Analysis of Origin of DNA Replication of Human Papovavirus BK

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We have analyzed the *cis*-acting sequence elements and properties of the origin of DNA replication of human papovavirus BK (BKV). The precise boundaries of the origin varied, depending on the cell type and the viral T antigen used for assay. The BKV minimal origin of replication consisted of an inverted repeat, T-antigen-binding site II, and a 20-base-pair AT block when assayed in monkey kidney CV1 and HeLa cells by using the BKV T antigen. This 76-base-pair minimal origin did not replicate in COS cells in the presence of the simian virus 40 (SV40) T antigen. Unlike that from the SV40 minimal origin, replication from the BKV minimal origin was not enhanced by BKV *ori*-flanking sequences in CV1 or HeLa cells, using the BKV T antigen. BKV *ori*-flanking sequences did activate the SV40 minimal origin of replication in COS cells and relieved the orientation-dependent property of this origin. Finally, the BKV T antigen was found to autoregulate activity of the BKV early transcriptional regulatory region. The BKV origin of replication shows similarities to and differences from those of the related viruses SV40 and polyomavirus, suggesting that the proteins involved in the initiation of replication interact with origin sequences differently in these viruses.

The well-defined structure and simplicity of the *Papovaviridae* make them attractive model systems for the study of eucaryotic DNA replication. The genomes of these small, circular, double-stranded DNA viruses assume a chromatin-like nucleoprotein structure within the host cell and replicate bidirectionally from a unique origin. A single viral gene product, the large T antigen, is required for replication; all other factors involved are encoded by the host genome. Sequences required for replication most likely include binding domains for those proteins involved in the initiation of viral DNA replication. Identification of sequences constituting papovavirus origins of replication, coupled with the recently developed *in vitro* replication systems (39, 62, 63, 69), may thus aid in the identification and characterization of cellular replication proteins.

The genomic organization of the *Papovaviridae* consists of a noncoding regulatory region located between protein-coding sequences transcribed in opposite directions. The noncoding regulatory region contains both the origin of replication and transcriptional regulatory elements. Structures within the origin region are highly conserved within the genus and include two inverted repeats and an AT-rich tract (52, 66). A sequence spanning this region has been identified as the minimal origin of replication in simian virus 40 (SV40) (8, 9, 11, 40, 60). Two areas within the SV40 minimal origin have been implicated in interactions with host cell replication factors (66, 71). Sequences flanking this region have been shown to augment, but are not required for, replication (11, 40). Similarly, the polyomavirus (Py) minimal origin of replication encompasses an inverted repeat, a palindrome, and an AT block (7, 34, 45). Unlike that of SV40, replication from the Py origin requires the presence of an enhancer element (13, 48, 52).

We have now mapped the sequence requirements for DNA replication from the origin of the human papovavirus BK (BKV), a virus originally isolated after passage through monkey kidney cells (19). Our results show that the BKV minimal origin contains two inverted repeats and an AT block. The level of replication from this minimal origin

equaled that of the intact wild-type origin in CV1 and HeLa cells, using the BKV T antigen. In contrast, this BKV minimal origin did not replicate in COS cells, using the SV40 T antigen, although the intact BKV origin was capable of replicating under these conditions. The precise boundaries of the BKV origin varied with the cell type and the T antigen used in the assay. BKV *ori*-flanking sequences activated the SV40 minimal origin of replication and altered the orientation-dependent properties of this origin. Finally, the BKV T antigen was found to autoregulate the BKV early promoter-enhancer. These data suggest the existence of variable mechanisms of replication activation from papovavirus origins of replication.

MATERIALS AND METHODS

Plasmid constructions. The construction of pSV2ABK3362 and of deletion mutants derived from this plasmid has been described earlier (15) (Fig. 1, A and B). All BKV noncoding regulatory region mutants are contained within an identical genetic background, a derivative of the pSV2CAT expression vector (23). The position of the BKV region within this vector allows the activity of the BKV early promoter-enhancer to be monitored by assaying chloramphenicol acetyltransferase (CAT) activity.

(i) Plasmids containing the SV40 minimal origin of replication. The SV40 minimal origin of replication, contained within the plasmid pOR1, was obtained from P. Tegtmeyer (11). The late boundary of the SV40 origin (*Nco*I site, nucleotide [nt] 39) in this plasmid is located directly adjacent to a polylinker containing *Sal*I and *Bam*HI sites. The SV40 minimal origin was excised from pOR1 as a *Hind*III-*Bam*HI fragment and was inserted into pSV2ABK3362 in place of the BKV noncoding regulatory region. The resultant plasmid, pOR1CAT, contains the SV40 minimal origin oriented with its early boundary (*Hind*III site, nt 5171) proximal to the CAT gene. An intermediate plasmid, pOR1XCAT, was constructed by conversion of the pOR1CAT *Hind*III site to an *Xho*I site. The SV40 minimal origin was excised from pOR1XCAT as an *Xho*I-*Sal*I fragment and was inserted into the *Xho*I site of Δ B3382-H3459 in both orientations to generate pBKSMO-R and pBKSMO-1 (Fig. 1C). The plasmid Δ B3382-H3459 was derived from unidirectional dele-

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FIG. 1. Construction of various BKV origin region deletion plasmids. (A) The noncoding regulatory region of BKV (nt 3362 to 3757) contains a palindrome (T-antigen-binding site I), an inverted repeat, another inverted repeat (T-antigen-binding site II), a 20-bp AT block, three direct 68-bp repeats (the middle one of which contains an 18-bp deletion) and late flanking sequences. This region is contained in pSV2ABK3362 as a *Hind*III (nt 3362)-*Bam*HI (nt 3757) fragment. PAL, Palindrome; IR, inverted repeat; AT, 20-bp AT block. Arrows indicate early mRNA start sites in CV1 and COS cells. (B) ΔB deletion mutants were constructed by digesting pSV2ABK3362 with *Bam*HI and digesting with *Bal* 31 towards the early side, while ΔH deletions were made in a similar way but starting at the *Hind*III site and digesting toward the late side. The plasmid names indicate the last BKV nucleotide remaining at the deletion endpoint. All deletion mutants contain *Xho*I linkers (CCTCGAGG) at the deletion endpoint, except mutants ΔH3377, ΔH3384, and ΔH3398, which contain a *Hind*III linker (CAAGCTTG). Internal deletion mutants were made by combining appropriate ΔH and ΔB deletion mutants. (C) Plasmid ΔB3382-H3459 was derived from unidirectional deletions of pSV2ABK3362 such that nt 3383 to 3458 of the BKV sequence in the latter were replaced by an *Xho*I linker (CCTCGAGG) in ΔB3382-H3459. The 81-bp SV40 minimal origin (SMO) of replication (nt 5171 to 39, with nt 5178 to 5208 deleted) was excised as an *Xho*I-*Sal*I fragment from pOR1XCAT and was inserted into the *Xho*I site of ΔB3382-H3459. The R orientation (pBKSMO-R) contains the SV40 origin with the 17-bp AT block furthest from the CAT gene, while the I orientation (pBKSMO-I) has the 17-bp AT block closest to the CAT gene. II, SV40 T-antigen-binding site II.

tions of pSV2ABK3362 such that nt 3383 to 3458 of the BKV sequence in the latter were replaced by an *Xho*I linker (CCTCGAGG) in ΔB3382-H3459.

(ii) **Plasmid containing the BKV minimal origin of replication.** The BKV minimal origin of replication was constructed by the polymerase chain reaction method (58) with a kit purchased from Perkin Elmer Cetus. The template for amplification was ΔB3675 (15), which contains the intact BKV origin of replication. The two oligodeoxynucleotide primers used to initiate the reaction were 5'-CTGCCTTACTACTTG AGAGAAAGGGTGGAGGCAGAGGCGG-3' and 5'-CTGG CCTTTTTTTTTTATAATATATAAGAGGCCGAGGCCGC C-3'. These primers together span the BKV region from nt 3385 to 3456, with 1 to 2 nt added on each side to form *Pvu*II-compatible ends. Reactions contained 40 ng of template DNA and 200 pmol of each phosphorylated primer, and the reaction conditions used were those recommended by the vendor. After 30 cycles of amplification, the products were resolved by electrophoresis on an 8% native polyacrylamide gel. The band corresponding to the 76-base-pair (bp) BKV minimal origin was eluted from the gel and purified by chromatography on a NACS mini-column (Bethesda Research Laboratories, Inc.). This 76-bp DNA fragment was subsequently inserted into the *Sal*I site of pTZ19U (U.S. Biochemicals) which had been filled in by using nucleotides and the large fragment of DNA polymerase I. The BKV inserts in recombinant plasmids were sequenced by using the dideoxy-sequencing method and Sequenase enzyme (U.S.

Biochemicals) (64). A plasmid containing the correct insert, pTZ19U-BK, was identified, and the BKV origin was excised as a *Sal*I fragment and purified as described above. This DNA fragment was then inserted into the *Xho*I site of vector pSV2AXho (15), a derivative of pSV2ABK3362 in which the BKV noncoding regulatory region has been replaced by an *Xho*I linker. The resultant plasmid, pBKmin, contains the BKV minimal origin of replication with its early boundary adjacent to the CAT gene of the vector.

(iii) **Plasmid expressing the BKV large T antigen.** The plasmid pRSV-BKT, which expresses the BKV early-coding region driven by the Rous sarcoma virus (RSV) long terminal repeat promoter, was derived from the previously described plasmid pRSV-CAT (22). The 2,852-bp *Stu*I-*Pvu*II BKV wild-type fragment containing the entire t- and T-antigen-coding regions and polyadenylation sequence was isolated and modified by the addition of *Bam*HI linkers. The unique *Hind*III site of pRSV-CAT, located at the junction between the RSV promoter and the CAT-coding sequences, was filled in and converted into a *Bgl*II site by the addition of a linker. The CAT-coding sequence of pRSV-CAT was then excised as a *Bgl*II-*Bam*HI fragment and replaced with the *Bam*HI fragment containing the BKV early-coding region.

Determination of in vivo replication. Monkey kidney CV1 cells were cared for and transfections were carried out as previously described (15). COS cells, transformed CV1 cells that constitutively express the SV40 large T antigen, were grown in Dulbecco modified Eagle medium (GIBCO Labo-

ratories) supplemented with 5% fetal calf serum (Irvine Scientific). L603 cells, a transformed human fibroblast cell line that constitutively expresses the BKV large T antigen (25), were grown in minimal essential medium supplemented with 10% fetal calf serum. These cells were generously provided by G. Barbanti-Brodano. HeLa cells, a human cervical carcinoma cell line, were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. For CV1 and HeLa cell experiments, the BKV T antigen was supplied by cotransfection of 3 μ g of pRSV-BKT with 7 μ g of a BKV plasmid. All DNAs tested for replication were prepared from *dam*⁺ *Escherichia coli* HB101 and thus were fully methylated at the sequence 5'-GATC-3'. Low-molecular-weight DNA was extracted from cells 44 to 48 h post-transfection by the method of Hirt (30). Replication was assayed by testing the sensitivities of these DNAs to digestion by *DpnI* and *MboI*. Both of these enzymes recognize the sequence 5'-GATC-3', but *DpnI* will cleave only fully methylated (unreplicated) DNA, whereas *MboI* cuts only completely unmethylated (two or more rounds of replication) DNA. To ensure complete digestion, all DNAs were digested with 5 U of either enzyme for 12 to 16 h at 37°C. The digestion products were electrophoresed on 1% agarose gels, transferred to nitrocellulose, hybridized, washed, and exposed to X-ray film as previously described (61). The probes used were nick translation products of pSV2ABK3362 or a fragment containing nt 3362 to 3475 of the BKV early regulatory region. Where appropriate, results were quantitated by measuring the density of the origin-containing DNA fragment on multiple exposures of autoradiograms by using an Ultrascan densitometer (LKB Instruments, Inc.). Three to seven independent transfections, using at least two different preparations of DNA, were carried out for each datum point.

CAT assays. Autoregulation of the BKV early regulatory region by the BKV T antigen was studied in CV1 cells by cotransfection of 7 μ g of Δ H and Δ B BKV deletion mutants with 3 μ g of either pRSV-BKT or pBR322 (control). The RSV promoter has been shown not to compete for limiting transcription factors with the BKV early promoter under these conditions (59). Replication of all plasmids was prevented by the addition of 10 μ g of ara-C (1-[β -D-arabinofuranosyl]cytosine; Sigma) per ml to the media immediately following transfection. Protein extracts were made 44 to 48 h posttransfection, and CAT activity was assayed as previously described (15). Luciferase activity (assayed as described in reference 14) and double indirect immunofluorescence (performed as described in reference 35) were used to ascertain the coexpression of both plasmids in the majority (>70%) of transfected cells (data not shown). The results represent the averages of eight independent transfections, using at least two different preparations of DNA.

RESULTS

The unique origin of DNA replication of a papovavirus is located within the viral noncoding regulatory region, which contains early- and late-gene regulatory elements in addition to the origin. Replication from the origin is absolutely dependent on the binding of the early-gene product large T antigen to sequences within the origin. Mutations of the noncoding regulatory region could thus affect replication by encroaching on the sequences required for the initiation of replication, by impairing T antigen production, or both. The ability of BKV mutants to support replication was assayed under conditions in which T antigen was provided in *trans*,

to circumvent possible variations in T antigen synthesis. The sequence requirements for the BKV minimal origin of replication were determined by analysis of deletion mutants of the BKV noncoding regulatory region (nt 3362 to 3757). The BKV mutants were contained within a common genetic background to avoid spurious effects of flanking sequences on replication. The vector employed for this purpose was pSV2ABK3362 (15), a derivative of the pSV2CAT expression vector (23) which contains the BKV noncoding regulatory region in place of the SV40 promoter-enhancer.

The BKV region of pSV2ABK3362 includes a palindrome containing T-antigen-binding site I, an inverted repeat, an inverted repeat containing T-antigen-binding site II, a 20-bp AT block, and the BKV enhancer (Fig. 2A). Two T-antigen-binding pentanucleotides are located within T-antigen-binding site I, four within T-antigen-binding site II, and one within T-antigen-binding site III (Fig. 2A). The ability of deletion mutants of this region to support replication was tested by transfection into a variety of cell types in the presence of either the BKV or the SV40 large T antigen. Low-molecular-weight DNA was extracted 44 to 48 h post-transfection and assayed for replication by determining both *DpnI* and *MboI* sensitivities. Transfected DNA is known to assemble into a typical chromatin structure in vivo during transient assay (4), and it has been shown that relative replication rates are constant from 36 to 48 h (40). Mutant origins were scored as either positive or negative for replication.

Early boundary of the origin lies within an inverted repeat and varies with cell type. Unidirectional deletion mutants extending from nt 3362 in the late direction (Δ H series) were used to define the early boundary of the BKV origin of replication in CV1, HeLa, and L603 cells in the presence of the BKV large T antigen. The results are shown in Fig. 2A. Mutants lacking two-thirds (Δ H3377) or all (Δ H3384) of the high-affinity T-antigen-binding site I were found to replicate in all three cell types. Similarly, origins lacking the first 7 bp of the inverted repeat (Δ H3388) were capable of replication under these conditions. Deletion of 15 bp from the early side of the inverted repeat (Δ H3396), however, resulted in an absence of replication in the human cell lines HeLa and L603. Interestingly, this deletion mutant was able to replicate in CV1 cells. Deletion of 2 more bp (Δ H3398), however, totally abolished replication in CV1 cells. The early boundary of the BKV origin of replication is thus located within an inverted repeat separating T-antigen-binding sites I and II.

Late boundary of the origin includes the AT block. Unidirectional deletion mutants extending from nt 3757 in the early direction (Δ B series) were used to define the late boundary of the BKV origin of replication in CV1, HeLa, and L603 cells in the presence of the BKV large T antigen. The BKV enhancer was found to be dispensable for replication, as mutants which contained variable portions of the enhancer were found to replicate in all three cell types (Fig. 2). Furthermore, a mutant completely lacking enhancer sequences (Δ B3454) also replicated in these cells. Deletion of the 20-bp AT block to nt 3434, however, abolished replication in both human and simian cells (Fig. 2). The late border of the BKV origin of replication in these cells is therefore located between nt 3454 and 3434 and includes a portion, if not all, of the 20-bp AT block.

BKV origin boundaries vary with cell type and T antigen. The SV40 and BKV large T antigens are 75% homologous on the basis of primary sequence data (57), and each protein can support replication of the heterologous virus (3, 37, 42). It was of interest to determine whether replication from the

FIG. 2. Replication of BKV unidirectional deletion mutants in COS, CV1, HeLa, and L603 cells. (A) Lines above (Δ H) and below (Δ B) the schematic of the BKV noncoding regulatory region indicate sequences remaining in various unidirectional deletion mutants. The last BKV nucleotide present in the deletion is shown and corresponds to the name of the mutant. The presence (+) or absence (-) of replication of these mutants in each of four cell types is indicated at the left. PAL, Palindrome; IR, inverted repeat; AT, 20-bp AT block. Sp1 consensus sequences ($\square$) and NF1-binding sites (---) are shown. An 18-bp deletion (∇) in the 68-bp BKV repeat unit is indicated. Small horizontal bars with numbers under them denote pentanucleotides to which T antigen binds. (B) Deletion mutants were transfected into cells, and low-molecular-weight DNA was isolated at 48 h and digested with *DpnI* (d) and *MboI* (m) to assay replication. The digested DNA fragments containing the BKV origins from appropriate unidirectional deletion mutants that define the endpoints of the BKV origin of replication in various cell types are shown.

BKV origin had the same sequence requirements when either the SV40 or BKV T antigen was used. The ability of BKV *ori* mutants to replicate in COS cells was therefore examined. The results from Δ H plasmids showed that BKV *ori* mutants lacking the high-affinity BKV T-antigen-binding site I were capable of replication, but deletion of 7 bp from the early side of the inverted repeat abolished replication under these conditions (Δ H3384 and Δ H3388, Fig. 2). Deletion mutants of the Δ B series demonstrated that the late boundary of the BKV origin included some enhancer sequences between nt 3454 and 3469 (Fig. 2). Replication from

the BKV origin in COS cells, with the SV40 T antigen, therefore requires additional sequences on both the early and late borders, compared with replication in HeLa, CV1, and L603 cells with the BKV T antigen.

Internal deletion mutants yield surprising results in different cell types. Unidirectional deletion data defined the BKV origin as consisting of approximately two inverted repeats and the 20-bp AT block, depending on the cell type and the T antigen. Internal deletions of this region were made to examine the importance of these features in the presence of wild-type flanking sequences. The results from

FIG. 3. Replication of internal deletion mutants of the BKV origin. (A) The lines represent sequences of the BKV noncoding regulatory region remaining in internal deletion mutants, with the extent of the gap representing deleted sequences replaced by an *Xho*I linker. The presence (+) or absence (–) of replication of these mutants in each of three cell types is indicated at the left. PAL, Palindrome; IR, inverted repeat; AT, 20-bp AT block. Sp1 consensus sequences (□) and NF1-binding sites (---) are shown. An 18-bp deletion (▽) in the 68-bp BKV repeat unit is indicated. Small horizontal bars with numbers under them denote pentanucleotides to which T antigen binds (B) Deletion mutants were transfected into cells, and low-molecular-weight DNA was isolated at 48 h and digested with *Dpn*I (d) and *Mbo*I (m) to assay replication. The digested DNA fragments containing the BKV origins are shown.

internal deletions of portions of the early inverted repeat confirmed that replication from the BKV origin in COS cells, with the SV40 T antigen, required more early sequences than did replication in human or simian cells with the BKV T antigen (Fig. 3). Furthermore, in the presence of T-antigen-binding site I, additional base pairs could be deleted from the early side of the inverted repeat without abolishing BKV T-antigen-stimulated replication in HeLa cells (compare ΔB3382-H3396, Fig. 3, with ΔH3396, Fig. 2). Deletion of the entire inverted repeat (ΔB3382-H3408, Fig. 3) abolished replication in all three cell types. These data indicate an absolute requirement for various portions of the early inverted repeat for replication from the BKV origin.

Results obtained with mutants containing internal deletions of portions of T-antigen-binding site II and the AT block revealed that these regions were essential for replication in COS cells (Fig. 3). These same mutants yielded unexpected results when their ability to support BKV T-antigen-stimulated replication was assayed in CV1 and HeLa cells. T-antigen-binding site II in ΔB3423-H3427 is disrupted by replacement of 3 bp of the bidirectional origin with an 8-bp linker (CCTCGAGG). This mutation removes the 2 3' bp of T antigen pentanucleotide 3 and places pentanucleotide 4 on the opposite side of the DNA helix relative to its position in the wild-type origin (57). Despite these rearrangements, this origin supported BKV T-antigen-stimulated replication in CV1 and HeLa cells (Fig. 3). A similar mutant (ΔB3423-H3442) in which the late 8 bp of the bidirectional origin (including 2 bp of T antigen pentanucleotide 3 and all of pentanucleotide 4) (57) and the adjacent 11 bp of the AT block were deleted failed to support replication. A more extensive deletion in this same region (ΔB3423-H3459) also failed to support replication (Fig. 3). These results suggest that although the configuration of T-antigen-binding site II is not absolutely critical, at least a portion of the AT block is required for replication.

The BKV minimal origin of replication supports replication in CV1 and HeLa cells but not in COS cells. The above unidirectional deletion data indicated that the BKV origin included two inverted repeats and the 20-bp AT block (Fig.

4). It was of interest to determine whether this region could support replication in the absence of BKV flanking sequences. A synthetic 76-bp oligonucleotide containing this BKV sequence (nt 3385 to 3456) was therefore synthesized and inserted into the plasmid pSV2AXho at the unique *Xho*I site, with the 20-bp AT block furthest from the CAT gene. Densitometric scanning showed that the level of replication from this minimal origin equalled that of the intact origin (pSV2ABK3362) when assayed in CV1 and HeLa cells, using the BKV T antigen (Fig. 4B). In contrast, the BKV minimal origin did not replicate in COS cells in the presence of the SV40 T antigen (Fig. 4B), confirming our earlier results which showed that replication in COS cells required additional sequences.

BKV flanking sequences activate the SV40 minimal origin of replication. The 81-bp SV40 minimal origin of replication, while having little activity on its own, is activated to various degrees by T-antigen-binding site I and the 21-bp repeats of the SV40 early promoter (1, 11, 16, 32, 38, 40, 45, 62). Given the similarities between SV40 and BKV, it was of interest to determine whether BKV flanking sequences could activate the SV40 minimal origin of replication. To test this, a segment of SV40 DNA spanning the minimal *ori* region (nt 5171 to 39, with nt 5178 to 5208 deleted) (11) was inserted in both orientations into the plasmid pSV2ABK3362 in place of the corresponding BKV region (nt 3383 to 3459; Fig. 1). The plasmids pBKSMO-R and pBKSMO-I were capable of supporting SV40 T-antigen-stimulated replication in COS cells and BKV T-antigen-supported replication in HeLa and CV1 cells (Fig. 5). Densitometric scanning revealed that in COS cells the BKV flanking sequences stimulated replication from the SV40 minimal *ori* 16- to 30-fold compared with replication of the minimal *ori* alone (pORICAT). Under these conditions a small effect of the orientation of the SV40 minimal *ori* was observed, with pBKSMO-R replicating approximately twice as efficiently as pBKSMO-I. In contrast, both orientations of the SV40 minimal *ori* replicated to equivalent levels in HeLa and CV1 cells, with the BKV T antigen.

BKV large T antigen autoregulates the BKV early pro-

FIG. 4. The BKV minimal origin of replication can replicate in HeLa and CV1 cells but not in COS cells. (A) The 76-bp BKV minimal origin of replication (nt 3385 to 3456) (■) and the origin boundaries defined by unidirectional deletion mutants (▨), with ΔB mutants defining the late boundary and ΔH mutants defining the early boundary, are shown above the schematic of the BKV noncoding regulatory region. Extra sequences required for replication in COS cells with the SV40 T antigen (□) are indicated. PAL, Palindrome; IR, inverted repeat; AT, 20-bp AT block; I, II, and III, T-antigen-binding sites. The Sp1 consensus sequence (□), NF1-binding site (---), and pentanucleotides to which T antigen binds (■) are shown. (B) Plasmid pSV2ABK3362 contains the intact BKV origin of DNA replication within the CAT vector, while plasmid pBKmin contains the 76-bp BKV minimum origin (nt 3385 to 3456) in the same vector. These two plasmids were transfected in parallel into HeLa, CV1, and COS cells. Low-molecular-weight DNA was extracted at 48 h and digested with *DpnI* (d) and *MboI* (m). The DNA fragments containing the BKV origin regions are shown. The intact BKV origin replicated in all three cell types, while the BKV minimal origin replicated in HeLa and CV1 cells, using the BKV T antigen, but did not replicate in COS cells, using the SV40 T antigen. Lanes contained DNA from pSV2ABK3362 (BK) or from pBKmin (min).

motor. Studies of SV40 have demonstrated a T-antigen-mediated shift in the 5' termini of the early mRNAs concomitant with the onset of replication in this virus (16, 21, 26, 54). This change in mRNA start sites is associated with early-gene autoregulation. The mRNA start sites directed by the BKV early promoter have been determined under replicating conditions in COS cells by using the SV40 T antigen (data not shown) and in CV1 cells by using the BKV T antigen (15). The mRNA 5' termini were found to be identical under both conditions and correspond in location to the downstream and upstream early mRNA start sites of SV40 (Fig.

1). The proportional use of the downstream and upstream start sites in BKV, unlike that in SV40, remains constant before and after replication (15). Gene expression from the BKV early regulatory region was therefore studied under nonreplicating conditions in CV1 cells to determine whether the BKV T antigen regulated its own promoter. The results from pSV2ABK3362 revealed that expression from the BKV early promoter decreased over 12-fold in the presence of BKV T antigen (Table 1). While promoter-enhancer sequences located to the late side of the AT block were not required for this effect (ΔB series, Table 1), the repression was partially relieved in a BKV mutant lacking T-antigen-binding site I (ΔH3377, Table 1). These data demonstrate that BKV T antigen represses early-gene expression and suggest a role for T-antigen-binding site I in the mechanism of repression.

DISCUSSION

We have used deletion mutants of the BKV noncoding regulatory region to define the BKV origin of replication in several cell types, using either the BKV or SV40 T antigen. Monkey kidney cell lines, known to be semipermissive for BKV (19, 37, 43), and human cell lines were tested for their ability to support replication of these mutants. Our results indicate that the sequences required for replication vary with the cell type and the T antigen. Overall, the BKV minimal origin of replication includes an inverted repeat, T-antigen-binding site II, and a 20-bp AT block (nt 3385 to 3456).

The sequence requirements for the BKV origin show similarities to and differences from those of related papovaviruses.

FIG. 5. BKV flanking sequences activated the SV40 minimum origin of replication in both orientations. Plasmids pBKSMO-R and pBKSMO-I, containing the 81-bp SV40 minimal origin of replication and BKV *ori*-flanking sequences, were transfected into COS, CV1, and HeLa cells. Low-molecular-weight DNA was extracted at 48 h and digested with *MboI* (m) and *DpnI* (d) to test for replication. The DNA digest fragments containing the SV40 origins are shown. BKV *ori*-flanking sequences activated the SV40 minimal origin in both orientations.

TABLE 1. Autoregulation of the BKV early regulatory region by BKV T antigen

Plasmid	Coplasmid ^a	Activity	Decrease (fold) ^b
pSV2ABK3362	pRSV-BKT pBR322	8 100	13
ΔB3675	pRSV-BKT pBR322	6 84	14
ΔB3645	pRSV-BKT pBR322	5 57	11
ΔB3634	pRSV-BKT pBR322	2 37	19
ΔB3566	pRSV-BKT pBR322	1 23	23
ΔB3516	pRSV-BKT pBR322	<1 4	13
ΔH3377	pRSV-BKT pBR322	25 82	3
ΔH3408	pRSV-BKT pBR322	25 20	1
ΔH3427	pRSV-BKT pBR322	19 12	<1

^a 7 μg of the plasmid and 3 μg of the coplasmid were cotransfected into the cells in these experiments.

^b Ratio of CAT activity in the absence of BKV T antigen to CAT activity in the presence of BKV T antigen.

The origin region of the *Papovaviridae* is highly conserved and contains (early-to-late direction) a high-affinity T-antigen-binding site, an inverted repeat, a GC-rich inverted repeat containing T-antigen-binding site II, and an AT-rich tract (52, 66). The orientation of the T-antigen-binding pentanucleotides within the origins is very similar (52) among these viruses and is identical in SV40 and BKV (57). Unidirectional deletion data for BKV, SV40, and Py locate the late boundaries for their replication origins at the late edge of the AT tract (12, 20, 40, 41, 45, 65; this study) and the early boundaries towards the late edge of the high-affinity T-antigen-binding site (7, 32, 34, 45, 48, 62; this study). For SV40, the early origin border is located at the junction of T-antigen-binding site I and the inverted repeat, with deletions on the early side of the inverted repeat severely decreasing replication (32, 40, 48, 62). In BKV, at least 7 bp of the corresponding inverted repeat can be deleted without affecting replication. It is interesting that the BKV inverted repeat contains 6 extra bp at this early boundary compared with SV40 (57), while the following 10 bp are highly conserved between the two viruses (8). This conserved region has been implicated in the binding of a monkey cell factor which may play a role in SV40 replication (66) and also contains the transition point between leading- and lagging-strand DNA synthesis in SV40 (27).

Replication of internally deleted BKV origin mutants shows striking differences from that of SV40. While SV40 is exquisitely sensitive to sequence and spacing changes within T-antigen-binding site II (6, 8–10, 17, 32, 40), BKV appears to be much less so. In this respect, BKV more closely resembles Py, which will also tolerate mutations within the origin of replication (41, 45, 67). These differences suggest

that the proteins involved in the activation of origin-dependent replication interact differently among the *Papovaviridae*. In addition, replication-competent BKV mutants containing deletions in T-antigen-binding site II indicate that a cruciform structure in this region is not required for BKV replication.

A majority of the SV40 and Py AT tracts is required for replication of these viruses (11, 12, 17, 20, 29, 41, 45, 62, 65). Similarly, a requirement for the BKV 20-bp AT tract for replication is indicated by the data presented here. The early portion of the AT block deleted in ΔB3423-H3442 is known to serve a spacer function in the analogous SV40 sequence (9), with deletions resulting in decreased replication (8). In addition, a region including the SV40 AT block has been implicated in the binding of a replication initiation factor (71). It is interesting that transcriptional regulatory elements are known to augment viral replication (11, 13, 38, 40, 52), often in a distance-dependent manner (31, 40, 68). The BKV 68-bp repeat contains an Sp1 consensus sequence (33) and a nuclear factor 1 (NF1)-binding site (51). The SV40 21-bp repeats, which contain Sp1-binding sites, are known to augment replication in that virus (1, 11, 40), and NF1 is absolutely required for stimulation of replication from the adenovirus origin of replication (49, 50, 53, 56, 68). These BKV transcriptional elements are apparently unable to compensate for the absence of the AT block in initiating BKV replication, even when moved into proximity with BKV T-antigen-binding site II.

Comparison of results obtained with unidirectional and internal deletion mutants of BKV reveals that the sequences required for BKV replication can vary, depending on the presence or absence of flanking sequences. Although sequences between nt 3388 and 3396 are required for replication in HeLa cells in the absence of T-antigen-binding site I, in the presence of this site a mutant lacking these sequences is still replication competent. These data imply that certain domains of the BKV origin are functionally redundant, similar to the SV40 origin (11, 28).

The boundaries of the BKV minimal origin of replication resemble those determined for SV40 (8, 9, 11, 20, 40, 60) and Py (7, 34, 45). While the SV40 minimal origin is capable of supporting a low level of replication (11), the corresponding region of Py is replication deficient (5). In contrast, the BKV minimal origin replicates to a level equivalent to that of a wild-type BKV origin. The BKV origin-flanking sequences do not augment replication in this virus as the corresponding sequences do in SV40 and Py (1, 7, 11, 16, 32, 34, 38, 40, 41, 62). In this respect, the BKV origin constitutes a simpler system than that of either SV40 or Py and may serve as a better model for in vitro studies of replication.

Because of the juxtaposition of replication and transcriptional control elements in the *Papovaviridae*, it has been speculated that early-gene transcription is required for replication of these viruses. The results presented here, in conjunction with those from an earlier study (15), indicate that transcription is not required for replication from the BKV origin. BKV deletion mutants lacking most or all of the 68-bp repeat region possess only background levels of transcriptional activity (15), yet are capable of replication.

BKV origin sequence requirements are different in COS cells. The variations in origin sequence requirements among different cell types and T antigens are quite striking. SV40 T-antigen-supported replication in COS cells consistently required more sequences than did BKV T-antigen-supported replication in CV1, HeLa, and L603 cells. Interestingly, sequence requirements in the BKV early inverted repeat,

bidirectional origin, and AT block closely resemble those of SV40 when both viral origins are assayed in COS cells (10, 29, 32, 40). *trans*-Acting host cell factors are required for replication (55, 66, 70, 71) and are thought to contribute to species specificity by determining the ability of papovaviruses to replicate in a given cell type (2, 18, 39, 46). The interaction of T antigen with host cell factors is thought to be important for stimulation of viral replication (24, 60, 62), and the abilities of the BKV and SV40 T antigens to support replication of the other virus vary, depending on the host cell (37, 39). Thus, the variations in sequence requirements most likely reflect differences between the SV40 and BKV T antigens or the availability of host cell replication factors or both.

BKV *ori*-flanking sequences activate the SV40 minimal origin. BKV origin-flanking sequences were found to increase replication from the SV40 minimal origin of replication 15- to 30-fold, in an essentially orientation-independent manner. In contrast, when examined with its own flanking sequences, replication from the SV40 minimal origin has strict orientation and spacing restrictions, requiring linkage to promoter-enhancer elements through the AT block and to T-antigen-binding site I through the early inverted repeat (5, 11, 20, 31, 38, 40, 60). Substitution of SV40 flanking sequences by the corresponding regions of BKV thus renders the SV40 minimal origin more similar to that of Py. The latter origin is capable of efficient replication in both normal and reversed orientations (52). It is interesting that, when driven by SV40 T antigen in COS cells, the SV40 minimal origin in normal orientation within BKV flanking sequences replicates twice as well as the inverted orientation.

Autoregulation of the BKV early promoter by BKV T antigen. Similar to that of SV40 and Py, the activity of the BKV early promoter-enhancer is decreased in the presence of BKV T antigen. In SV40, this autoregulation is mediated by the binding of T antigen to sites I and II and is accompanied by a shift in the preponderance of early mRNA start sites from 21 to 26 nt downstream of the TATA box before viral replication to 10 to 15 nt upstream of the TATA box after replication (17, 21, 26, 40, 54). A similar shift in early mRNA start sites before and after replication has been observed with Py (7). Decreased expression of SV40 early genes late in infection is thought to result, in part, from the blocking of downstream mRNA initiation (26, 47) and inefficient translation of upstream mRNAs, which contain an extra AUG in their leader sequences (36). Similar to that of SV40, BKV T-antigen-binding site I appears to be involved in repression, and the mRNA start sites directed by the BKV early promoter-enhancer are located in positions analogous to those of SV40 (15). The use of downstream and upstream BKV mRNA start sites, however, appears to remain constant before and after replication (15; data not published). Early-gene regulation in BKV may therefore involve other mechanisms, such as the displacement of enhancer-binding transcription factors by T antigen, as has been postulated for SV40 (44).

Summary. In conclusion, we have mapped the *cis*-acting sequence elements of the BKV origin of replication under a variety of conditions. Our results show that the specific sequences required for replication vary with the cell type and the T antigen, probably reflective of differences in host cell factors and T antigen function. Requirements for replication from the BKV origin of replication show similarities to and differences from those of SV40 and Py. The most striking differences are the ability of a BKV minimal origin to support replication at wild-type levels in CV1 and HeLa cells

and the inability of this origin to replicate in COS cells. These data suggest that despite their high overall degree of similarity, these viruses may employ different mechanisms of replication activation.

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