JOURNAL OF VIROLOGY, Jan. 1982, p. 319–325
0022-538X/82/010319-07$02.00/0

Vol. 41, No. 1

Transformation of Hamster Kidney Cells by Fragments of BK Virus DNA

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Received 22 June 1981/Accepted 6 October 1981

Hamster kidney cells were transformed, with comparable efficiency, by circular or linear molecules of complete BK virus (BKV) genome and by agarose gel-purified fragments of BKV DNA obtained by single or double digestions with various restriction endonucleases. Only fragments containing the complete early region of BKV DNA displayed transforming activity. Analysis by blot hybridization of the arrangement of viral DNA sequences in a cloned cell line transformed by a 3.8-kilobase fragment, obtained after sequential digestion of BKV DNA with *Hha*I and *Bam*HI, showed the presence of seven viral integrations into the cellular DNA. Apparently all of the integrated viral molecules contained the entire early region of BKV DNA. Large T antigen, small t antigen, and the 56,000-dalton nonviral Tau antigen were detected in transformed cells by immunoprecipitation. The pattern of integration of viral sequences in transformed cells was constant over many generations. Likewise, large T antigen was always detected in transformed cells at various passage levels. These results may suggest that all of the sequences of the early region coding for large T antigen are required for transformation by BKV. Alternatively, subgenomic segments of the BKV DNA early region may be unable to transform because the appropriate polyadenylation site, necessary to obtain a complete functional transcriptional unit, is removed by the restriction enzyme cleavage.

Transformation by papovaviruses requires the continuous presence and expression of the early region of the viral genome in transformed cells (31). Transformation with fragments of the viral genome, however, has shown that different portions of the early region are needed to induce and maintain transformation by polyoma virus or simian virus 40. Indeed, transformation of rat cells was obtained with fragments of polyoma virus DNA extending from 65.4 to 100 map units (22) or from 45.0 to 1.4 map units (11). In addition, a fragment of polyoma virus DNA spanning 58.0 to 100 map units produced tumors in newborn hamsters (7). Treisman et al. (32) recently demonstrated that a modified polyoma virus genome, encoding only the middle T antigen (Ag), is able to transform cultured rat cells. These results indicate that expression of the middle T Ag is sufficient for establishing and maintaining transformation and oncogenesis by polyoma virus. On the contrary, transformation by simian virus 40 was obtained only with fragments of the viral DNA spanning 14.9 to 72.0 map units (1) or 14.9 to 82.7 map units (10) which contain the complete early region. Studies with simian virus 40 *tsA* mutants (3, 4, 15, 17, 23, 30), mapping in the sequences of the early region coding for the unique carboxy terminal portion of large T Ag, and with 54/59 deletion mutants (26), mapping in the coding sequences for small t Ag, indicated that, although small t Ag may be required for the expression of some properties of the transformed phenotype (2, 9, 18, 28), the induction and maintenance of the transformed state is essentially dependent on the presence and continuous expression of large T Ag.

BK virus (BKV), a human papovavirus, is oncogenic and transforms cells of various animal species cultured in vitro (see references 12 and 24 for reviews). The expression of small t Ag seems to be unnecessary for BKV transformation and oncogenicity. In fact, MM virus (29), a transforming (19) and oncogenic (8) strain of BKV, does not encode small t Ag (25) because it carries a 262-base pair deletion in the early region (33) which removes sequences coding for the carboxy terminus of small t Ag, including the donor site for the small t Ag splice junction. The deletion in the MM virus genome is located within the intervening sequences for large T Ag

mRNA (33), so that MM virus codes for a normal large T Ag. BKV *tsA* mutants are not available to test the role of BKV large T Ag in the induction and maintenance of transformation. On the other hand, transformation with fragments of BKV DNA was not attempted to determine directly which portion of the early region is necessary to induce transformation. We report here that in vitro transformation can be obtained with fragments of BKV DNA containing a complete early region.

The preparation of hamster kidney (HK) cells, the techniques of DNA transfection, indirect immunofluorescence, gel electrophoresis, production of ^{32}P-labeled BKV DNA probes by nick translation and blot hybridization have been described (5, 6). In blot hybridization experiments, molecular weights of cellular DNA restriction fragments were measured, using *Eco*RI-generated fragments of SPP1 phage DNA (20) or BKV DNA fragments produced by digestion with various endonucleases as internal standards. Band intensity was determined by screening autoradiographs with a Kipp and Zonen densitometer. Immunoprecipitation was carried out by the method of Kress et al. (16).

BKV DNA was cleaved with various restriction endonucleases and fragments obtained after a single or a sequential digestion with two enzymes (Fig. 1) were used for transformation. The results of 13 experiments of transformation with BKV DNA and BKV DNA fragments are summarized in Table 1. Transformation was obtained with supercoiled BKV DNA (form I) and with linear molecules of BKV DNA (form III) produced by *Eco*RI or *Bam*HI which cleave BKV DNA once in the late region at 0 and 97.9 map units, respectively. *Eco*RI and *Bam*HI linear molecules have cohesive ends and therefore tend to circularize or to join in linear tandems. These linear molecules treated with the single strand-specific endonuclease S1, which eliminates cohesive ends and therefore inhibits end-to-end joining, were still capable of transforming HK cells. A *Hha*I-*Bam*HI 3.8-kilobase (kb) fragment, extending clockwise from 97.9 to 72.2 map units, induced transformation of HK cells. In addition, transformation was obtained with a *Hha*I-*Eco*RI 3.7-kb fragment (0 to 72.2 map units clockwise) and with a *Pvu*II 3.2-kb fragment (9.9 to 72.3 map units clockwise). The efficiency of transformation of the fragments was comparable to that of circular and linear molecules of the complete BKV genome. HK

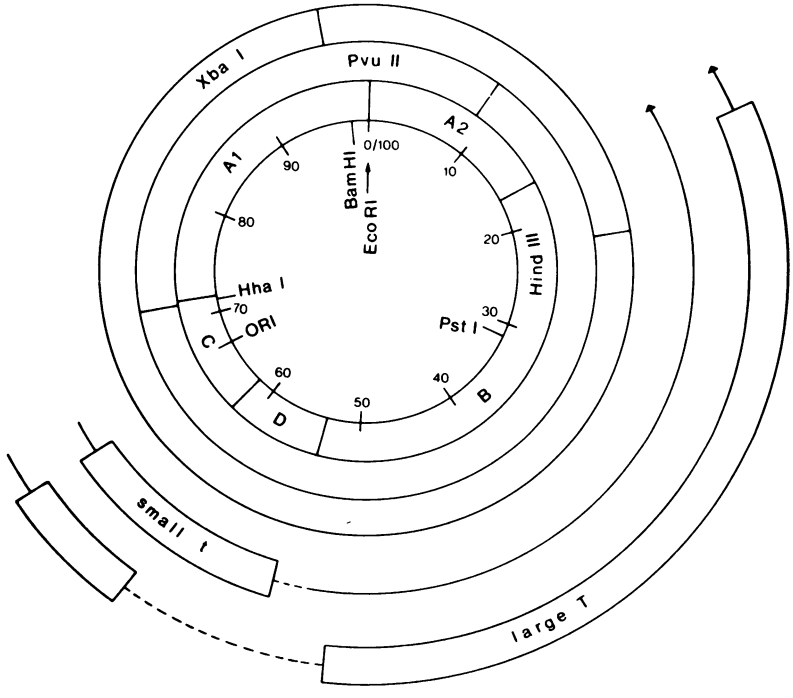

FIG. 1. Physical map of the BKV genome (12, 24) with the cleavage sites of the enzymes used in these experiments. Cleavage sites of enzymes which cut BKV DNA once are marked within circle 1. The letters within circle 2 label the four *Hin*dIII fragments A, B, C, and D. *Hin*dIII fragment A is represented after cleavage with *Eco*RI, generating fragments A1 and A2. Numbers inside circle 1 indicate map units, starting from the *Eco*RI site taken as the zero position.

TABLE 1. Transformation by BKV DNA and BKV DNA fragments

BKV DNA spanning map units (kb)	Digested with:	μg of DNA/dish (genome equivalent)	Colonies/dish	No. of colonies/μg of genome equivalent
0–100 (5.2) form I		1	26, 16, 25, 14	20.2
		2	45, 57, 36, 37	21.8
		2	38, 38, 15, 46	17.1
0–100 (5.2) form III	*Eco*RI	1	20, 16	18.0
		2	24, 38	15.5
	*Eco*RI + S1	1	23, 12, 13	16.0
97.9–97.9 (5.2) form III	*Bam*HI	1	25, 13, 16	18.0
		2	30, 45	18.7
	*Bam*HI + S1	1	18, 15, 13	15.3
97.9–72.2 (3.8)	*Hha*I + *Bam*HI	1	22, 17, 19	19.3
		2	34, 18, 33	14.2
72.2–97.9 (1.4)	*Hha*I + *Bam*HI	1	0, 0, 0	0
		2	0, 0, 0	0
0–72.2 (3.7)	*Hha*I + *Eco*RI	0.5	21, 18	39.0
		1	22, 20	21.0
		2	34, 60, 53, 5	19.0
		2.5	38, 42	16.0
		3	42, 45	14.5
72.2–100 (1.5)	*Hha*I + *Eco*RI	0.5	0, 0	0
		1	0, 0, 0, 0	0
		2	0, 0, 0, 0, 0, 0	0
9.9–72.3 (3.2)	*Pvu*II	1	23, 12, 17	17.3
		2.5	41, 16, 39, 42	14.7
72.3–9.9 (2.0)	*Pvu*II	1	0, 0, 0	0
		2.5	0, 0, 0	0
31.9–72.2 (2.1)	*Hha*I + *Pst*I	1.5	0, 0, 0, 0	0
72.2–31.9 (3.1)	*Hha*I + *Pst*I	1.5	0, 0, 0, 0	0
22.7–96.7 (3.8)	*Xba*I	1	0, 0	0
		2	0, 0	0
		4	0, 0	0
96.7–22.7 (1.4)	*Xba*I	2	0, 0, 0, 0	0
22.7–72.7 (2.6)	*Hha*I + *Xba*I	1	0, 0, 0, 0	0
		2	0, 0, 0, 0	0
		4	0, 0	0

cells transformed by fragments or complete molecules of BKV DNA showed indistinguishable phenotypic properties. They had morphological alterations, grew to high saturation density in medium containing 1% serum and induced tumors in 100% of suckling hamsters inoculated subcutaneously with 1.5×10^6 cells. The *Hha*I-*Bam*HI 1.4-kb fragment (72.2 to 97.9 map units clockwise), the *Hha*I-*Eco*RI 1.5-kb fragment (72.2 to 0 map units clockwise), and the *Pvu*II 2.0-kb fragment (72.3 to 9.9 map units clockwise) did not show transforming activity. All BKV DNA transforming fragments contained the sequences of the entire early region of the BKV genome coding for small t Ag (64.0 to 53.5 map units counterclockwise) and for large T Ag (64.0 to 17.2 map units counterclockwise) (Fig. 1). It was therefore of interest to test whether BKV DNA fragments covering only a fraction of the early region had transforming activity. A *Hha*I-*Pst*I 2.1-kb fragment (31.9 to 72.2 map units clockwise), a *Xba*I 3.8-kb fragment (22.7 to 96.7 map units clockwise) and a *Hha*I-*Xba*I 2.6-kb fragment (22.7 to 72.2 map units clockwise) were all devoid of transforming activity (Table 1). All these nontransforming fragments are lacking the region of BKV DNA, including the 3′ untranslated portion of both large T and small t Ag mRNA's and their polyadenylation sites.

In each experiment, several cell lines were established by isolating colonies transformed by different fragments and by complete circular or linear BKV DNA. In all cell lines BKV T Ag was detected in 95 to 100% of cells by indirect immunofluorescence. One cell line transformed by the *Hha*I-*Bam*HI 3.8-kb fragment was cloned (L578) and studied in detail.

Large T Ag (97,000 daltons [97K]), small t Ag (17K), and nonviral Tau Ag (56K) (27) were detected in the homogenate of L578 cells at passages 18, 48, and 84 by immunoprecipitation with a specific serum to BKV tumor Ags (Fig. 2).

Line 578 DNA (culture passage 16) either uncut or digested with *Hin*cII, *Eco*RI, *Hin*dIII, and *Hha*I did not hybridize to a ^{32}P-labeled probe of BKV DNA *Hha*I-*Bam*HI 1.4-kb fragment, indicating that the *Hha*I-*Bam*HI 3.8-kb

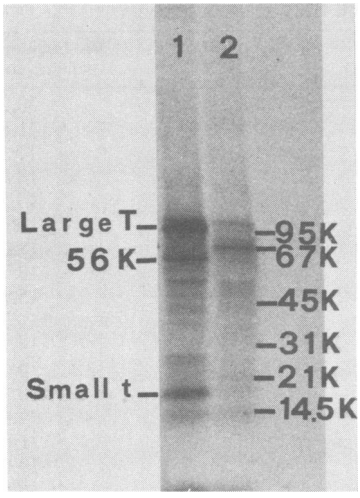

FIG. 2. Immunoprecipitation of L578 cell homogenate with hamster serum to BKV tumor Ags (lane 1) and with normal hamster serum (lane 2). Three bands corresponding to 97,000 (97K), 56,000 (56K), and 17,000 (17K) daltons appear in lane 1, whereas they are absent in lane 2. Molecular weight markers are from top to bottom: phosphorylase B (95K), bovine serum albumin (67K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21K), and lysozyme (14.5K).

fragment, used for transformation, was not contaminated with the remaining BKV genome. L578 DNA was then blot hybridized to the ^{32}P-labeled *Hha*I-*Bam*HI 3.8-kb fragment. Cellular DNA digested with *Hin*cII, an enzyme which does not cut BKV DNA, shows seven bands of hybridization (Fig. 3A, lane 2), suggesting the presence of at least seven integrations of viral DNA sequences into the cellular DNA. Digestion of cellular DNA with *Bam*HI and with *Bam*HI plus *Hha*I (two enzymes which should not cleave integrated viral sequences) produced five bands of hybridization (Fig. 3A, lanes 3 and 4). Cleavage of cellular DNA with *Eco*RI yielded eight bands (Fig. 3A, lane 7) and cleavage with *Eco*RI plus *Hha*I produced a very similar pattern, but one more band was present (Fig. 3A, lane 6). Since *Hha*I should behave as a noncutter enzyme on integrated viral sequences, the bands in the *Eco*RI and *Eco*RI-*Hha*I digests, exceeding the number of bands in the *Hin*cII digest, may represent integrations of viral sequences which had maintained the *Eco*RI site. Digestion of L578 DNA with *Hin*dIII (Fig. 3A, lane 8; Fig. 4A, lanes 6 and 8) released free fragments B and D. Fragments A and C were absent, suggesting that the integrations represented by the other nine bands present in the *Hin*dIII digest of cellular DNA (Fig. 4A, lanes 6 and 8) occurred in the latter two fragments. By densitometric analysis of the hybridization bands in Fig. 3A, lane 8 and in Fig. 4A, lanes 6 and 8, the amount of viral sequences present in fragments B and D of *Hin*dIII digest of cellular DNA corresponds approximately to seven genome equivalents, suggesting that the *Hin*dIII sites at 16.8, 53.5 and 61.8 map units were conserved and that *Hin*dIII fragments B and D were released free from all seven integrations of the transforming fragment. Indeed, the *Hin*dIII digest of L578 DNA, when hybridized to BKV DNA *Hin*dIII fragment B, showed the presence of only one prominent band (Fig. 3B, lane 3), which comigrates with BKV DNA *Hin*dIII fragment B (Fig. 3B, lane 2), confirming that no viral insertions occurred in BKV DNA sequences corresponding to *Hin*dIII fragment B. A sequential digestion of L578 DNA with *Eco*RI and *Hin*dIII released, in addition to fragments B and D, fragment A2 (Fig. 3A, lane 10). The A2 band in the *Hin*dIII digest of cellular DNA represents about two viral genome equivalents, suggesting that two insertions of the transforming fragment had retained the restriction site for *Eco*RI. *Eco*RI-*Pst*I cleavage of L578 DNA probed with *Hin*dIII-*Eco*RI fragment A2 and *Hin*dIII fragment B also indicated that two viral integrations had maintained the *Eco*RI site.

When L578 DNA was cleaved with *Pvu*II and hybridized to the ^{32}P-labeled *Hha*I-*Bam*HI 3.8-kb fragment, 11 bands were detected (Fig. 4A, lane 3), 4 of which moved faster than the *Hha*I-*Bam*HI 3.8-kb marker fragment (Fig. 4A, lanes 1 and 10), suggesting that 4 of the viral integrations had retained the cleavage site for *Pvu*II at 9.9 map units. Fourteen bands were observed when L578 DNA was cleaved with *Pst*I (Fig. 4A, lane 9) indicating that the *Pst*I site at 31.9 map units, approximately in the middle of *Hin*dIII fragment B, was conserved in all the integrated viral sequences. *Eco*RI-*Pvu*II cleavage of L578 DNA yielded 13 bands (Fig. 4A, lane 4), 1 of which comigrated with the 0.5-kb fragment (0 to 9.9 map units) generated by *Eco* RI-*Pvu*II digestion of BKV DNA (Fig. 4A, lanes 2 and 5). About two viral genome equivalents were present in the *Eco*RI-*Pvu*II 0.5-kb band of cellular DNA, suggesting that two viral integrations had retained the cleavage sites for both *Eco*RI and *Pvu*II. Hybridization of *Pvu*II-*Hin*dIII cleaved L578 DNA to BKV DNA fragment A2 showed that a cellular DNA fragment (Fig. 4B, lane 1) comigrated with the 0.4-kb fragment (9.9 to 16.8 map units) resulting from *Pvu*II-*Hin*dIII digestion of BKV DNA (Fig. 4B, lane 2). The amount of DNA contained in the 0.4-kb cellular band corresponds to about four viral genome equivalents, consistent with four integrations having maintained the *Pvu*II site at 9.9 map units. The other bands present in the *Pvu*II-*Hin*dIII digest

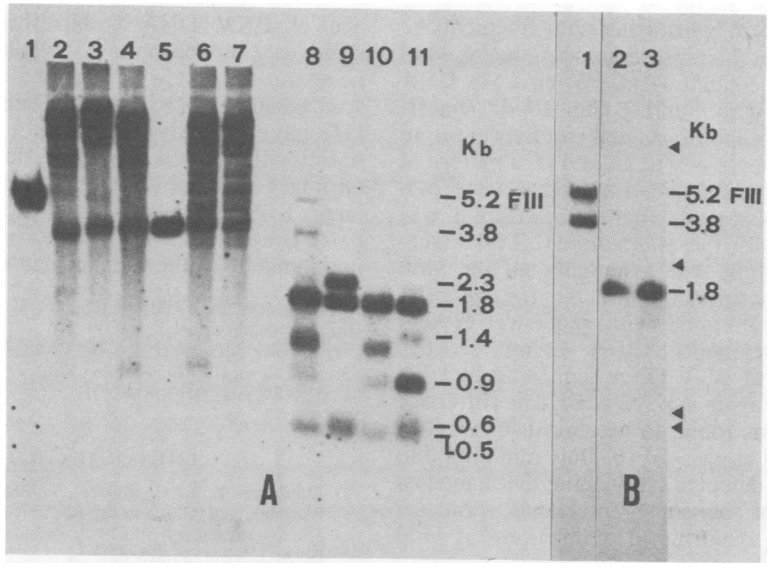

FIG. 3. (A) Blot hybridization of the ^{32}P-labeled *Hha*I-*Bam*HI 3.8-kb fragment of BKV DNA to L578 DNA cleaved with *Hin*cII (lane 2), *Bam*HI (lane 3), *Bam*HI plus *Hha*I (lane 4), *Eco*RI plus *Hha*I (lane 6), *Eco*RI (lane 7), *Hin*dIII (lane 8), and *Eco*RI plus *Hin*dIII (lane 10). Molecular weight standards are BKV DNA (form III) (5 genome equivalents, 5.2 kb, lane 1), *Hha*I-*Bam*HI 3.8-kb fragment (2.5 genome equivalents, lane 5), BKV DNA cleaved with *Hin*dIII (7.5 genome equivalents; fragments A of 2.3 kb, B of 1.8 kb, C of 0.6 kb, and D of 0.5 kb, lane 9), BKV DNA sequentially cleaved with *Eco*RI and *Hin*dIII (7.5 genome equivalents; fragments A1 of 1.4 kb and A2 of 0.9 kb, lane 11). (B) Blot hybridization of ^{32}P-labeled BKV DNA *Hin*dIII fragment B with L578 DNA cleaved with *Hin*dIII (lane 3). Lane 1 contains 2.5 genome equivalents of BKV DNA (form III) (5.2 kb) and of the *Hha*I-*Bam*HI 3.8-kb fragment. Lane 2 contains 5 genome equivalents of BKV DNA cleaved with *Hin*dIII: only *Hin*dIII fragment B (1.8 kb) hybridizes with the probe; the relative position of fragments A, C, and D is indicated by arrows.

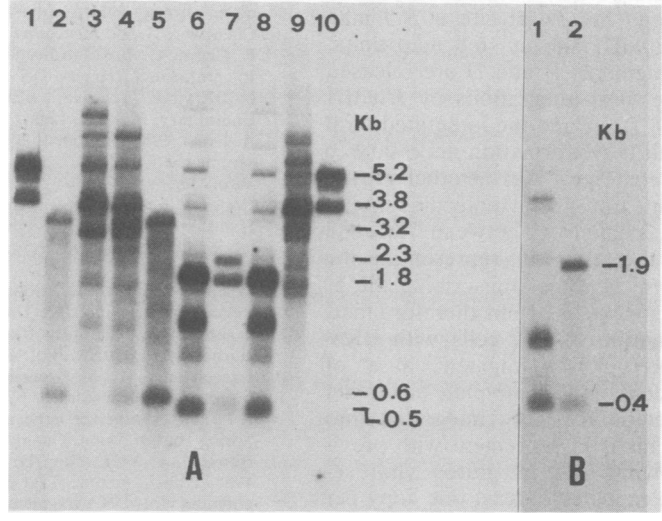

FIG. 4. (A) Blot hybridization of ^{32}P-labeled *Hha*I-*Bam*HI 3.8-kb fragment to L578 DNA cleaved with *Pvu*II (lane 3), *Eco*RI plus *Pvu*II (lane 4), *Hin*dIII (lanes 6 and 8), and *Pst*I (lane 9). Molecular weight and band density markers are BKV DNA (form III) (5.2 kb) and *Hha*I-*Bam*HI 3.8-kb fragment (5 genome equivalents, lanes 1 and 10), BKV DNA cleaved with *Eco*RI plus *Pvu*II (2.5 genome equivalents, lane 2; 5 genome equivalents, land 5), BKV DNA cleaved with *Hin*dIII (2.5 genome equivalents; fragments A of 2.3 kb, B of 1.8 kb, C of 0.6 kb, and D of 0.5 kb, lane 7). (B) Blot hybridization of ^{32}P-labeled *Hin*dIII-*Eco*RI fragment A2 to L578 DNA cleaved with *Pvu*II plus *Hin*dIII (lane 1). Lane 2 contains BKV DNA (2.5 genome equivalents) cleaved with *Pvu*II plus *Hin*dIII: the bands hybridizing with the probe correspond to two fragments of 1.9 and 0.4 kb, resulting from *Pvu*II cleavage of *Hin*dIII fragment A at 9.9 map units.

of cellular DNA hybridizing with fragment A2 (Fig. 4B, lane 1) represent junctions of viral sequences to cellular DNA. When L578 DNA was cleaved with *Bam*HI plus *Hha*I, *Hin*cII, *Bam*HI, *Hha*I, and *Eco*RI and blot hybridized to *Hin*dIII fragments A2, B, C, and D, a pattern of hybridization was obtained which was very similar to that observed when hybridization was carried out with the *Hha*I-*Bam*HI 3.8-kb fragment, suggesting that essentially all the viral integrations hybridizing with the *Hha*I-*Bam*HI 3.8-kb fragment contain viral sequences present in *Hin*dIII fragments A2, B, C, and D. The arrangement of BKV DNA sequences in L578 was analyzed again at passage 84. The pattern of integration was found to be almost identical to that observed at passage 16. Only minor modifications were detected, suggesting that a modest rearrangement of some integrated viral sequences had occurred. No deletions of viral bands were observed.

From the results of blot hybridization analysis it can be concluded that one site of the viral insertions maps in various points of the integrated viral DNA between 97.9 and 16.8 map units clockwise. In fact, since the *Eco*RI site was conserved in two integrations and the *Pvu*II site was conserved in four integrations, two insertions map between the *Bam*HI site at 97.9 map units and the *Eco*RI site at 0 map units and two insertions map between the *Eco*RI site and the *Pvu*II site at 9.9 map units, whereas three insertions map between the *Pvu*II site at 9.9 map units and the *Hin*dIII site at 16.8 map units. Since *Hin*dIII fragments B and D are released free from all the viral integrations by *Hin*dIII digestion of L578 DNA and the integrated viral DNA contains all the information necessary to code for a complete large T Ag, the other sites of insertion of the integrated viral molecules should map in *Hin*dIII fragment C between 72.2 and 64.0 map units, the latter site representing the region of the viral genome transcribing the 5′ end of the early mRNA. It seems therefore that, during transformation of HK cells with BKV DNA *Hha*I-*Bam*HI 3.8-kb fragment, a set of viral sequences containing a complete and intact T Ag coding region is stably integrated into cellular DNA. This is in agreement with previous results showing that integrated viral sequences corresponding to at least one copy per cell of the entire early region were always found in BKV-transformed rabbit, hamster, and mouse cells (5, 13, 21) and in BKV-induced tumors (6).

The results of this study may suggest that all sequences of the early region coding for large T Ag are necessary to induce and maintain transformation by BKV. Alternatively, subgenomic segments of the BKV DNA early region may be unable to induce transformation because cleavage of BKV DNA within the early region removes the appropriate polyadenylation site necessary to obtain a complete functional transcriptional unit. On the other hand, Yogo et al. (34) have recently described a BKV-induced hamster tumor cell line which does not contain an intact early region and is negative for BKV large T Ag, suggesting that a complete BKV early gene region may not be required for the maintenance of the transformed state.

We thank W. Rossi-Pusinanti and C. Mussi for their excellent technical assistance.

This work was supported by Consiglio Nazionale delle Ricerche, Progetto Finalizzato Virus, grants 80.00577.84, 81.00258.84, and 204121/84/81821.

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