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Integrated and free viral DNA in hamster tumors induced by BK virus

(blot-transfer hybridization/tumor tissues/cultured tumor cells)

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ABSTRACT **BK virus (BKV)-induced tumors in hamsters were investigated for the presence of viral DNA by the blot-transfer hybridization technique. Several viral genomes per cell were found in tumor tissues and in their derived cell lines and clones. Most of the detected viral genomes were integrated into the cellular DNA, but some tumors also contained free viral DNA sequences. Integration patterns were different from each other, and many different integration sites were available on the cellular or on the viral DNA or on both. Typical features of integration patterns were found in ependymomas, which were the most frequent (72%) among BKV-induced tumors. Readily detectable viral DNA sequences were only found in neoplastic tissues, but traces of BKV DNA were also present in the apparently normal portion of the brain of an animal that had developed an ependymoma and in the brain (but not in the liver) of another animal 15 days after virus inoculation. A cell line and a single-cell clone derived from a tumor had hybridization patterns considerably simpler than the pattern of the original tumor, lacking several integrated viral genomes and all free viral sequences.**

BK virus (BKV) is a recently discovered human papovavirus originally isolated by Gardner *et al.* (1) from the urine of an immunosuppressed patient. It is related both antigenically and biochemically to the papovavirus simian virus 40 (2–7). Like all papovaviruses, BKV is oncogenic in rodents, inducing several types of tumors when injected into newborn hamsters and mice (8–12). When injected intracerebrally, this virus induced tumors in 88% of inoculated hamsters and 29% of inoculated mice, and all tumors were intraventricular ependymomas (11). Furthermore, when injected intravenously into hamsters, BKV induced tumors in 82% of the inoculated animals, and 72% of the tumors were cerebral ependymomas (12). The two other most frequent types of tumors induced in hamsters inoculated intravenously with BKV were pancreatic insulinomas (12%) and osteosarcomas (10%). BKV large tumor antigen was detected in BKV-induced tumors (11, 12), indicating that at least a portion of the viral genome was being expressed in tumor cells. It was therefore of interest to search for BKV DNA sequences in tumor tissues and to analyze such sequences in order to determine their physical state (free or integrated, complete or incomplete viral genomes) and their mode of integration.

We present here the results of an analysis of BKV DNA sequences in several BKV-induced hamster tumors by the blot-transfer hybridization technique originally devised by Southern (13). With this technique, viral DNA sequences are visualized as hybridizable bands after digestion of tumor cell DNA by restriction endonucleases and fractionation by agarose gel electrophoresis.

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MATERIALS AND METHODS

Virus growth, purification and inoculation into Syrian golden hamsters, pathology of tumors, and cultivation of tumor cells have been described (12).

Cellular DNA Purification. Tissues were minced with scissors, homogenized in TD (25 mM Tris·HCl, pH 7.4/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/5 mM D-glucose) (14) in a Dounce homogenizer equipped with a loose-fitting piston, and digested overnight at 37°C with proteinase K (Merck) at 50 μg/ml in 20 mM EDTA/0.5% $NaDodSO_4$. The viscous lysate was then gently extracted with phenol/chloroform (1:1, vol/vol), dialyzed, treated with pancreatic RNase (Worthington) and again with proteinase K, reextracted with phenol/chloroform, and dialyzed (to be described in detail elsewhere). DNA purification from *in vitro* cultured cells followed the same procedure but with the omission of the homogenization step.

^{32}P-Labeled BKV DNA Probe. Viral DNA was extracted from CsCl-purified virions by disrupting the virions in $NaDodSO_4$ at 50°C and digesting with proteinase K. Superhelical BKV DNA was then purified in a CsCl/ethidium bromide density gradient (15). Labeling with deoxyribonucleoside [^{32}P]triphosphates by nick-translation was catalyzed by *Escherichia coli* DNA polymerase I (Boehringer) and will be described in detail elsewhere. The ^{32}P-labeled probe had a specific radioactivity of 1×10^8 to 2×10^8 cpm/μg of input DNA on the day of synthesis.

Blot-Transfer Hybridization. Cellular DNA (25 μg) was digested with restriction endonucleases (Miles) in the appropriate reaction mixtures at 37°C for 3 hr. The reaction was stopped with EDTA, and DNA was precipitated with ethanol, dissolved in a small volume of 1:5 diluted electrophoresis buffer (E buffer, 40 mM Tris·HCl, pH 7.8/5 mM sodium acetate/1 mM EDTA) (16). Electrophoresis was performed in 0.8% agarose gels (Bio-Rad) for digested DNAs and 0.6% gels for undigested DNAs, in horizontal slabs 0.5 cm thick and 20 cm long, with 20 μg of DNA for each sample. Electrophoresis was at 1.5 V/cm for 15 hr at room temperature. After electrophoresis, DNA was denatured in the gel, transferred to a nitrocellulose membrane, and hybridized to the ^{32}P-labeled BKV DNA probe (to be described in detail elsewhere). The nitrocellulose sheet was finally laid against a preflashed Kodak Royal X-Omat film in the presence of a $CaWO_4$ intensifying screen (Ilford Fast Tungstate) (17).

Tumors and Cell Lines. The following hamster tumors induced by BKV injected intravenously were analyzed: four ependymomas (nos. 121, 140, 156, and 158), six osteosarcomas (nos. 208, 239, 275, 284, 297, and 317), and three pancreatic insulinomas (nos. 208, 209, and 210). In addition, analysis of

Abbreviation: BKV, BK virus

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BKV DNA sequences was performed on cell lines T117, T133, and T135 derived from three different ependymomas and on cell line T284 established from osteosarcoma no. 284, as well as on a single-cell clone obtained from line T284.

RESULTS

Ependymomas. Restriction endonuclease *Eco*RI cuts BKV DNA at a single site (18). When DNA from four cerebral ependymomas was digested with *Eco*RI and analyzed by blot-transfer hybridization to a BKV [^{32}P]DNA probe, the patterns shown in Fig. 1*a* were obtained. Each DNA gave rise to many hybridization bands, and the patterns from the four tumors were different. Furthermore, most of the *Eco*RI-generated segments containing BKV sequences were either faster or slower than linear BKV DNA. This is what would be expected for viral genomes integrated into cellular DNA, because digestion with *Eco*RI would then generate, for one inserted viral genome, two segments each containing a viral sequence joined to a cellular sequence. The length of these two segments would in general be different from that of linear viral DNA. Alternatively, all hybridization bands appearing in Fig. 1*a* could represent free (nonintegrated) viral DNA molecules smaller or larger than normal BKV DNA.

This possibility was ruled out by two kinds of experiment. In the first one, uncut DNAs from the same four tumors were electrophoresed on agarose gel and analyzed by the same technique (Fig. 2*a*). No hybridization band was visible in the region of the *Eco*RI-generated bands: instead, all BKV sequences were found in the region of high molecular weight DNA, suggesting that they were integrated into the cellular genome. Electrophoresis of uncut DNA however, is not the best way of separating free viral sequences from cellular DNA because very large DNA could trap the nonintegrated sequences during migration. We therefore digested ependymoma DNAs with *Hin*dII, an enzyme that does not cut BKV DNA but does cut cellular DNA into segments whose average length should exclude aggregation artefacts. Again, practically all of the hybridization was found in the region of very large DNA segments (Fig. 2*b*), indicating that most viral sequences were indeed integrated in the cellular DNA. Faint, faster migrating bands were also visible, which could represent nonintegrated viral DNA or integrated, incomplete viral genomes. Intensities of such bands, however, corresponded to much less than one genome per cell.

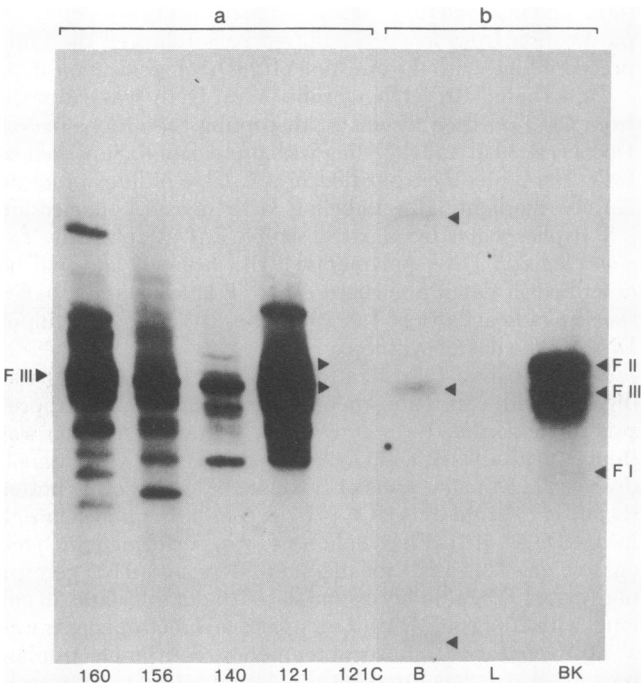

FIG. 1. Blot-hybridization of ^{32}P-labeled BKV DNA to *Eco*RI-digested DNAs (20 μg each) from neoplastic and normal tissues of BKV-inoculated animals. (*a*) DNAs from ependymomas nos. 160, 156, 140, and 121; sample 121C was DNA from the normal portion of the brain from which ependymoma 121 was removed. (*b*) B and L were DNAs from brain and liver, respectively, of a hamster killed 15 days after BKV inoculation. BK was a control that contained 50 pg each of nicked-circular (F II) and linear (F III) BKV DNA, with traces of superhelical BKV DNA (F I). Molecules migrating ahead of F III were defective BKV genomes present in the viral preparation, which did not contain hamster DNA sequences, as shown by sample L. Autoradiographs were overexposed in order to reveal faint bands in samples from nontumor tissues (arrows). *a* and *b* represent separate experiments. The position of control BKV DNA was determined in each experiment.

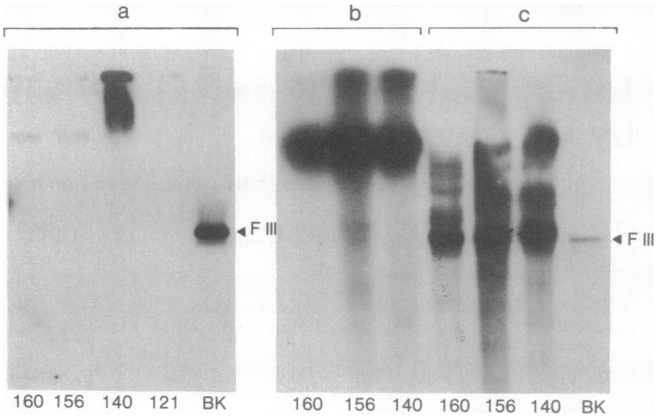

FIG. 2. Blot-hybridization of ^{32}P-labeled BKV DNA to DNAs from ependymomas. (*a*) Uncut DNAs (20 μg each). (*b*) *Hin*dII-digested DNAs (20 μg each). (*c*) *Pst* I-digested DNAs (20 μg each). BK controls contained 50 pg each of *Eco*RI-linearized BKV DNA. *b* and *c* were run on the same gel; *a* represents a separate experiment. The autoradiographic exposure time was different in the two experiments.

A common feature of all four *Eco*RI patterns obtained with DNA from ependymomas was the presence of a strong hybridization band in the position corresponding to full-size linear BKV DNA. Considering that almost no free viral DNA was detected in these four tumors, such bands must also come from integrated viral genomes. Comparison of their intensities with those of known amounts of linear BKV DNA (data not shown) suggested that they represent several viral genome equivalents per cell. The simplest interpretation of this result is that the full-size linear viral DNA molecules were generated by the cutting of tandemly integrated viral genomes by *Eco*RI. If this were the case, then digestion of ependymoma DNA with another enzyme that cuts BKV DNA at a different unique site should again cleave out linear BKV molecules from tandem integrations. We chose *Pst* I, which cuts BKV at a single position (6) quite far from the *Eco*RI site. A strong hybridization band was again visible in each pattern at the position of full-size BKV linear DNA (Fig. 2*c*). We conclude that tandem integrations of viral genomes into the cellular DNA did in fact occur in BKV-induced ependymomas.

Lane 121c in Fig. 1*a* shows hybridization to *Eco*RI-digested DNA from the normal portion of the brain from which ependymoma 121 was removed. Two faint bands were visible after prolonged autoradiographic exposure, suggesting that the presence of BKV DNA was not rigorously confined to the neoplastic tissue in the affected animal. However, the very low

intensity of the observed bands compared to the pattern of an equal amount of DNA from the corresponding tumor (no. 121) indicates that only a small fraction of cells in the surrounding brain carried viral DNA. The possible meaning of this finding is discussed in conjunction with results from a related experiment (see below).

Osteosarcomas. Fig. 3*a* shows the result of an experiment in which DNAs from six osteosarcomas were digested with *Eco*RI and analyzed as above. Again, each pattern consisted of several bands covering a wide range of electrophoretic mobilities. Comparison with the corresponding *Eco*RI patterns of ependymomas (Fig. 1) showed that some features were consistently different in the two types of tumors. The most obvious difference was the absence, in four of the patterns from osteosarcomas (nos. 239, 275, 297, and 208) of a heavy hybridization band at the position of linear BKV DNA, whereas this was always the main band with ependymomas. In fact, only two osteosarcomas gave bands of outstanding intensity: sample 284 and sample 317. Surprisingly, these strong bands were found to originate from free DNA.

Fig. 3*b* shows that uncut DNA from sample 284 gave one major band faster than linear BKV DNA and two bands of lower intensity migrating ahead. Such bands indicate the presence of free, presumably defective, viral DNA molecules in this sample (see below). Uncut DNA from sample 317 gave a strong hybridization band of free DNA slightly slower than linear BKV DNA, with a mobility comparable to that of nicked circular BKV DNA (which, in 0.6% agarose, migrated rather close to the linear form). No bands migrating like free DNA were seen in any of the other four osteosarcomas, with either undigested or *Hin*dII-digested DNA (data not shown).

In an attempt to characterize further the free DNA molecules, we digested DNA from osteosarcomas 284 and 317 with *Hin*dIII, an enzyme that cuts BKV DNA at four sites (18). Obviously, because integrated viral DNA molecules were present in both samples together with free viral DNA, such an analysis cannot give straightforward information exclusively on free DNA molecules. Any conclusion must rely on the assumption that the most intense bands in the *Hin*dIII pattern come from the predominant bands in *Eco*RI or uncut-DNA patterns—i.e., free DNA. Fig. 3*c* shows that, besides regular BKV DNA-*Hin*dIII segments A and B, sample 284 gave a strong band migrating faster than B. By comparison with intensities of A and B segments in the control sample, such a band could represent a segment A carrying a deletion, and this would be consistent with the observation that free DNA molecules in sample 284 migrated faster than linear BKV DNA (Fig. 3 *a* and *b*). Sample 317, on the contrary, showed two "normal" A and B *Hin*dIII segments in large amounts, suggesting that free viral DNA molecules in this tumor were normal in this part of the genome. It should be remembered that A covers most of the BKV late region and B covers a large fraction of the early region (6, 18). Analysis for segments C and D was not sufficiently precise, due to their small size and, in sample 317, to the presence of degraded material migrating in this part of the gel. Free DNA in sample 284 seemed to lack segment D. All other bands in both *Hin*dIII patterns presumably came from the splitting of segments from integrated genomes as a result of the integration event.

Insulinomas. DNAs from three pancreatic insulinomas were analyzed for the presence of BKV sequences. After digestion with *Eco*RI, they gave rise to a relatively small number of hybridizable bands compared to ependymomas and osteosarcomas (Fig. 4*a*). Two of the tumors (nos. 209 and 210) contained free viral DNA, as indicated by hybridization bands seen with uncut DNAs (indicated by arrows in Fig. 4*b*). Unfortunately, bands were very faint with this type of tumor, and a more precise analysis could not be performed due to the limited amount of material.

Presence of BKV DNA Before Tumor Appearance. BKV DNA could be detected in the brain, but not in the liver, of inoculated hamsters before the appearance of tumors. BKV was injected subcutaneously into suckling hamsters and the animals were killed after 15 days. Organs from these animals were examined histologically and found to be normal. DNA was purified from liver and brain of one animal and analyzed by blot-transfer hybridization after *Eco*RI digestion. The results of such an experiment are shown in Fig. 1. Three hybridization bands were visible in the DNA extracted from the brain after prolonged autoradiographic exposure (Fig. 1*b*, lane B), one migrating like linear BKV DNA, one migrating faster, and one migrating slower. No bands were visible in DNA from the liver

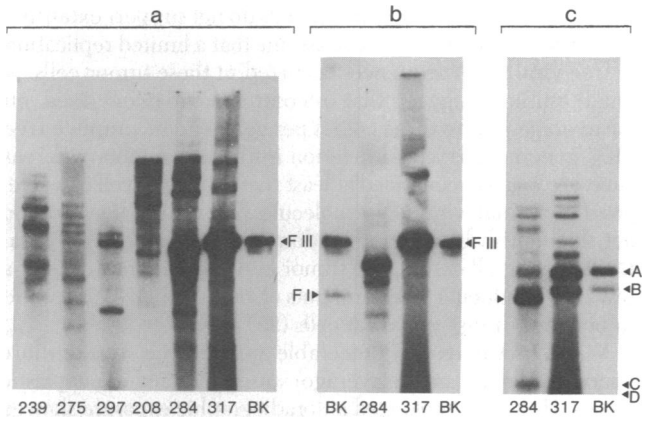

FIG. 3. Blot-hybridization of ^{32}P-labeled BKV DNA to DNAs from osteosarcomas. (*a*) *Eco*RI-digested DNAs (20 μg each). (*b*) Uncut DNAs from tumors 284 and 317. (*c*) *Hin*dIII-digested DNAs from tumors 284 and 317. Bands A, B, C, and D indicate hybridization to the four BKV DNA-*Hin*dIII segments (50 pg total). The heavy band marked by an arrow in *c* most likely represents a defective segment A from the free DNA in tumor 284. *a* and *c* represent separate 0.8% agarose gels; *b* represents a 0.6% gel.

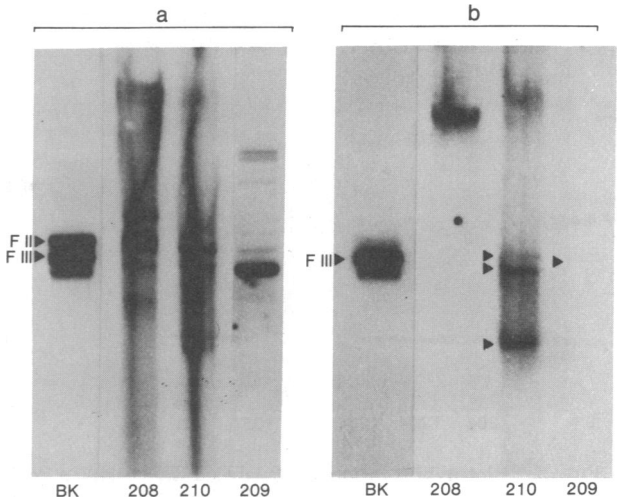

FIG. 4. Blot-hybridization of ^{32}P-labeled BKV DNA to DNAs from pancreatic insulinomas (tumors 208, 209, and 210). (*a*) *Eco*RI-digested DNAs (20 μg each). (*b*) Undigested DNAs (20 μg each); arrows point to bands of nonintegrated DNA. BK control samples contained 50 μg each of nicked circular and linear BKV DNA (*a*) or 50 pg of linear BKV DNA (*b*). The band migrating ahead of F III was due to defective BKV genomes (see legend to Fig. 1).

of the same animal (Fig. 1*b*, lane L). Possible implications of this finding in connection with the tropism of BKV tumorigenic action for brain tissue are discussed below.

Cultured Tumor Cells. In view of the complexity of hybridization patterns observed with virus-induced tumors (particularly with ependymomas and osteosarcomas), it is possible that cells within the same tumor were heterogeneous with respect to the mode of viral integration. To investigate this problem, cell lines were established from three ependymomas (nos. 117, 133, and 135) and from osteosarcoma 284. DNA was purified from *in vitro* cultured cells and analyzed as above. Fig. 5*b* shows the hybridization patterns obtained when ^{32}P-labeled BKV DNA was hybridized to *Eco*RI-digested DNAs from the three ependymoma cell lines. Patterns were similar to those observed with tissues from the same type of tumors (Fig. 1), all of them showing comparable complexity and a strong band migrating like linear BKV DNA. Unfortunately, comparison with the original tumor tissues was impossible in this case because, due to their small size, the whole tumors were used to establish the cell cultures. Comparison of original tumor, cultured tumor cells, and a single-cell clone derived from the established line was possible, however, for osteosarcoma 284 and is shown in Fig. 5*a*. The *Eco*RI-generated patterns of the cultivated cells were strikingly simpler than the pattern of the original tumor. The heavy band of free DNA had disappeared in both patterns of cultured cells, as well as most of the bands from integrated genomes. Some of the bands seen in line T284 were also present in the original tumor, suggesting that a selection of cell types or of chromosomes containing integrated viral genomes had taken place. However, this was probably not the only cause of variation because two of the bands of line T284 were not present in detectable amounts in the original tumor and at least one of the bands of clone T284c13 was not present in the cell line from which the clone was derived (arrows in Fig. 5*a*). Absence of free viral sequences in lines T284 and T284c13 was confirmed by the absence of free-migrating bands when uncut DNAs were analyzed (data not shown).

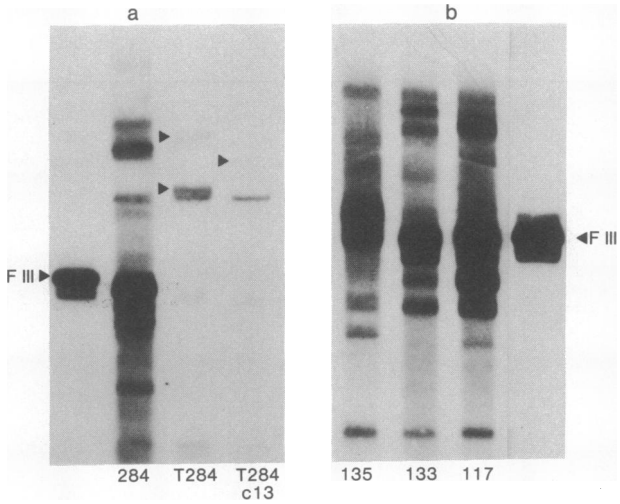

FIG. 5. Blot-hybridization of ^{32}P-labeled BKV DNA to *Eco*RI-digested DNAs from cultured tumor cells. (*a*) DNAs from osteosarcoma 284, from cultured cells from the same tumor (T284), and from a single-cell clone derived from the established line (T284c13). (*b*) DNAs from cell lines established from three ependymomas (nos. 117, 133, and 125). Controls contained 50 pg of linear BKV DNA (F III), including some defective viral genomes (see legend to Fig. 1). Arrows point to new hybridization bands arising during culture (see text). *a* and *b* represent separate experiments.

DISCUSSION

We have investigated the presence and state of viral DNA in BKV-induced hamster tumors and their derived cell lines and clones. BKV DNA sequences were found in all tumors examined; most of them were integrated in the cell genome and some were in a free state. The precise number of viral genome equivalents per cell could not be estimated in tumors for several reasons: (*i*) the relative amounts of neoplastic and stromal cells in tumor tissues were not known; (*ii*) tumor cells could be heterogeneous with respect to the distribution of viral DNA (see below); and (*iii*) the nature of the hybridization technique used, involving transfer of DNA from agarose gels to nitrocellulose and autoradiographic exposure, did not allow a precise quantitation of hybridizable sequences. However, the presence of many bands in all *Eco*RI-generated hybridization patterns shows that differently integrated BKV genomes were present in each tumor. Comparison of band intensity with hybridization bands of known amounts of viral DNA suggested that, on the average, each tumor cell contained at least one and in most cases several integrated viral genomes.

Integration patterns observed were all different from each other, and digestion with *Eco*RI generated segments that covered a wide size range and contained different lengths of viral DNA (as judged by intensity of hybridization bands). All these observations suggest that viral integration could take place at many different sites on cellular and viral DNA, in agreement with what was found with cells transformed *in vitro* by simian virus 40 (19–21), polyoma (22), and BKV (unpublished data). In spite of the great variability of hybridization patterns, however, one feature was common to all ependymomas examined and absent in osteosarcomas and in insulinomas: all ependymomas had tandem integrations of full-size linear BKV DNA, whereas osteosarcomas and insulinomas had patterns suggesting individual integrations (or short tandems of defective molecules).

The presence of free viral sequences in osteosarcomas and insulinomas was unexpected. Because hamster cells are nonpermissive for BKV replication (23, 24), free viral DNA molecules could not be propagated as infectious viral particles, as supported by the finding that BKV structural antigens were never detected in BKV-induced tumors (12). One possible explanation is that free viral DNA sequences were actually replicated as integrated viral genomes and then occasionally excised (see ref. 22 for a detailed discussion of this possibility). Alternatively, although hamster cells do not support extensive BKV DNA synthesis, one could assume that a limited replication of free viral DNA sequences occurred in these tumor cells. A rough estimate suggests that osteosarcoma 284 contained, on the average, one to three copies per cell of an incomplete free BKV genome carrying a deletion in the late region, whereas osteosarcoma 317 contained at least five copies per cell of a free, possibly normal, viral DNA molecule. Our results, however, do not distinguish between a random distribution of free viral DNA among all cells in the tumor and the presence of only a few cells producing large amounts of free viral DNA, as found in polyoma-transformed rat cells (25).

Viral DNA in readily detectable amounts (i.e., one or more genomes per cell, on the average) was found only in neoplastic tissues. However, prolonged autoradiographic exposure showed that the normal portion of the brain in which an ependymoma had developed contained small traces of BKV DNA. Contamination by neoplastic tissue due to incomplete removal of the tumor was made rather unlikely by the comparison of hybridization patterns of ependymoma and surrounding brain (Fig. 1). This finding correlates well with the presence of traces of BKV DNA in the brain of an animal 15 days after virus in-

jection. Three bands were visible in this case (Fig. 1), two of them corresponding to an integrated viral genome and one to either a tandemly integrated or a free viral genome. No BKV DNA sequences could be detected in the liver of the same animal (Fig. 1) or in various organs, including brain, of noninoculated animals (data not shown). The presence of BKV DNA sequences in apparently normal cerebral tissues before and after the appearance of tumors could lead to speculation that BKV DNA can persist in the brain of inoculated animals long after inoculation, possibly because immunological protection is less efficient in this organ. The persistence of viral DNA in brain cells could be one of the causes of the high incidence of ependymomas among BKV-induced tumors.

Analysis of the mode of integration of viral DNA in virus-induced tumors should offer, in principle, a valuable tool to investigate the problem of the clonal origin of tumor cells. We found that *Eco*RI-generated hybridization patterns observed with DNA from tumor tissues had more bands than did patterns from cultured tumor cells, and that single-cell clones isolated from such cultured tumor cells had even simpler patterns. These results would be compatible with a multiclonal origin of BKV-induced tumors because they suggest that selection during culture leads to disappearance of certain cell types and of the corresponding viral genomes. However, considerable caution should be used in evaluating these results because other events besides cell selection could cause loss of viral genomes from tumor cells. Selective loss of chromosomes or rearrangement of integrated viral genomes could also give rise to new integration patterns, provided that similar changes took place in all cells of the same culture. Alternatively, some cells, after developing an aneuploid karyotype or chromosomal aberrations during culture, could have acquired some selective advantage and rapidly outgrow other cells. That rearrangement of integrated viral sequences could in fact take place in cultivated tumor cells was shown by the appearance in clone T284c13 of at least one new band not present in line T284, from which the clone was derived. Also, two of the bands of line T284 were apparently not present in the original tumor. Interestingly, free viral DNA present in osteosarcoma 284 was lost in cultivated tumor cells and in the derived single-cell clone. In conclusion, we think that, although identical integration patterns of tumor and clones would unambiguously prove the monoclonal nature of the tumor, the variations we observed are not convincing evidence for multiclonality. Our findings, however, do show that loss and rearrangement of viral DNA sequences can occur in cultured tumor cells, suggesting that such cells give only an incomplete account of the state of viral DNA in virus-induced tumors.

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