restore replication. An analysis of point mutations within the enhancer suggests that the replication and transcription functions of the BKV enhancer can be separated.

MATERIALS AND METHODS

Plasmid constructions. Plasmids p33-1 and p34-2, containing the entire genomes of the Gardner and Dunlop strains of BKV, respectively, cloned into the *Bam*HI site of pBR322 were used as parental constructs. The *Hin*dIII C origin fragments of Gardner and Dunlop were cloned into the *Hin*dIII site of puc 19 to generate pBKori(Gardner) and pBKori(Dunlop), respectively (see Fig. 1). Plasmid pBKcorBKen+L(D) was constructed by digesting pBKori (Dunlop) with *Bsu*36I and religating the large fragment. Plasmid pBKcorBKen was obtained by filling in the ends of the *Hin*fI fragment (BKV, nt 3284 to 3519), using Klenow, and cloning into the *Sma*I site of puc 19. In this construct the late side of the enhancer is next to the *Kpn*I site of the polylinker; plasmid pBKcorBKen(R) contains the identical fragment cloned into puc 19 in the reverse orientation. pBKcorBKen, containing a single enhancer, served as the parental plasmid for all deletion and insertion constructs described below.

A series of plasmids with deletions on the late side of the enhancer were generated from pBKcorBKen (see Fig. 3). Deletion plasmids pΔ47L, pΔ43L, pΔ38L, pΔ22L, and pΔ19L were created by deleting from the *Nco*I, *Bsm*I, or *Bsu*36I site in the BKV enhancer to the *Nde*I site in the vector, followed by either mung bean nuclease treatment or filling in with Klenow. Another deletion of the enhancer, pΔ56L, was created by cloning the *Hin*fI-*Alu*I fragment (BKV nt 3284 to 3465) into the *Sma*I site of puc 19. For convenience, nt 3456 in the enhancer was changed from A to T by site-directed mutagenesis (31), transforming a *Hae*III site into a *Stu*I site. Digestion with *Stu*I, which separates the enhancer from the A-T-rich stretch, was used to generate pBKcor, which is devoid of any of the 68-bp enhancer sequences; pBKcor contains BKV nt 3284 to 3453 cloned in the *Sma*I site of puc 19. A 26-bp deletion from the early side of the enhancer was constructed using this engineered *Stu*I site; pΔ26E deletes BKV nt 3454 to 3480. A series of internal deletions and insertions in the enhancer of pBKcorBKen were generated utilizing unique restriction sites *Nco*I, *Bsm*I, and *Bsu*36I in the enhancer. Plasmid pΔN-M(−) deletes nt 3475 to 3504; pΔI-N(−) deletes nt 3476 to 3482; pΔI-B deletes nt 3480 to 3486; pΔI-M(−) deletes nt 3501 to 3503. Plasmid pI-N(+) inserts CATG between nt 3479 and 3480; pΔB-M inserts 51 bp between nt 3484 and 3504; pI-M(+) deletes nt 3501 to 3508 and inserts a G.

Plasmids containing distally placed enhancer elements were constructed from replication-defective plasmid pMLΔ56L, which contains the same origin as pΔ56L cloned into pMLtet (35). The *Hae*III fragment containing the BKV enhancers served as the insert in all constructs (see Fig. 7). In pBKen(A) the enhancers were inserted into the *Bam*HI site, placing them 372 bp from their natural position. In pBKen(E) the enhancers reside 3.7 kilobases from their natural location. In pBKenTag(E) the enhancers abut the early boundary of T-antigen-binding site I at the *Stu*I site, 94 bp from their natural position. Plasmid pMLBKcorBKen +L(Gardner) contains a BKV origin with a single, intact 68-bp repeat and late-side sequences from the Gardner strain.

Plasmid pBKcorBKen+L(G)CAT was used as the parental construct for site-directed mutagenesis of the enhancer and was made from pBKCAT-1 (5) by digesting completely with *Bsu*36I and religating the large fragment.

All constructs were propagated in *Escherichia coli* XL-1 (3) and purified by two rounds of equilibrium centrifugation on cesium chloride gradients. All constructs were verified by dideoxy-chain termination sequencing (47) of the BKV inserts, using the double-stranded plasmid DNA as a template (30).

Cells and transfections. COS-1 cells (20) were grown in Dulbecco modified Eagle medium with 7.5% fetal calf serum and gentamicin. Cells under passage 10 were seeded 24 h before transfection at a density of 6×10^5 cells per 100-mm dish. Cells were cotransfected with 5 μg of the test plasmid and 5 μg of an internal standard plasmid (pBR325 or pRSV β-globin) by calcium phosphate precipitation, followed by a 60-s 15% glycerol shock (21). The inclusion of a nonreplicating internal standard plasmid was used to correct for differences in transfection efficiency.

DNA extractions and Southern blotting. At 48 h after transfection, cells were washed three times and DNA was extracted by the method of Hirt (23). DNA was extracted once with phenol and twice with chloroform and ethanol precipitated. DNA was treated with DNase-free RNase (20 μg), and half of the sample was digested with *Dpn*I (10 U) and an appropriate restriction enzyme that linearized the transfected plasmid; the other half was digested with *Mbo*I (10 U) and *Pst*I. To ensure complete digestion, reactions were incubated for 16 to 20 h at 37°C. Digests were electrophoresed through 0.8% agarose gels and transferred onto nitrocellulose by the method of Southern (51). The *Hin*dIII C fragment of BKV (Dunlop) was used as the nick-translated probe on both *Dpn*I and *Mbo*I blots. Additionally, the *Mbo*I blots were simultaneously probed with either the *Eco*RV-*Sal*I fragment of pBR325 or with the *Hin*dIII-*Bgl*II B fragment of pRSV β-globin. Blots were hybridized in 50% formamide–1× Denhardt solution–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate, washed at 65°C in 0.2% sodium dodecyl sulfate–0.2× SSC, and visualized by autoradiography at −80°C. Intensities of replicative bands were quantitated by scanning densitometry and normalized to the intensity of the internal standard bands.

In vitro replication assay conditions. In vitro replication assays were performed as described previously (33, 34). HeLa cells grown in suspension were used to make cell extracts (5 μg of protein per μl, determined by Bio-Rad assay). Reactions (30 μl) included 2 μg of immunoaffinity-purified SV40 large T antigen (Molecular Biological Resources Inc.), 0.1 μg of DNA template, 1 μl of [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol), and 80 μg of extract. After incubation at 37°C for 4 h, DNA was isolated. Half of the sample was codigested for 6 h with 10 U of *Dpn*I and 10 U of *Eco*RI, and the other half was left undigested. Both samples were run on 1.5% agarose gels and then air dried on NuBond film (FMC Corp.) at 37°C and autoradiographed at −80°C. The linear *Dpn*I-resistant bands were excised from the gel and quantitated by counting in scintillation fluid.

CAT assays. Chloramphenicol acetyltransferase (CAT) activity was determined as previously described (21). In

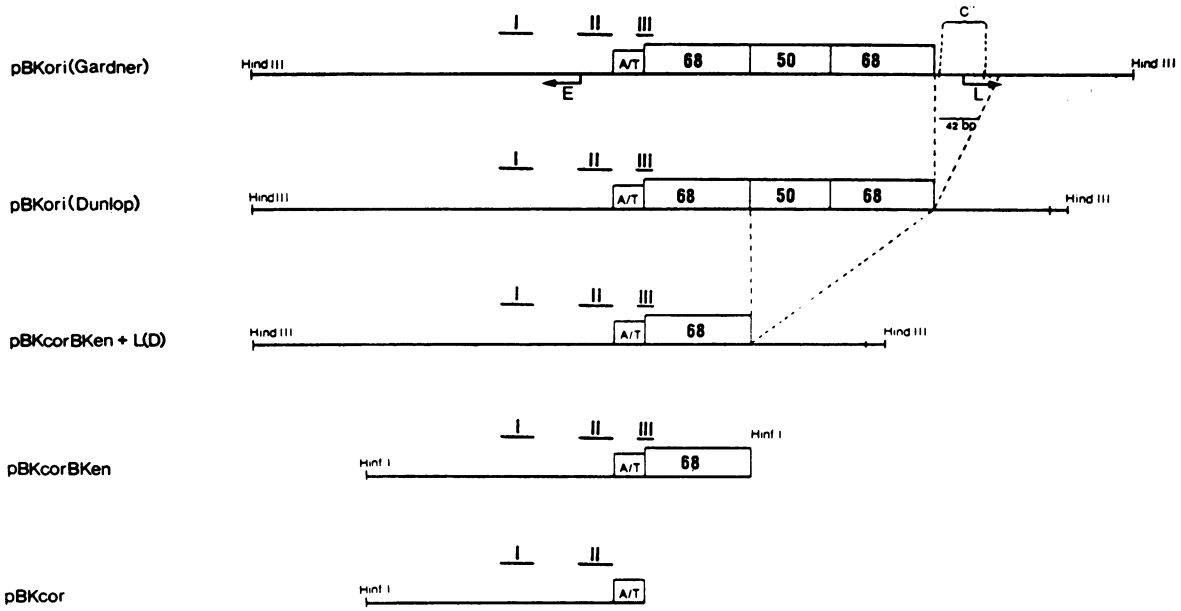

FIG. 1. Diagram of BKV origins from prototype viral strains and single-enhancer derivatives. Shown are the Gardner [pBKori(Gardner)] and Dunlop [pBKori(Dunlop] origins containing the early (E) transcription start site, T-antigen-binding sites (I, II, and III), an A-T-rich stretch, three enhancer repeats of 68, 50, and 68 bp, and late (L) transcription start sites. The Gardner strain contains a 42-bp stretch adjacent to the late side of enhancers that is not present in Dunlop. This stretch includes the 30-bp element c, which augments early transcription. Single enhancer origins that either include late-side sequences [pBKcorBKen+L(D)] or delete them (pBKcorBKen) were derived from the Dunlop strain. Also shown is the enhancerless BKV origin plasmid pBKcor.

each reaction an equal amount of protein from the transfected cell extracts was used. Following thin-layer chromatography, acetylated forms of [^{14}C]chloramphenicol were quantitated by scintillation counting.

Site-directed mutagenesis. Oligonucleotide site-directed mutagenesis was performed by the method of Kunkel (31). Oligonucleotides (18-mers) were obtained from the Wistar Institute DNA synthesis facility. Mutations were confirmed by DNA sequencing (47).

RESULTS

Multiple enhancers and late-side sequences are dispensable in replication. We and others have previously shown that plasmids containing the complete BKV origin replicate in transfected cell lines which provide BKV or SV40 T antigen and that the BKV early promoter is functional in such plasmids (5, 36). To define *cis* elements involved in the replication of BKV, a series of origin constructs (Fig. 1) were tested for their ability to replicate in COS-1 cells, an African Green monkey kidney cell line which has been transformed by replication-defective SV40 and supplies endogenous SV40 T antigen (20). Replication was monitored 48 h after transfection by observing the appearance of either *Mbo*I or *Dpn*I endonuclease-generated bands. Input plasmid DNA is methylated, making it sensitive to *Dpn*I but resistant to the isoschizomer *Mbo*I. Conversely, plasmid DNA that has undergone replication in mammalian cells is nonmethylated, making it resistant to *Dpn*I and sensitive to *Mbo*I. To quantitatively adjust for differences in transfection efficiency among samples, the intensity of the replicative bands was normalized to that of the nonreplicative internal standard.

Replication of plasmids containing the complete origins from the prototype Gardner and Dunlop strains of BKV is demonstrated by the *Dpn*I-resistant and *Mbo*I-generated bands in Fig. 2 [pBKori(Gardner) and pBKori(Dunlop)]. Both plasmids contain three T-antigen-binding sites, the three enhancer repeats of 68, 50, and 68 bp, and a stretch of sequences on the late side of the last enhancer repeat. They differ only in that the Gardner strain contains an additional 42-bp stretch that juxtaposes the third enhancer on the late side (49, 57) (Fig. 1). This stretch, not present in the Dunlop strain, was previously shown to contain an element denoted "c," which augments early transcription (11). Plasmid pBKcorBKen+L(D), which contains a single 68-bp enhancer and late-side sequences from the Dunlop strain (Fig. 1), replicated as well as the Dunlop and Gardner plasmids containing three enhancers (Fig. 2). Since late-side sequences can influence transcription (e.g., region element c of the Gardner strain), it was important to know whether any late-side sequences could also influence replication. Plasmid pBKcorBKen, which contains a single 68-bp enhancer but no late-side sequences (Fig. 1), replicated in COS-1 cells as well as the Gardner and Dunlop plasmids (Fig. 2). Replication was not altered when the orientation of this single enhancer origin was reversed in plasmid pBKcorBKen(R) (Fig. 2) or when different vectors were used (not shown). It is noted that when pBKcorBKen and all of its derivatives were digested with either *Mbo*I after transfection or *Dpn*I before transfection, one or more minor bands corresponding to partial digestion products were seen. This was observed reproducibly with different plasmid preparations and extended times of digestion. Thus, replication of a plasmid containing a single enhancer and no late-side sequences is as efficient as one containing three enhancers with late-side sequences.

The *rep* element of the BKV enhancer is required for DNA replication. To examine the involvement of the enhancer sequences in replication, a series of deletions were produced on the late side of the single enhancer of pBKcorBKen (Fig.

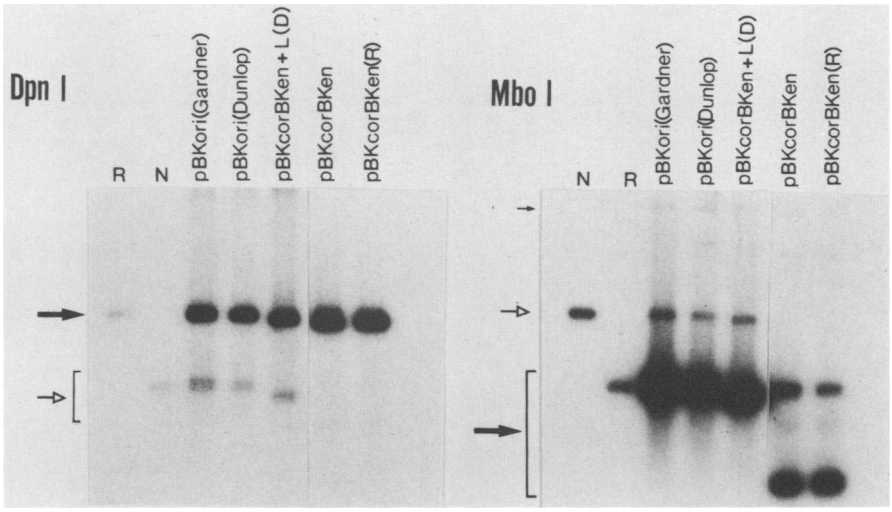

FIG. 2. Replication of plasmids containing multiple- and single-enhancer repeats. BKV plasmids and a nonreplicative internal standard plasmid (pRSV β-globin) were transfected into COS cells, and Hirt DNA was extracted after 48 h. Half of the Hirt DNA from each transfection was codigested with *Dpn*I and *Bam*HI and the other half was digested with *Mbo*I and *Pst*I and analyzed by Southern blotting. Both *Dpn*I and *Mbo*I filters were probed with the *Hin*dIII C origin fragment of BKV of the Gardner strain; additionally, the *Mbo*I filter was coprobed with the *Eco*RV-*Sal*I fragment of pRSV β-globin. Bands indicative of replication are denoted by a bold arrow, unreplicated input plasmid bands are indicated by an open arrow, and internal standard DNA is indicated by a thin arrow. To better visualize the internal standard bands in the *Mbo*I blot, the film was overexposed. Lanes with marker DNAs representing the positions of replicative (R) and nonreplicative (N) bands are shown.

3) and tested in COS-1 cells. Sequential deletion of the enhancer resulted in a decrease in replication (Fig. 4 and 5). When compared with the single-enhancer construct pBKcor-BKen, replication of plasmids pΔ19L, pΔ22L, and pΔ38L, which eliminate 19, 22, and 38 bp from the late side of the enhancer, was reproducibly reduced by 40 to 50% (Fig. 4 and 5). It is noted that the difference in the sizes of the *Mbo*I-generated bands in Fig. 4 is due to slight size differences in the plasmids as well as the loss of an *Mbo*I site in the construction of the deletions. Replication of plasmids pΔ43L and pΔ47L, which delete 43 and 47 bp of the enhancer, was reduced in both cases by 50 to 80% (Fig. 4). Neither pΔ56L, which deletes 56 bp of the enhancer, nor the enhancerless plasmid pΔ69L (same as pBKcor) exhibited any detectable replication (Fig. 4 and 5). To more precisely map specific sequence requirements for replication, plasmid pΔ26E, which deletes 26 bp from the early side of the enhancer, was constructed (Fig. 3). pΔ26E exhibited no apparent replica-

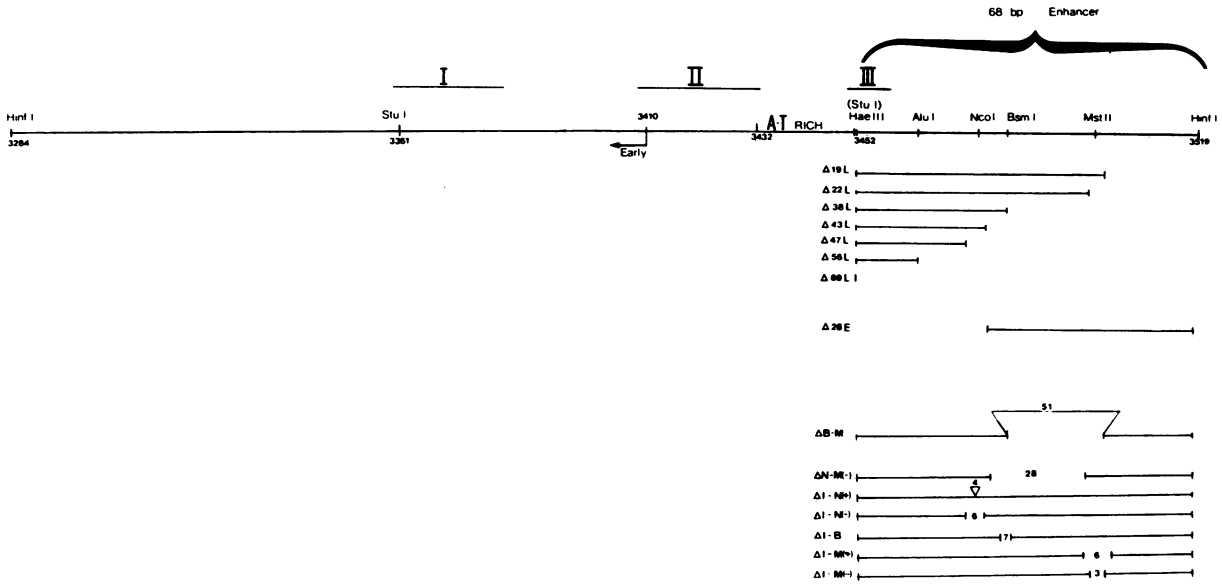

FIG. 3. Diagram of origins with deletions and insertions in the BKV enhancer. All constructs were derived from the parental plasmid pBKcorBKen (top) and contain T-antigen-binding sites I, II, and III, and the A-T-rich stretch. Deletions of the enhancer made from the early side (ΔE), from the late side (ΔL), or internally (ΔI) are shown. Deletion and insertion sizes are given in base pairs.

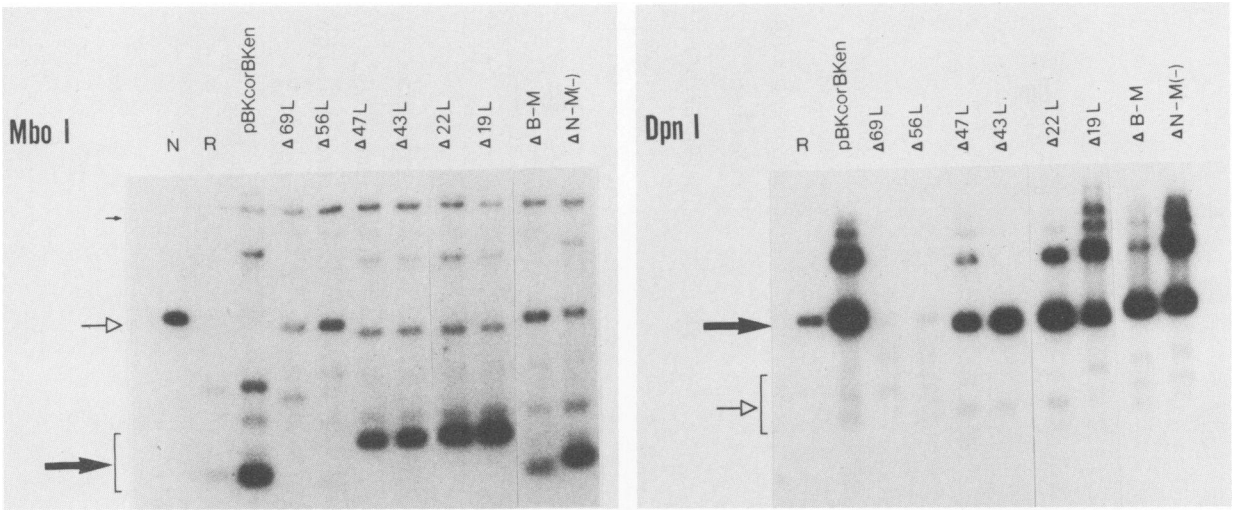

FIG. 4. Replication of BKV origin plasmids with deletions and insertions in the BKV enhancer. Constructs depicted in Fig. 3 were transfected into COS-1 cells, and Hirt DNA was analyzed as described in the legend to Fig. 2. Marker bands representing the positions of replicative (R) and nonreplicative (N) bands are shown. Arrows are the same as in Fig. 2.

tion, similar to the enhancerless plasmid pΔ69L (Fig. 5). Together these results demonstrate that the first 21 bp of the enhancer contain an element essential for BKV replication. We refer to these 21 bp on the early side of the BKV enhancer as the "*rep*" element.

Constructs shown in Fig. 3, which deleted large internal regions [pΔN-M(−)] or small regions [pΔI-M(−), pΔI-B, pΔI-N(−), and pΔI-M(+)] or created large insertions (pΔB-M) or small insertions [pΔI-N(+)] in the enhancer, reduced replication by only 5 to 10% (Fig. 4 and 6). These findings confirmed the fact that a critical function for replication is contained within the *rep* element and that alterations outside this element have no major effect on replication. Additionally, these results show that stereospecific alignment between different sections of the BKV enhancer does not appear to be crucial for replication.

These results demonstrate that a single, complete BKV enhancer is required for optimal replication of the BKV origin in COS-1 cells and that at least the first 21 bp of the enhancer are required for replication to occur. Although the remaining 47 bp of the enhancer appear to increase the level of replication by two- to fivefold in conjunction with *rep*, they alone are not sufficient to support replication.

Distal placement of the BKV enhancer fails to restore replication. It was important to know whether replication could be restored by the distal insertion of complete BKV enhancers into the replication-defective plasmid pMLΔ56L, which contains the BKV origin with 12 bp of the enhancer

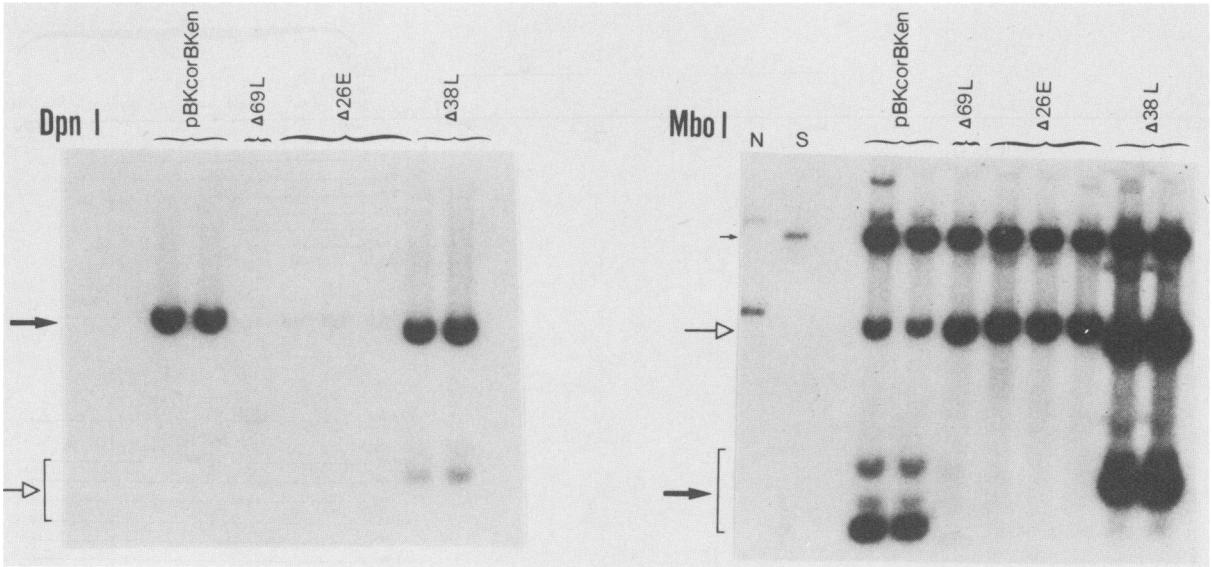

FIG. 5. Replication of BKV origin plasmids with deletions and insertions in the BKV enhancer. Constructs depicted in Fig. 3 were transfected into COS-1 cells, and Hirt DNA was analyzed as described in the legend to Fig. 2. Bands indicative of unreplicated input DNA for pΔ69L and pΔ26E on the *Dpn*I blot were seen after overexposure of the film. Marker bands representing the positions of internal standard (S) and nonreplicative (N) bands are shown. Arrows are the same as in Fig. 2.

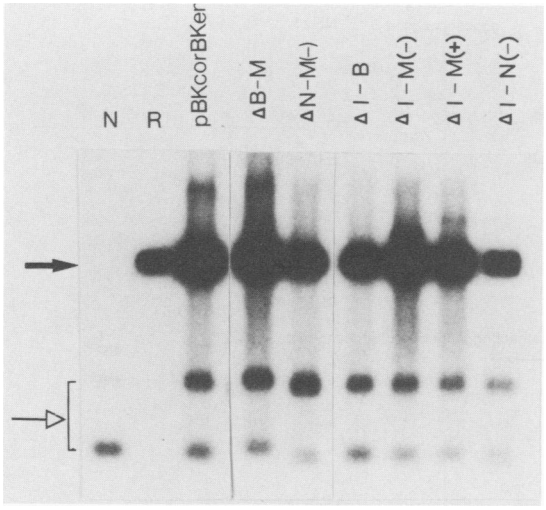

FIG. 6. Replication of BKV origin plasmids with internal alterations producing changes in the stereospecific alignment of the BKV enhancer. Constructs depicted in Fig. 3 were transfected into COS-1 cells, and Hirt DNA was analyzed by Southern blotting as described in the legend to Fig. 2. The *Dpn*I blot is shown. Marker bands representing the positions of replicative (R) and nonreplicative (N) bands are shown. Arrows are the same as in Fig. 2.

juxtaposed to the A-T-rich stretch (Fig. 7). In pBKen(A) the enhancer insert is positioned 2,969 bp from its natural position. In pBKen(E) the enhancer insert is positioned on the early side of the regulatory region, 198 bp from its natural location, and in pBKenTag(E) the enhancer insert abuts the early boundary of T-antigen-binding site I, 94 bp from its natural position. The extent of plasmid replication 48 h after transfection into COS-1 cells was determined by the appearance of *Mbo*I bands. The control plasmid pMLBKcorBKen+L(Gardner), which contains an intact single-enhancer origin, produced the expected *Mbo*I band (Fig. 7, lane 1), whereas pMLΔ56L (Fig. 7, lane 2) did not. Each plasmid with a distally inserted BKV enhancer failed to produce an *Mbo*I band, indicating that replication was not restored (Fig. 7, lanes 3 through 5). Of note is the fact that the distal placement of the SV40 enhancer in pMLΔ56L also failed to augment replication (not shown). These results clearly indicate that the ability of the BKV enhancer to support replication is position dependent.

Point mutagenesis of factor-binding sites in the BKV enhancer reduced transcription but not replication. We wanted to determine whether the requirement of the BKV enhancer in replication is linked to its ability to stimulate transcription. Depicted in Fig. 8 are the landmark elements of the BKV enhancer, which either are binding sites for cellular proteins or have been shown to be important in the transcription of other genes. These include a sequence for an Sp1-like site (14), an NF1 site (11, 12, 37, 42), an adenovirus E1A core (11, 45), and a stretch of DNA which could potentially form a Z-DNA structure (41). Since enhancers appear to function through the binding of factors involved in transcription, we generated point mutations in the known NF1 and the putative Sp1 factor-binding sites to determine whether these elements play distinct roles in transcription and replication. We mutated the Sp1-like site (GGGAGGAG to GTGAG GAG), which is present in the *rep* element required for replication. In SV40, replication and transcription are both stimulated by the G-C-rich 21-bp-repeat elements which bind Sp1 (14, 15, 19). Although our deletion analysis showed that the NF1 site in the enhancer is not absolutely required for

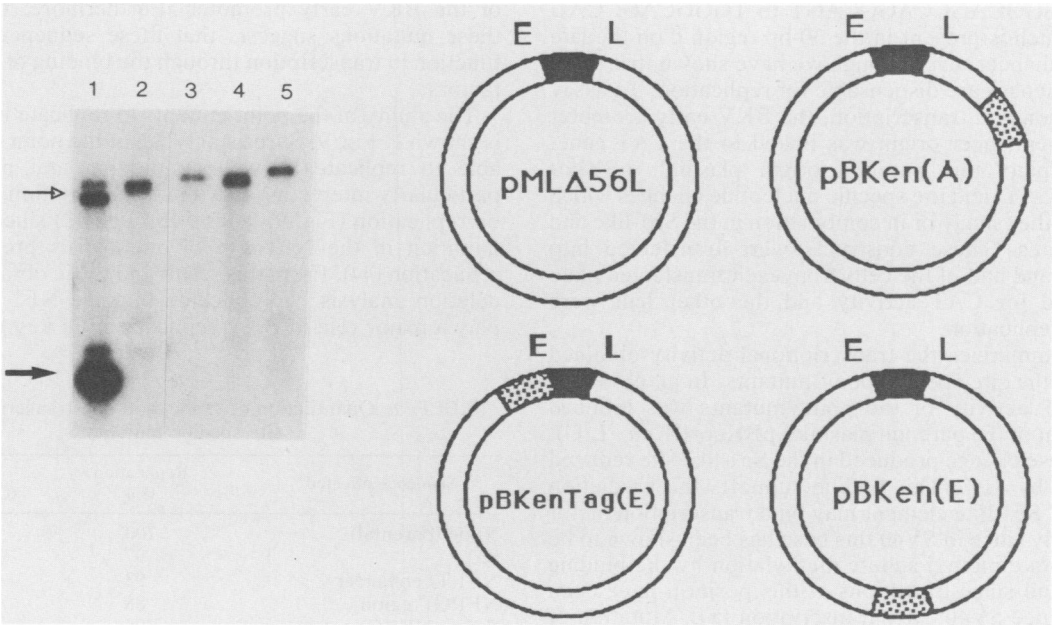

FIG. 7. Analysis of BKV origin plasmids containing replication-defective enhancers and distally inserted BKV enhancers. Constructs depicted were analyzed for replication by *Mbo*I digestion as described in the legend to Fig. 2. pMLBKcorBKen+L(Gardner) (lane 1) contains a BKV origin with a single complete 68-bp repeat and late-side sequences; nonreplicative pMLΔ56L (lane 2) contains a BKV origin with 12 bp of the enhancer. Insertion of BKV enhancers into pMLΔ56L: 372 bp on the late side of the origin generated pBKen(A) (lane 3); 3.7 kb away generated pBKen(E) (lane 4); abutting T-antigen site I generated pBKenTag(E) (lane 5). Arrows are the same as in Fig. 2. E, Early side; L, late side.

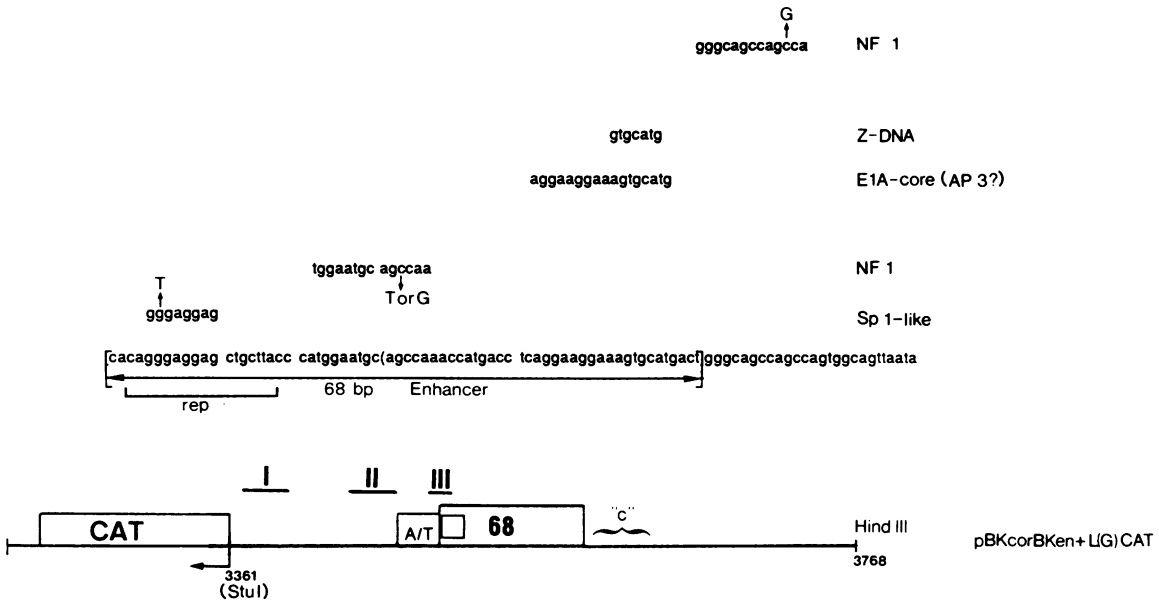

FIG. 8. Diagram of BKV enhancer landmarks and specific point mutations. Point mutations shown were made in the Sp1-like site and the NF1 sites present in both the enhancer and region c of parental plasmid pBKcorBKen+L(G)CAT. The small box in the 68-bp enhancer represents the *rep* element. Nonmutated landmark sites include the adenovirus E1A core and Z-DNA elements. Shown are the A-T-rich stretch and T-antigen-binding sites I, II, and III. The CAT gene was used to monitor transcription from the BKV early promoter.

BKV replication, we chose to mutate this site (TGGAATG CAGCCAA to TGGAATGCAG[G/T]CAA) and examine how these alterations affect both replication and transcription in the context of the complete enhancer. NF1, which is known to bind to the BKV enhancer (12, 37, 42) and is required for the replication of adenovirus DNA (9, 44), has also been shown to be the CAAT-binding protein involved in the transcription of certain genes (26). We also mutated the NF1 site (TGGGCAGCCAGCCAGT to TGGGCAGCCAG GCAGT), which is present in the 30-bp region c on the late side of the enhancer, even though we have shown that these late-side sequences are dispensable for replication. To assay both replication and transcription, the BKV early promoter of the single-enhancer origin was linked to the CAT gene. Figure 8 depicts this parental origin plasmid, pBKcor BKen+L(G)CAT, and the specific nucleotide changes which were made either singly or in combination in the Sp1-like and both NF1 sites. These constructs were transfected into COS-1 cells, and half of the cells from each transfected plate were assayed for CAT activity and the other half were assayed for replication.

Table 1 summarizes the transcriptional activity obtained with each of the site-specific point mutants. In all cases the level of CAT activity of the point mutants was reduced relative to that of the parental plasmid, pBKcorBKen+L(G). The single-base change produced in the Sp1-like site reduced transcription by 81%. This finding supports the prediction that the BKV Sp1-like element may bind transcription factor Sp1, especially since in SV40 this base has been shown to be protected from dimethyl sulfate methylation by the binding of Sp1 (19) and since transitions at this position have been shown to reduce SV40 early transcription (54). Mutation of the base in the enhancer NF1 site, which is a contact point for NF1 binding and which is a conserved base in CAAT box sequences (10, 26), caused CAT activity to be reduced by 50%. This result suggests a potential involvement of NF1 in the transcription of the BKV early promoter and is in agreement with the recent findings of others (11). Consistent with the finding that region c augments early transcription, mutation of the NF1 site present in this element caused CAT activity to be reduced by 25%. This finding suggests that the stimulation of transcription by region c could involve the binding of an NF1/CAAT-binding protein. As expected, combinations of mutations in the above sites resulted in comparable reductions in CAT activity. These results show that the Sp1-like and NF1 sites are involved in transcription of the BKV early promoter. Furthermore, the nature of these mutations suggests that these sequences potentially function in transcription through the binding of the predicted factors.

The ability of the point mutants to replicate in COS-1 cells is shown in Fig. 9. Surprisingly, all of the point mutants were able to replicate as well as the parental plasmid. It is particularly interesting that the NF1 mutation had no effect on replication (Fig. 9, lanes 5 through 12) since an identical mutation in the NF1 site of adenovirus prevented DNA replication (44). From this result and those obtained from the deletion analysis, we conclude that the NF1 site does not play a major role in BKV replication. Of key importance is

TABLE 1. Quantitation of replication and transcription of BKV site-specific mutants[a]

Sequence affected	Replication (%)	Transcription (CAT activity [%])
None (parental)	100	100
Sp1	97	19 ± 12
NF1(T) enhancer	93	54 ± 23
NF1(G) region c	88	25 ± 11
Sp1 + NF1(G) enhancer	106	17 ± 13
Both NF1 sites	88	32 ± 27

[a] Levels of replication and CAT activity of the mutants are expressed as a percentage of the parental plasmid. The intensity of replicative bands was normalized to the internal standard. The range of CAT activity is noted. Results are based on the determination obtained from three to five experiments.

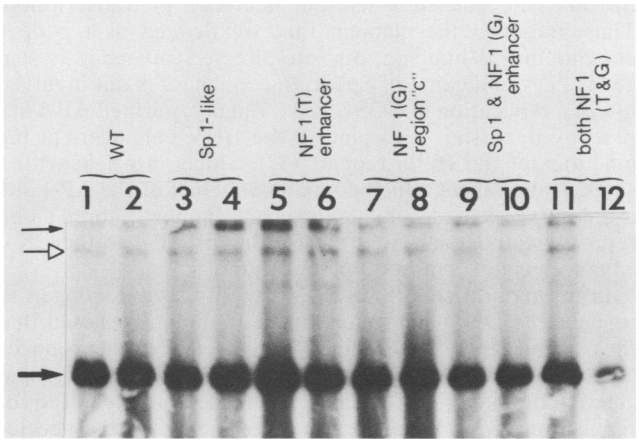

FIG. 9. Replication of BKV origin plasmids containing point mutations in the enhancer. Constructs depicted in Fig. 8 were transfected into COS-1 cells, and Hirt DNA was analyzed by Southern blotting as described in the legend to Fig. 2. Locations of the point mutations made in the enhancer of the BKV origin plasmid depicted in Fig. 8 are noted above lanes. Constructs were tested in duplicate. The *Mbo*I blot is shown. Positions of replicative and nonreplicative bands are shown as in Fig. 2. Arrows are the same as in Fig. 2. WT, Wild type.

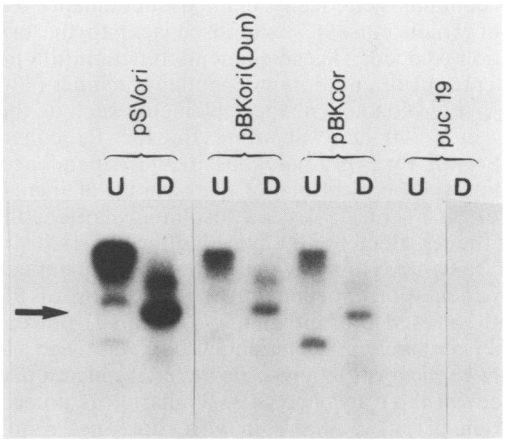

FIG. 10. In vitro replication of BKV origins. An in vitro replication system containing a HeLa cell extract, immunoaffinity-purified SV40 T antigen, and BKV origin constructs was incubated at 37°C for 4 h. Half of the ^{32}P-labeled products were codigested with *Eco*RI and *Dpn*I. Both the digested (D) and undigested (U) samples were electrophoresed through 1.5% agarose gels, dried, and autoradiographed. Plasmids represented are pBKori(Dunlop), a BKV origin with three enhancers; pBKori, an enhancerless BKV origin; pSVori, an SV40 origin-positive control; and puc 19, a negative control. Replicative bands are indicated by the arrow.

the fact that mutations in the Sp1 and NF1 sites which greatly reduced transcription had no effect on replication (Fig. 9). To rule out the possibility that the point mutants were altered in their rates of replication such that at the end of a 48-h transient assay they could reach the same level of replication as plasmids with the wild-type origin, we examined replication at 24, 36, and 48 h posttransfection. The replication rate of the mutated origins was the same as that of the wild-type origin, further demonstrating that these mutations had no effect on replication (data not shown). These findings suggest that the requirements for the BKV enhancer in replication are different from those involved in transcription.

Replication of BKV in vitro does not require any enhancer sequences. It has been shown that replication of SV40 in vivo is dependent on the presence of either the 21-bp-repeat elements or the 72-bp enhancer, but neither of these elements is required for replication in vitro (33, 34). We examined whether the BKV enhancer is also dispensable for in vitro replication. Plasmid pBKori(Dunlop), containing the complete origin of BKV with three 68-bp enhancers, and pBKcor, an enhanceless plasmid, were assayed for their ability to replicate in vitro. In vitro replication of BKV does not require any enhancer elements since pBKcor and pBK ori(Dunlop) replicated to virtually the same extent (Fig. 10). It is noted that replication was dependent on the presence of large T antigen in the extract (not shown) and that the BKV origin replicated less efficiently than did the SV40 origin. The negative control, puc 19, failed to replicate. Thus, as with SV40, replication of BKV in vitro does not require the presence of any enhancer elements.

DISCUSSION

The data presented here show that the 68-bp enhancer of BKV is directly involved in DNA replication in COS-1 cells. Although an origin containing one copy of the 68-bp repeat is able to replicate equally well as one that contains three repeats, the elimination of all enhancer sequences completely abolishes replication. Replication from BKV origins occurs when only the first 21 bp of the enhancer are juxtaposed to the A-T-rich stretch. We refer to these 21 bp of the BKV enhancer as the *rep* element. Deletion of 9 bp of the *rep* element from the late side reduced replication to undetectable levels, but origins containing only the *rep* element could replicate at 20 to 40% of the level of origins with a complete enhancer. Juxtaposing the remaining 47 bp of the enhancer to the A-T-rich stretch in the absence of *rep* would not support replication. The distal placement of a complete enhancer to an origin with a partially deleted *rep* element could not restore replication, demonstrating a positional requirement of the *rep* element. Additionally, internal deletions or insertions in sequences outside the *rep* element that changed the helical register in the enhancer had little or no effect on the level of replication, further demonstrating that they are nonessential for replication. It has been similarly shown that stereospecific alignment within the BKV enhancer is not critical for transcription of the early promoter (12). Further analysis is needed to delineate the boundaries of the *rep* element and critical nucleotides within this element which are essential for replication. Additionally, although the known variants of BKV differ with respect to their enhancer sequence arrangements (18, 49, 57), the element that we defined as *rep* is conserved in all sequenced variants.

In contrast to the results obtained in vivo, enhancerless BKV origins were able to replicate in vitro. Using immunoaffinity-purified SV40 T antigen and a HeLa cell extract capable of supporting replication in vitro, we found that the enhancerless origin could replicate to the same level as an origin containing three enhancer repeats. Thus, whatever function the BKV enhancer serves in stimulating replication in COS-1 cells is not required for replication in vitro.

It is important to compare these results on BKV replication with those obtained with SV40 and Py. Replication of Py is dependent on the presence of its enhancer element upstream of the site of initiation of replication. The Py en-

hancer contains genetically defined subdomains α and β, either of which must be juxtaposed next to the origin for replication to occur. These elements function in a position-dependent, but orientation-independent, manner (40). Additionally, heterologous enhancer elements such as the 72-bp repeats of SV40 can substitute for the Py enhancer in replication of Py (4). Analogous to the dependence of Py replication on the positions of the α and β elements, replication of BKV in COS-1 cells absolutely requires the presence of the *rep* element juxtaposed to the A-T-rich stretch; it remains to be determined whether the *rep* element exhibits a similar orientation independence. Replication of SV40 requires the presence of either the G-C-rich 21-bp repeats or the 72-bp enhancers. Deletion of both of these elements prevents replication in vivo; however, replication in vitro can occur in their absence (34). Similarly, as noted above, replication of BKV origins in vitro does not require any enhancer sequences. In SV40, juxtaposing either the 21-bp repeats or the 72-bp enhancer elements to the A-T-rich stretch supports replication equally well. In contrast, replication of BKV in vivo absolutely requires the presence of the *rep* element juxtaposed to the A-T-rich stretch. Juxtaposing the remaining 47 bp of the BKV enhancer to the A-T-rich stretch does not restore replication. This demonstrates a specific requirement for these particular 21 bp of the BKV enhancer, which is in sharp contrast to the replication requirements of SV40.

Through site-directed mutagenesis of the Sp1-like and NF1 sites of the 68-bp repeat, as well as the NF1 site in region c, we were able to reduce the level of transcription without affecting replication. Mutation of the Sp1-like and both NF1 sites reduced the level of transcription by two- to fivefold, but had no effect on replication. Biochemical and genetic data suggest that both the enhancer and region c bind NF1 since purified NF1 will footprint in these regions (37). Our mutation in the NF1 site in region c, previously shown to reduce NF1 binding to the adenoviral origin of replication by 10-fold (44), reduced transcription from the BKV early promoter by 4-fold. It is interesting that the mutation in the NF1 site in region c appeared to exert a greater effect on transcription that did the mutation which affected the NF1 site in the enhancer, even though the mutation in this latter site was estimated to reduce NF1 binding to a synthetic NF1 site by 250- to 400-fold (48). Our results on the replication of the point mutants indicate that the binding of NF1 to the BKV enhancer is not involved in replication. Indeed, the genetic analysis clearly shows that replication can occur despite deletion or mutation of both the NF1 site in the 68-bp enhancer and the NF1 site of region c. Additionally, plasmids with the 21-bp *rep* element deleted which have the remaining 47 bp of the enhancer containing the NF1 site juxtaposed to the A-T-rich stretch did not replicate. Similarly, we believe that factor binding to the Sp1-like site in the *rep* element is not critical for replication. In fact, it is important to note that there are somewhat conflicting findings as to whether a factor(s) actually does bind to the Sp1-like site (12, 37). Markowitz and Dynan (37) showed that neither HeLa cell proteins from heparin-agarose column fractions nor affinity-purified Sp1 from HeLa cells binds to the Sp1-like site in the BKV enhancer. However, Deyerle and Subramani (12) found weak protection of this site, using heparin-agarose-purified HeLa cell protein fractions. It is possible that a factor other than Sp1 binds here or that the binding of Sp1 requires interaction with other factors. In agreement with our study, linker-scanning mutation analysis by Deyerle and Subramani (12) showed that mutations in the Sp1-like site caused a fivefold decrease in transcription. Thus, although the element that we defined as *rep* does contain an Sp1-like site, our site-directed mutagenesis suggests that the binding of Sp1 to the enhancer is not involved in BKV replication in COS-1 cells. Finally, purified AP-1 has recently been shown to bind to the BKV enhancers at the junctions of the 68-bp repeats (37). Since an origin with a single 68-bp repeat which contains only half of the AP-1 site is able to replicate as efficiently as an origin with three 68-bp repeats, we postulate that the binding of AP-1 to the BKV enhancer is not required for replication.

In the natural situation, BKV uses its own T antigen to replicate its DNA in a human host. It should be noted that these studies were done using a simian cell line that produces SV40 T antigen. Although the first BKV isolate was propagated in monkey (Vero) cells (18), *cis* elements required for replication of BKV could differ, depending on the species from which the cell line was derived or the available T antigen.

We favor the hypothesis that the mechanism by which the BKV enhancer stimulates transcription is different from the mechanism by which it augments replication. Our analysis shows that elements of the BKV enhancer defined as important for transcription were not essential for replication. These results are critical not only in understanding how elements classically defined as transcriptional enhancers can augment replication, but also in distinguishing between elements of the BKV enhancer involved in replication or transcription. The *rep* element that we defined as critical for the replication from BKV origins could function either by binding a cellular factor involved in DNA replication or by assuming a certain DNA conformation required for replication. Since *rep* appears to be dispensable for replication in vitro, it may be involved in determining chromatin structure. For example, *rep* could prevent histones from binding to the origin and allow the replication machinery better access to the origin. Since chromatin structure does not appear to be important in vitro, *rep* would not be needed. Alternately, *rep* could bind a specific factor required for the initiation of DNA replication (56). Replication in vivo would require the binding of a cellular factor to *rep* for the efficient formation of an initiation complex; however, in vitro proteins such as T antigen and polymerase α-primase could form an initiation complex on naked DNA without the aid of a putative *rep*-binding protein.

The greatest divergence among the papovaviruses BKV, SV40, and JC virus occurs in their enhancer regions (16). This may in part explain their host specificities and tissue tropisms. Since these viruses have evolved complex control regions with the origin of replication overlapping the early and late promoters, it is often difficult to examine one function of this region without perturbing another. We have genetically defined elements of the BKV enhancer involved in DNA replication and suggest that the role of the enhancer in replication can be uncoupled from its role in transcription. Further analysis of the *cis* elements and *trans*-acting factors involved in the regulation of this region may not only provide a model for the mechanism of enhancer action in both replication and transcription, but also help in understanding how BKV regulates its life cycle.

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LITERATURE CITED

1. **Arthur, R. R., K. V. Keerti, S. J. Baust, G. W. Santos, and R. S. Saral.** 1986. Association of BK viruria with hemorrhagic cystitis in recipients of bone marrow transplants. N. Engl. J. Med. **315**:230–234.
2. **Baty, D., H. A. Barrera-Daldana, R. D. Everett, M. Vigneron, and P. Chambon.** 1984. Mutational dissection of the 21 bp repeat region of the SV40 early promoter reveals that it contains overlapping elements of the early-early and late-early promoters. Nucleic Acids Res. **12**:915–932.
3. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL 1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques **5**: 376–379.
4. **Campbell, B. A., and L. P. Villarreal.** 1985. Host species specificity of polyomavirus DNA replication is not altered by simian virus 40 72-base-pair repeats. Mol. Cell. Biol. **5**:1534–1537.
5. **Caputo, A., G. Barbanti-Brodano, E. Wang, and R. P. Ricciardi.** 1986. Transactivation of BKV and SV40 early promoters by BKV and SV40 T-antigens. Virology **152**:459–465.
6. **Chesters, P. M., J. Heritage, and D. J. McCance.** 1983. Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. J. Infect. Dis. **147**: 676–684.
7. **DeLucia, A. L., S. Deb, K. Partin, and P. Tegtmeyer.** 1986. Functional interactions of the simian virus 40 core origin of replication with flanking regulatory sequences. J. Virol. **57**: 138–144.
8. **de Villiers, J., W. Schaffner, S. Lupton, and C. Tyndall.** 1985. Enhancer function is essential for polyomavirus DNA replication. Nature (London) **312**:242–246.
9. **de Vries, E., W. van Driel, M. Tromp, J. van Boom, and P. C. van der Vliet.** 1985. Adenovirus DNA replication in vitro: site-directed mutagenesis of the nuclear factor I binding site of the Ad2 origin. Nucleic Acids Res. **13**:4935–4952.
10. **de Vries, E., W. van Driel, S. J. L. van den Heuvel, and P. C. van der Vliet.** 1987. Contactpoint analysis of the HeLa nuclear factor I recognition site reveals symmetrical binding at one side of the DNA helix. EMBO J. **6**:161–168.
11. **Deyerle, K. L., J. A. Cassill, and S. Subramani.** 1987. Analysis of the early regulatory region of the human papovavirus BK. Virology **158**:181–193.
12. **Deyerle, K. L., and S. Subramani.** 1988. Linker scan analysis of the early regulatory region of the human papovavirus BK. J. Virol. **62**:3378–3387.
13. **Dorries, K., G. Loeber, and J. Meixensberger.** 1987. Association of polyomavirus JC, SV40, and BK with human tumors. Virology **160**:268–270.
14. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35**:79–87.
15. **Everett, R. D., D. Baty, and P. Chambon.** The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter. Nucleic Acids Res. **11**:2447–2464.
16. **Frisque, R. J., G. L. Bream, and M. T. Cannella.** 1984. Human polyomavirus JC virus genome. J. Virol. **51**:458–469.
17. **Gardner, S. D.** 1973. Prevalence in England of antibody to human polyomavirus (B.K.). Br. Med. J. **1**:77–78.
18. **Gardner, S. D., A. M. Field, D. V. Coleman, and B. Hulme.** 1971. New human papovavirus [BK] isolated from urine after renal transplantation. Lancet **i**:1253–1257.
19. **Gidoni, D., W. S. Dynan, and R. Tjian.** 1984. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature (London) **312**:409–413.
20. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
21. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
22. **Hertz, G. Z., and J. E. Mertz.** 1986. Bidirectional promoter elements of simian virus 40 are required for efficient replication of the viral DNA. Mol. Cell. Biol. **6**:3513–3522.
23. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
24. **Howley, P. M.** 1980. Molecular biology of SV40 and the human polyomaviruses BK and JC, p. 489–514. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
25. **Innis, J. W., and W. A. Scott.** 1984. DNA replication and chromatin structure of simian virus 40 insertion mutants. Mol. Cell. Biol. **4**:1499–1507.
26. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
27. **Jones, N. C., P. W. J. Rigby, and E. B. Ziff.** 1988. *trans*-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
28. **Keller, J. M., and J. C. Alwine.** 1985. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication. Cell **36**:381–389.
29. **Kenney, S., V. Natarajan, D. Strike, G. Khoury, and N. P. Salzman.** 1984. JC virus enhancer-promoter active in human brain cells. Science **226**:1337–1339.
30. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using miniprep plasmid DNA for sequencing double stranded templates with Sequenase™. Biotechniques **6**:544–547.
31. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
32. **Lee-Chen, G.-J., and M. Woodworth-Gutai.** 1986. Simian virus 40 DNA replication: functional organization of regulatory elements. Mol. Cell. Biol. **6**:3086–3093.
33. **Li, J. J., and T. J. Kelly.** 1984. Simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA **81**:6973–6977.
34. **Li, J. J., K. W. C. Peden, R. A. F. Dixon, and T. Kelly.** 1986. Functional organization of the simian virus 40 origin of replication. Mol. Cell. Biol. **6**:1117–1128.
35. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
36. **Major, E. O., and P. Matsumura.** 1984. Human embryonic kidney cells: stable transformation with an origin-defective simian virus 40 DNA and use as hosts for human papovavirus replication. Mol. Cell. Biol. **4**:379–382.
37. **Markowitz, R., and W. Dynan.** 1988. Binding of cellular proteins to the regulatory region of BK virus DNA. J. Virol. **62**: 3388–3398.
38. **Mason, D. H., Jr., and K. K. Takemoto.** 1976. Complementation between BK human papovavirus and a simian virus 40 tsA mutant. J. Virol. **17**:1060–1062.
39. **Milanesi, G., G. Barbanti-Brodano, M. Negrini, D. Lee, A. Corallini, A. Caputo, M. P. Grossi, and R. P. Ricciardi.** 1984. BK virus-plasmid expression vector that persists episomally in human cells and shuttles into *Escherichia coli*. Mol. Cell. Biol. **4**:1551–1560.
40. **Muller, W. J., C. R. Mueller, A.-M. Mes, and J. A. Hassell.** 1983. Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virol. **47**:586–599.
41. **Nordheim, A., and A. Rich.** 1983. Negatively supercoiled simian virus 40 DNA contains Z-DNA segments within transcriptional enhancer sequences. Nature (London) **303**:674–678.
42. **Nowock, J., U. Borgmeyer, A. W. Püschel, R. A. W. Rupp, and A. E. Sippel.** 1985. The TGGCA protein binds to the MMTV-LTR, the adenovirus origin of replication, and the BK virus

enhancer. Nucleic Acids Res. **13**:2045–2060.
43. **Ondek, B., A. Shepard, and W. Herr.** 1987. Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. EMBO J. **6**:1017–1025.
44. **Rosenfeld, P. J., E. A. O'Neill, R. J. Wides, and T. J. Kelly.** 1987. Sequence-specific interactions between cellular DNA-binding proteins and the adenovirus origin of DNA replication. Mol. Cell. Biol. **7**:875–886.
45. **Rosenthal, N., M. Kress, P. Gruss, and G. Khoury.** 1983. BK viral enhancer element and a human cellular homolog. Science **222**:749–765.
46. **Ryder, K., A. L. DeLucia, and P. Tegtmeyer.** 1983. Binding of SV40 A protein of the BK virus origin of DNA replication. Virology **129**:239–245.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
48. **Schneider, R., I. Gander, U. Müller, R. Mertz, and E. L. Winnacker.** 1986. A sensitive and rapid gel retention assay for nuclear factor I and other DNA-binding proteins in crude nuclear extracts. Nucleic Acids Res. **14**:1303–1317.
49. **Seif, I., G. Khoury, and R. Dhar.** 1979. The genome of human papovavirus BKV. Cell **18**:963–977.
50. **Shah, K. V., R. W. Daniel, and R. M. Warszawaski.** 1973. High prevalence of antibodies to BK virus, an SV40-related papovavirus, in residents of Maryland. J. Infect. Dis. **128**:784–787.
51. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–517.
52. **Takahashi, K., M. Vigneron, H. Matthes, A. Wildeman, M. Zenke, and P. Chambon.** 1986. Requirement of sterospecific alignments for initiation from the simian virus 40 early promoter. Nature (London) **319**:121–126.
53. **Veldman, G. M., S. Lupton, and R. Kamen.** 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. **5**:649–658.
54. **Weiher, H., M. König, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**: 626–631.
55. **Wildeman, A. G., M. Zenke, C. Schatz, M. Wintzerith, T. Grundström, H. Matthes, K. Takahashi, and P. Chambon.** 1986. Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. **6**:2098–2105.
56. **Yamaguchi, M., and M. L. DePamphilis.** 1986. DNA binding site for a factor(s) required to initiate simian virus 40 DNA replication. Proc. Natl. Acad. Sci. USA **83**:1646–1650.
57. **Yang, R. C. A., and R. Wu.** 1979. Comparative study of papovavirus DNA: BKV [MM], BKV [WT] and SV40. Nucleic Acids Res. **7**:651–658.
58. **Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.