Functional Similarity Between the Early Antigens of Simian Virus 40 and Human Papovavirus BK

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The functional properties of the early antigens of simian virus 40 (SV40) and human papovavirus BK (BKV) were investigated. Infection of African green monkey kidney cells with BKV permitted the bidirectional replication of an early temperature-sensitive mutant (tsA) at a nonpermissive temperature. Conceivably, an early gene product (T-antigen) of BKV can substitute functionally for the defective SV40 T-antigen. On the other hand, SV40 DNA replication remained undetectable in human embryonic kidney cells preinfected with BKV, suggesting that BKV early antigens alone are not sufficient to provide for the replication of SV40. Preinfection of African green monkey kidney cells with BKV restored the normal pattern of late lytic SV40 transcription, suppressing the overproduction of early DNA by an SV40 tsA mutant at the nonpermissive temperature. Furthermore, preinfection of African green monkey kidney cells with BKV supported the growth of adenovirus type 2, providing a "helper function" similar to that provided by SV40 for the growth of human adenovirus in monkey kidney cells.

The classification of papovavirus BK (BKV) as a human virus is based on several criteria. Isolations of this agent have been made repeatedly from the urine of renal transplant recipients and from the urine and a tumor of patients with the Wiskott-Aldrich syndrome, a rare genetic disorder manifested by deficiencies in both cellular and humoral immunity (9, 16, 28). BKV neutralizing antibodies are found in approximately 70% of adults, suggesting that most individuals have been infected by this agent. In contrast, less than 3% of persons have simian virus 40 (SV40) neutralizing antibodies (8, 24). In spite of these distinctions, BKV appears to be very closely related to the simian papovavirus SV40. Both viruses consist of a protein capsid with a nucleoprotein core. The supercoiled double-stranded DNAs of BKV and SV40 are about 3.4 × 10^6 and 3.6 × 10^6 daltons, respectively, and exhibit extensive nucleotide sequence homology, especially in the late gene region (12, 14, 20). Less stringent techniques, however, which allow for greater base mismatch, indicate that there is extensive homology in the early regions (19). BKV and SV40 show significant immunological cross-reactivity between their T-antigens. Several studies have shown that SV40 T-antigen is a DNA binding which is essential for the initiation of viral DNA replication (3, 29). In addition, T-antigen or a portion of this molecule appears to play an important role in the control of early and late viral transcription (15, 23) and in providing the "helper function" for the growth of adenovirus in monkey kidney cells (22).

Physical characterizations of the SV40 T-antigen in lytically infected cells have shown that there are two major polypeptides of approximately 90,000 and 17,000 daltons (21, 30). A comparison by tryptic peptide analysis of the T-antigens of these two viruses indicated that there is an extensive amino acid homology in the large T-antigen as well as in the smaller T-antigen (4, 25). A recent study (18) demonstrated that coinfection of monkey kidney cells with BKV and an early mutant of SV40 (tsa58) allows the latter to replicate to a significant titer at the nonpermissive temperature. One interpretation of these results is that the early BKV antigen can substitute for the SV40 tsA58 T-antigen which is known to be defective at 41°C. The present study was undertaken both to define the level of this complementation and to determine whether BKV infection could also substitute for the other early SV40 functions, including regulation of the early versus late SV40 transcription ratio and establishment of the helper function for growth of adenovirus in monkey cells.

MATERIALS AND METHODS

Cell cultures and virus strains. Primary cultures of African green monkey kidney cells (AGMK) as well as a continuous monkey kidney cell line (BSC-1) were used in these experiments. Primary cultures of human embryonic kidney (HEK) cells were purchased from

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Microbiological Associates and were used after two to four subcultures. The virus strains used in this study included the original isolate (prototype strain of human papovavirus BKV), which was grown and titrated in secondary HEK cells. SV40 strain 776 and a temperature-sensitive mutant, tsA58, kindly supplied by Peter Tegtmeier, were grown and assayed in AGMK cells. The adenovirus type 2 (Ad2) used in experiments to study adenovirus helper function was kindly provided by Heiner Westphal and was titrated in HEK cells.

Analysis of viral DNAs in cells coinfected with BKV and SV40. Because the BKV lytic cycle is approximately twice as long as that of SV40 (17), effective complementation was obtained by preinfecting cells with BKV before infection with SV40 mutant tsA58. AGMK cells or HEK cells were infected with BKV at a multiplicity of approximately 1 PFU/cell for 24 h, followed by infection with several different multiplicities (1, 3, and 10 PFU/cell) of the SV40 mutant. The coinfected cells were incubated at 40°C, labeled with 32P (50 Ci/ml) for a period of 24 h, and subsequently lysed with 0.6% sodium dodecyl sulfate-0.02 M EDTA. To each lysate was added a sample of form I SV40 and BKV DNAs labeled with [3H]thymidine as internal standards. Form I viral DNA was prepared by the method of Hirt (10). The purified 32P- and 3H-labeled viral DNAs were digested with the HindII + III enzyme which cleaves SV40 DNA into 11 fragments and BKV DNA into 4 fragments. This digest was analyzed by electrophoresis on 3.5% polyacrylamide gels (ratio of acrylamide to N,N-methylenebisacrylamide, 20:1). BKV- and SV40-specific DNA fragments were located by autoradiography and excised from the gels. The relative yield of BKV and SV40 DNAs in each infection was determined from the ratio of 32P to 3H radioactivity.

Assay of SV40 tsA58 transcription. Three flasks of AGMK cells, one of which had been preinfected with BKV (10 PFU/cell) for 24 h, were inoculated with SV40 tsA58 (5 to 10 PFU/cell) for 46 h at 32°C. One control culture was maintained at 32°C, and another control culture and the BKV-preinfected cells were shifted to 41°C for 2 h. Each culture was then labeled at the growth temperature (32 or 41°C) for 45 min with 100 μCi of [5,6-3H]uridine (50 Ci/mmoll; Amersham/Searle, Arlington Heights, Ill.) per ml. Cytoplasmic RNA was isolated, purified, and analyzed by hybridization to the separated strands of restriction fragments of SV40 DNA (15) which had been transferred from agarose gels to nitrocellulose filters by the Southern transfer procedure (26). The amount of labeled RNA homologous to the separated strands of each SV40 fragment was determined either by densitometric analysis of fluorographs made from the blots or by cutting out the bands from the nitrocellulose paper and counting them in a liquid scintillation counter.

Analysis of adenovirus proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ad2 helper function provided by papovavirus BKV in AGMK cells was determined by the enhanced synthesis of several adenovirus-specific proteins, such as hexon, 72K, and protein V. These peptides are produced in low quantities in monkey kidney cells infected with low Ad2 multiplicities when compared with their production in human cells under similar conditions (6). The procedure described earlier was followed in this study. In short, BSC-1 cells were preinfected with BKV (multiplicity of infection, 10) for 2 days before inoculation with Ad2 at multiplicities of 1.0 and 2.5 PFU/cell. The infected cultures were pulse-labeled with 20 μCi of [35S]methionine (865 Ci/mmol; Amersham/Searle) per ml in methionine-free medium between 30 and 32 h after Ad2 infection and then lysed in buffer containing 0.125 M Tris, pH 7.5, 0.28 M 2-mercaptoethanol, and 2% sodium dodecyl sulfate. Analysis of labeled peptides was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20% acrylamide and 0.1% N,N-methylene bisacrylamide in buffer containing 0.65 M Tris, 0.59 M boric acid, pH 8.7, and 0.2% sodium dodecyl sulfate). Labeled peptides from Ad2 virus were run in parallel as size markers.

RESULTS

Replication of tsA58 DNA at nonpermissive temperatures in coinfected with BKV. Based on plaquing experiments, BKV replicates approximately 5 to 10% as efficiently in BSC-1 cells as it does in HEK cells (1; Lai, unpublished data). It was therefore possible to demonstrate the presence of newly replicated BKV DNA in the Hirt supernatant fraction of BKV-infected BSC-1 cells. The restriction enzyme cleavage map of prototype BKV DNA with HindII + III is shown in Fig. 1. The HindII + III fragments of BKV are distinguishable in size from HindII + III fragments of SV40 DNA. Cleavage with this enzyme was used to separately analyze and quantitate both viral genomes in the infected cells. Figure 2A shows that when BSC-1 cells were infected with the early SV40 mutant tsA58 at 41°C, there was no detectable replication of SV40 DNA. In contrast, BSC-1 cells preinfected with BKV for 48 h and subsequently infected

![Fig. 1. Restriction enzyme cleavage maps of SV40 and BKV DNA. The physical map of SV40 DNA shows the cleavage sites of HindII + III (inner circle) and of BamHI + HpaII (outer circle). Shown for comparison is the HindII + III (HindII) cleavage map of BKV (11). The unique EcoRI site in both genomes serves as the reference point (0.0) for both viruses.](http://jvi.asm.org/Downloadedfrom)}
with SV40 mutant tsA58 at 41°C allowed the replication of SV40 DNA. Furthermore, an increase in the multiplicity of infecting tsA58 resulted in both an increase in the amount of replicating tsA58 DNA and a concomitant decrease in the replicating BKV DNA. There appears to be a "competition" for replication between the two papovaviruses. The nature of the competition is discussed further below. In a separate experiment, we employed a pulse-labeling procedure (5) to determine the origin and direction of SV40 DNA replication complemented by BKV infection (Table 1). Analysis of the pulse-label distribution in the mutant tsA58 DNA indicated that HindIII + III fragment G contained the highest amount of label and that the flanking fragments showed decreased amounts similar to the pulse-label distribution of the wild-type virus described by Danna and Nathans (5) and Lai (Methods Enzymol., in press). These results show that the normal origin of SV40 is functional in the BKV-complemented replication. These findings indicate that BKV infection of BSC-1 cells complements the early defect of the SV40 tsA mutant, most likely by providing an early BKV function.

To determine whether BKV preinfection is sufficient to support the replication of tsA58 DNA at 41°C in other cells, we analyzed the replication of the SV40 mutant in BKV-infected HEK cell cultures, which are normally nonpermissive for the growth of SV40 (Fig. 2B). In this set of experiments, HEK cells were similarly preinfected with BKV for 48 h at 37°C and superinfected with increasing concentrations of tsA58 at 41°C. After 24 h at the nonpermissive temperature, cells were labeled with 32P (see above) and examined as described for the replication of BKV and SV40 DNA. At none of the tsA58 input multiplicities of infection did we detect any SV40 DNA replication, although BKV replication was readily observed. Nevertheless, as the ratio of SV40 to BKV input virus increased, we detected a decrease in the amount of replicating BKV DNA. This is similar to the decrease in BKV replication in monkey kidney cells with increasing tsA58 multiplicities described above, suggesting a possible competition for replication function which was not accompanied by a detectable enhancement of tsA58 replication.

Table 1. Pulse-label distribution of viral DNA in cells coinfect ed with BKV and SV40 tsA58

<table>
<thead>
<tr>
<th>Virus</th>
<th>HindIII + III fragment</th>
<th>Relative amt of label</th>
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<tbody>
<tr>
<td>BKV</td>
<td>A</td>
<td>1.33</td>
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<tr>
<td></td>
<td>B</td>
<td>2.65</td>
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<td></td>
<td>C</td>
<td>1.0</td>
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<td></td>
<td>D</td>
<td>1.0</td>
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<tr>
<td>SV40</td>
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<td>0.86</td>
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<tr>
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<td>B</td>
<td>2.38</td>
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<td></td>
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<td></td>
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<td></td>
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*Infection of AGMK cells with BKV and SV40 mutant tsA58 at 40°C was carried out as described in the text. The coinfected cells were pulse-labeled with [3H]thyminde for 7 min, and the distribution of radioactivity was analyzed by the method of Danna and Nathans (26). The relative amount of pulse-label in each fragment was determined by using uniformly 32P-labeled DNA fragments as reference standards.
of viral DNA replication, a shift of SV40 tsA-infected monkey kidney cells from the permissive (32°C) to the nonpermissive temperature (41°C) results in the overproduction of early SV40 RNA (15, 23). From these observations it was concluded that an autoregulation of the early SV40 protein(s) is modulated at the level of transcription. We therefore investigated whether BKV infection of monkey kidney cells could regulate the transcription of a coinfecting SV40 tsA mutant. In a control experiment (Fig. 3), the ratio of late to early tsA58 RNA in cells infected at the permissive temperature (32°C) for 48 h and labeled for 1 h at this same temperature was determined by annealing labeled cytoplasmic RNA to separated strands of SV40 DNA restriction fragments immobilized on nitrocellulose filters (see above). The relative quantities of early and late SV40 RNA were determined by a densitometric analysis of fluorographs made from the separated strand blots. A total of 80% of the pulse-labeled SV40-specific RNA annealed to the late DNA template (Fig. 3b). In parallel cultures shifted from 32 to 41°C at from 46 to 48 h postinfection and labeled for 1 h with [3H]thymidine, late SV40 RNA represented only 30% of the virus-specific fraction (Fig. 3c). Thus, the increase in the ratio of cytoplasmic early to late SV40 RNA was approximately 3.5-fold in cells shifted to the nonpermissive temperatures. To investigate the effect of BKV preinfection on the transcription of SV40 tsA58 at the nonpermissive temperature, AGMK cells were preinfected with BKV for 24 h at 37°C, then infected with tsA58 at 32°C for 48 h, and finally shifted to 41°C. After 2 h of incubation at this nonpermissive temperature, cells were pulse-labeled for 45 min with [3H]thymidine and the cytoplasmic RNA was extracted. The ratio of early to late RNA determined from hybridization of this cytoplasmic RNA preparation to the separated strands of SV40 DNA (15:85; Fig. 3d) was similar to that found in cells infected with tsA58 at 32°C (20:80) or in cells infected with wild-type SV40 at either temperature (5:95). Under the stringent hybridization conditions employed in this experiment, BKV RNA does not anneal to a significant extent with the SV40 filters. Thus, we conclude that BKV preinfection suppresses the overproduction of early tsA58 RNA at the nonpermissive temperature and effectively restores the autoregulatory effect of T-antigen.

Adenovirus helper effect of BKV. Because SV40 T-antigen is immunologically cross-reactive with BKV T-antigen, it was of interest to determine whether BKV could also provide adenovirus helper function. To evaluate this property, we assayed the synthesis of late adenovirus proteins (hexon, 100K, and 72K, as well as protein V) after infection with adenovirus. This assay takes advantage of the fact that these late Ad2 proteins are synthesized at elevated levels in monkey cells infected with Ad2 at a low multiplicity of infection in the presence of a helper papovavirus. This method has proven to be sensitive for assaying helper function (6).

Figure 4 presents data which indicate that BKV, like SV40, enhances the synthesis of late adenoviral proteins in AGMK cells. Cells were preinfected for 2 days with BKV, then inoculated with Ad2, and finally labeled with [35S]methionine at from 30 to 32 h postinfection with Ad2. The cell lysates were prepared as described above and subjected to electrophoresis in polyacrylamide gels. When compared with the marker Ad2 virion proteins and the uninfected cellular extract, Ad2-specific proteins in cells infected with 1.0 PFU of adenovirus per cell

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**Fig. 3.** Determination of ratios of early versus late cytoplasmic SV40 RNA in singly infected or coinfectected AGMK cells. Strands of the two SV40 DNA fragments (fragments A and B; Fig. 1) generated by cleavage with restriction enzymes HpaII and BamHI were separated by agarose gel electrophoresis (15) and transferred to nitrocellulose filters by the Southern transfer procedure (26). Positions of the four DNA strands are shown in a; subscripts E and L denote the early and late strands of fragments A and B. Hybridization experiments with nitrocellulose filters containing the separated strands of these fragments were performed as described in the text with various [3H]RNA preparations. After annealing, the nitrocellulose strips were washed (15), incubated with RNase (25 μg/ml) for 1 h, dried, treated with 2,5-diphenyloxazole-toluene, and exposed to Kodak SB5 film at −70°C. Sample fluorographic patterns as well as densitometric tracings of these films are shown in b through d. (b) Cytoplasmic RNA from cells infected with tsA58 at 48 h postinfection at 32°C; (c) cytoplasmic RNA from cells infected with tsA58 at 32°C, shifted at 46 h postinfection to 41°C, and pulse-labeled at 41°C at 48 to 49 h postinfection; (d) cytoplasmic RNA from cells preinfected for 24 h with BKV and then infected with tsA58 and labeled following the protocol in (c).

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A diagram is included to illustrate the experimental setup and results, with labeled strands and subscripts indicating early (E) and late (L) strands for SV40. The figure details the hybridization procedures and the resulting patterns of transcriptional activity in response to BKV preinfection.
were essentially undetectable, although they became detectable when the multiplicity of infection increased to 2.5 PFU/cell. If the cells were also infected with BKV (at 10 PFU/cell at 48 h before adenovirus infection), the synthesis of several Ad2 proteins, including hexon (120K), 100K, 72K, and protein V (48K), was present at enhanced levels. The enhancement could be detected at both multiplicities of Ad2 although it appeared more significant at the higher Ad2 multiplicity of infection. We conclude from this analysis that BKV infection in monkey kidney cells enhances the synthesis of late Ad2-specific proteins, presumably by providing a helper func-
tion similar to that which has been demonstrated for SV40 (22).

DISCUSSION

In this study, we have investigated the biological properties of BKV T-antigen in primate cells; in particular, we have focused on the functional similarity between the BKV and SV40 T-antigens. Mason and Takemoto have found that preinfection of monkey kidney cells with BKV leads to an increase in the plaques formed by SV40 tsA mutants at the nonpermissive temperature (18). Here, we show that BKV preinfection enhances SV40 tsA DNA replication at 41°C, a defect which has been directly related to a deficiency in the function of T-antigen at this temperature (2, 3, 29-31). In addition, there is evidence that an increase in SV40 tsA DNA replication is accompanied by a proportional decrease in BKV DNA synthesis. Although the basis for this apparent competition is not clear, a reasonable explanation would be that SV40 preferentially sequesters a molecular species required for the replication of both papovaviral DNAs. One candidate for such a molecule is the BKV T-antigen itself.

In coinfected HEK cells, an increase in the multiplicity of infection of SV40 tsA58 also resulted in a decrease in the synthesis of BKV DNA. In contrast to the situation in coinfected monkey kidney cells, however, this decrease in BKV DNA replication was not accompanied by a coincident detectable rise in SV40 DNA replication. Although a number of explanations could be entertained, it seems possible that the SV40 genome also competes for BKV T-antigen in coinfected HEK cells. If our assumption that BKV and SV40 DNA compete for a similar molecular species active in replication is correct, then the result in coinfected HEK cells would suggest that this competition can occur whether or not effective complementation takes place. The absence of detectable SV40 replication in these cells may indicate the deficiency of HEK cell-specific molecules which can functionally interact with the SV40 DNA-T-antigen complex.

We have also demonstrated in this study that the overproduction of early SV40 RNA characteristic of SV40 tsA mutants grown at the restrictive temperature can be suppressed if cells are preinfectected with BKV. It is believed that late in the SV40 lytic cycle (after the onset of viral DNA replication) T-antigen is the primary regulator of the ratio of early versus late transcriptional initiation events. Thus, it seems likely that BKV T-antigen (and not a secondary effect related to the resumption of tsA DNA replication) is also responsible for the restoration of a normal SV40 transcriptional pattern.

Finally, we have demonstrated at the level of protein synthesis that BKV preinfection can enable Ad2 to overcome the normal host range restriction in AGMK cells; i.e., BKV provides the helper function. SV40 helper function has been demonstrated for Ad2-SV40 hybrid genomes containing only those SV40 DNA sequences encoding the carboxy-terminal end of T-antigen (7, 32). The proteins made by these cells have U-antigen activity, which is thought to be a subset of T-antigen. Because it was shown that BKV induces a protein which cross-reacts immunologically with U-antigen in infected cells (27), it is perhaps not surprising that this virus also provides helper function for Ad2. A similar observation has been made recently in studies with BKV-transformed AGMK cells which were infected by adenovirus (1).

Although the early antigens of BKV and SV40 showed immunological cross-reactivity, it seemed at the time these studies were initiated that the DNA sequence which encoded these proteins had diverged considerably (12, 14, 20). Whereas most of the close sequence homology exists in the late regions of these SV40-BKV genomes, less stringent hybridization techniques have shown that there is significant DNA sequence similarity in the early regions of BKV and SV40 genomes, filigreed with divergent sequences (19). From an examination of portions of this sequence for the early BKV gene region (5a), it now appears that the considerable divergence in nucleotide sequence has frequently occurred in inconsequential third base positions or in nucleotide substitutions which have a minimal effect on the protein structure. Thus, we speculate that in spite of nucleotide drift there has been an evolutionary conservation of a significant portion of the protein sequence for T-antigen. It is presumably this conservation which allows for the complementation of BKV and SV40 T-antigens as demonstrated in this study.

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LITERATURE CITED


