Hybrid Genomes of the Polyomaviruses JC Virus, BK Virus, and Simian Virus 40: Identification of Sequences Important for Efficient Transformation

BRIGITTE BOLLAG, WEI-FONG CHUKE, AND RICHARD J. FRISQUE*

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

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Hybrid viral genomes were used to investigate the influence of specific polyomavirus sequences on the transforming behavior of JC virus (JCV). One set of chimeric DNAs was made by exchanging the regulatory regions between JCV and simian virus 40 (SV40) or JCV and BK virus (BKV). A second set of constructs was produced that expressed hybrid JCV-BKV T proteins under the control of either JCV or BKV regulatory signals. Transformation of Rat 2 cells with the parental and chimeric DNAs indicated that both the JCV regulatory signals and the sequence encoding the amino terminus of T protein contributed to the restricted transforming behavior of this virus. Analysis of the viral proteins in the transformed rat cells indicated that the large T antigens of JCV and BKV were less stable than their SV40 counterpart, that small t protein was produced in JCV transformants, and that the subpopulation of T antigen that forms a stable complex with cellular p53 protein was smaller in JCV-transformed cells than in SV40- or BKV-transformed cells.

The polyomavirus JC virus (JCV) readily infects its human host (30) and in immunocompromised individuals may cause the fatal demyelinating brain disease progressive multifocal leukoencephalopathy (32). JCV also induces a high incidence of tumors in hamsters (33, 50) and rats (29), and it is the only human virus known to cause solid tumors in primates (19, 37). Although it functions efficiently in vivo, JCV, unlike the closely related polyomaviruses simian virus 40 (SV40) and BK virus (BKV), is highly restricted in its lytic and transforming behavior in tissue culture (for a review, see reference 49). Our laboratory has been investigating the molecular basis for the unique biology of JCV and has taken advantage of the abundant information available for SV40 and BKV. A comparison of the three viral genomes at the nucleotide level indicates that while the coding regions share extensive homology, the JCV regulatory region is distinct and exhibits an unusual arrangement of the promoter-enhancer signals (9). A number of studies have emphasized the importance of these transcriptional control elements on host range, tissue tropic, and oncogenic properties of the polyomaviruses (7, 11, 14, 17, 52). Recent studies involving the analysis of JCV variants isolated from the brain tissues of different patients with progressive multifocal leukoencephalopathy indicate that alterations also occur frequently within their promoter-enhancer sequences (25). Some of these variants exhibit altered antigenic, oncogenic, and host range properties (31, 33; M. T. Cannella and R. J. Frisque, unpublished data). While these results suggest that the JCV regulatory region may be responsible for the restricted behavior of the virus in vitro, a recent report emphasizes the importance of JCV coding sequences (presumably those specifying T protein) on its narrow host range (1). These results support the suggestion that differences identified previously in the carboxy-terminal sequences of the JCV, SV40, and BKV T proteins might contribute to the different host cell specificities of the viruses (9); alterations of these sequences in SV40 have been shown recently to

limit the ability of this virus to replicate in certain cells (24, 34, 48).

This paper describes our investigation of the restricted transforming capacity of JCV in vitro. In these initial studies, we have not relied on a mutagenesis approach to identify JCV sequences contributing to this reduced activity. Since mutagenesis of the SV40 genome has generally resulted in the reduced ability of the virus to transform cells (for a review, see reference 28), this approach would be impractical for studying JCV, which already has a low transforming activity. Furthermore, although deletion and insertion mutants have been useful in identifying SV40 regulatory and early coding sequences that affect transformation efficiency, this approach has its shortcomings (44). For example, alterations in coding sequences might indirectly affect transforming activity because of changes in the secondary and tertiary structures of T protein. Such results might lead to the discrepancies that have been reported concerning the influence of specific regions of the T antigen on transformation (3, 4, 35, 41, 42, 44). We have attempted to minimize these problems by using chimeric polyomavirus genomes. The constructs used, which include JCV genomes with regulatory or coding sequences replaced by those of SV40 or BKV (1) and hybrid genomes containing chimeric early coding regions, still retain intact regulatory regions and encode full-length early proteins. Although it is not known whether this approach has altered the higher-order structure of the chimeric T proteins, such manipulations do not appear to have adversely affected the transforming activity of the proteins; most chimeric DNAs demonstrated enhanced levels of transformation. Determination of the transforming activities of these chimeric viral genomes has enabled us to identify two regions of the JCV genome which are responsible for its restricted transforming behavior in vitro.

MATERIALS AND METHODS

Cells. Rat 2 cells (47) were cultured in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), penicillin (99 U/ml), and streptomycin (73 U/ml), at 37°C in an atmosphere containing 10% CO₂.

* Corresponding author.

Recombinant DNAs. All viral DNAs were cloned into pBR322 at their unique *EcoRI* sites. The pMad1-TC clone represents prototype JCV DNA isolated from virus that had been passaged in tissue culture (8). The parental BKV plasmid, pBKV-9, was made with DNA extracted from the prototype Gardner strain of BKV (1). pSV40 contains the DNA of the small-plaque variant of SV40 (strain 776).

The regulatory region hybrid plasmids pM-1(BKV), pBKV(M-1), pM-1(SV40), and pSV40(M-1) were constructed by exchanging the intact regulatory regions of pMad1-TC, pBKV-9, and pSV40. The naming scheme of the hybrid viruses is as follows. The first half of the name indicates the virus which donates the coding sequences, and the second half (in parentheses) indicates the virus which donates the regulatory sequences. Structures and characterizations of these chimeric DNAs have been described elsewhere (1).

T-antigen hybrid plasmids were constructed by using the parental plasmids pMad1-TC and pBKV-9 or the regulatory region hybrid plasmids pM-1(BKV) and pBKV(M-1). Each DNA was digested with *Pst*I to generate two fragments. The small fragment, containing the carboxy-terminal coding sequences of the viral large T and VP1 proteins (nucleotides 1722 to 3355) and pBR322 sequences, was exchanged between the two plasmids of each set. This yielded the T-antigen hybrid plasmids pMRMBT, pMRBMT, pBRMBT, and pBRBMT. The naming scheme is as follows. M and B denote JCV (Mad 1) and BKV-9 sequences, respectively. R follows the letter representing the regulatory sequences, and T follows the two letters representing the amino and carboxy portions of large T antigen. Additional details related to these constructions are included in Results.

Several recombinant DNAs containing altered viral sequences were used in these studies. pM-1(Δ 98) was constructed by partially digesting pMad1-TC with *Sac*I to remove one of two copies of the 98-nucleotide-pair tandem repeat of the JCV enhancer. pM-1(Δ Nco) was produced by digesting pMad1-TC with *Nco*I to yield two fragments. The large fragment, missing nucleotides 4980 to 275, was recircularized by ligation to yield a JCV recombinant DNA lacking the entire regulatory region (1). The plasmid pdl892 contains the DNA of a viable mutant of SV40 that has a 19-nucleotide-pair deletion (nucleotides 35 to 53) within the SV40 regulatory region (38). pBRB Δ T was constructed by digesting pBKV-9 with *Pst*I and *Nsi*I to generate three fragments with compatible sticky ends. The short *Nsi*I-*Pst*I fragment between nucleotides 2897 and 3447 of BKV-9 was removed, and the remaining two fragments were ligated back together. This yielded a mutant pBKV-9 DNA that lacked sequences near the carboxy terminus of large T but retained the poly(A) signal for the early coding region. The predicted sequence of this large T protein would include the first 439 amino acids of the authentic BKV-9 protein followed by 97 amino acids from an alternate reading frame at the carboxy end of the sequence.

DNA transfection. Culture plates (60 mm) were seeded with 4×10^5 Rat 2 cells and were grown overnight to 30 to 50% confluence in DMEM plus 10% FBS. The cells were transfected with 1 μ g of recombinant DNA per plate by the calcium coprecipitation technique as modified by Wigler and co-workers (55). When the cells reached confluence, the serum concentration in the medium was changed to 5% and the culture medium was replaced every 5 days. Transformation was scored by the appearance of dense foci on the monolayer of transfected cells (46). The foci were counted after the cells were fixed with 3.7% formaldehyde in phos-

phate-buffered saline and were stained with hematoxylin for 1 h.

Transformed Rat 2 cell lines were isolated from dense foci growing on a monolayer of untransformed Rat 2 cells and were cloned from single cells by limited dilution. All cells from each line expressed viral T antigen as detected by immunofluorescent staining (the truncated T antigen present in CBRB Δ T cells was only detected as the degraded peptide, T', by immunoprecipitation).

Immunofluorescent staining. A sparse layer of cells growing on glass cover slips was fixed for 90 s with a 1:1 mixture of methanol and acetone. T antigen was detected after the cells were incubated for 30 min with SV40 anti-T serum from tumor-bearing hamsters and, after being washed thoroughly, for an additional 30 min with fluorescein-conjugated goat-anti-hamster immunoglobulin G serum (1).

Saturation density. For each transformed cell line, 12 60-mm plates were each seeded with 10^4 cells and grown overnight in medium supplemented with 10% FBS. After 1 day, the serum concentration for six plates was reduced to 1% and was kept at this concentration for the duration of the experiment. The second set of six plates continued to be fed with DMEM containing 10% FBS. The medium was replaced on all plates every third day. For each cell line in the two groups, the cells from two plates were counted with a hemacytometer at three times: at 10, 17, and 28 days for cells in the high serum concentration, and at 14, 22, and 36 days for cells in the low serum concentration. The greatest number of cells per square centimeter attained for each cell line was considered its saturation density. After reaching this density, patches of cells started to come off the plates or showed signs of dying or both.

Growth in soft agarose. Parental and transformed Rat 2 cells were suspended in medium containing 10% FBS–0.25% agarose and were seeded onto 60-mm plates having a bottom layer of 0.5% agarose medium (three plates, 10^5 cells per plate). Fresh suspension medium was added to the cells every 7 days, and after 3 weeks colonies with diameters exceeding 0.1 mm were counted in 75 to 150 fields (0.8 mm²) selected at random (20).

Immunoprecipitation. A modified immunoprecipitation procedure (5) was used to detect viral proteins in the transformed cells. Cells (5×10^5) were seeded onto 35-mm plates and were grown for 1 day. The cells were washed with phosphate-buffered saline and were incubated in 2 ml of medium consisting of 85% DMEM lacking methionine, 10% complete DMEM, 5% FBS, and 100 μ Ci of [³⁵S]methionine (specific activity, >1,000 Ci/mmol). After 12 to 16 h, the cells were washed twice with cold STE (0.15 M NaCl–0.02 M Tris hydrochloride [pH 7.2]–1 mM EDTA). All immunoprecipitation procedures were carried out at 4°C or on ice. Cells were lysed by adding 0.5 ml of RIPA buffer (1% Triton X-100–1% sodium deoxycholate–0.1% sodium dodecyl sulfate [SDS]–0.02 M Tris hydrochloride [pH 7.2]) containing 50 Kallikrein inhibitor units of aprotinin and 1 mM phenylmethylsulfonyl fluoride dissolved in ethanol and rocking the plates for 15 min. Lysates were collected and clarified by centrifugation at $25,000 \times g$ for 30 min and, after removing 3 μ l to determine trichloroacetic acid-insoluble radioactive counts, were preabsorbed with 30 μ l of a 10% suspension of fixed *Staphylococcus aureus* bacteria in RIPA buffer for 20 min. The bacteria were removed by centrifugation at $13,600 \times g$ in a microfuge for 25 s, and the preabsorbed lysates were incubated for 45 min with 5 μ l of hamster antiserum directed against SV40 T antigen or with 7 μ l of the monoclonal antibody Pab 122 (12), which recognizes cellular p53 pro-

tein. Anti-T-antigen and PAb 122 antibodies were used in excess. Immune complexes containing cellular p53 and viral T proteins were collected by using 40 μ l of a 10% fixed *S. aureus* suspension in RIPA buffer plus 0.1% bovine serum albumin. The mixture was incubated for 20 min and was washed once with high-salt buffer (1.0 M NaCl–0.5% Triton X-100–10 mM Tris hydrochloride [pH 7.2]) containing 0.5 mM phenylmethylsulfonyl fluoride and 50 Kallikrein inhibitor units of aprotinin per ml and was washed twice with RIPA buffer. The washed pellets were suspended in 20 to 40 μ l (on the basis of trichloroacetic acid-insoluble counts) of electrophoresis sample buffer (as recommended by Sigma Chemical Co., St. Louis, Mo.). Samples were heated at 90°C for 2 min and were analyzed by electrophoresis on SDS–10% and –15% polyacrylamide gels with the Laemmli buffer system (16). Protein molecular weight markers were detected by Coomassie brilliant blue staining. Gels were treated with En³Hance (New England Nuclear Corp., Boston, Mass.), and the [³⁵S]methionine-labeled proteins were visualized by fluorography.

RESULTS

Transformation of Rat 2 cells by regulatory region hybrid viral DNAs. The availability of regulatory region hybrid polyomavirus genomes (1) made it possible to examine whether JCV regulatory or early coding sequences were responsible for the restricted transforming behavior of this virus in vitro. To compare the influence of these viral sequences on the transforming efficiencies of JCV, BKV, and SV40, a cell line had to be found which could be transformed, at a detectable frequency, by all three polyomaviruses. Rat 2 cells have proven useful for investigating the transforming potential of SV40 and BKV wild-type and mutant viruses. Both viruses efficiently induce dense focus formation on a contact-inhibited monolayer of these cells. On the basis of our results with BHK-21 cells (data not shown), we expected that at least some of the chimeric genomes would transform the Rat 2 cells. To test this possibility, 8×10^5 Rat 2 cells in 60-mm plates were transfected with 1 μ g of the regulatory region hybrid or parental DNAs. The cells were maintained at confluence until focus formation was complete. Cells were fixed and stained at that time, and dense foci were counted (Fig. 1). The number of foci counted per plate was a direct indication of the transforming efficiency of each DNA. The parental DNAs pSV40 (>900 foci per plate) and pMad1-TC (<1 focus per plate) were at the high and low extremes of transforming activity, while pBKV-9 (161 foci per plate) demonstrated a moderate activity (Fig. 1A). The hybrid DNAs had activities that were intermediate between those of the two parental DNAs used to construct each chimeric genome (Fig. 1B and C). These experiments also revealed that while both regulatory and T-antigen-coding sequences of JCV contributed to the restricted transforming behavior of this virus, the latter sequences had a larger influence. Constructs containing the JCV regulatory signals [e.g., pSV40(M-1) and pBKV(M-1); Fig. 1B] induced significantly more foci than did the reciprocal constructs containing JCV T-antigen-coding information [e.g., pM-1(SV40) and pM-1(BKV); Fig. 1C].

Isolation and characterization of transformed Rat 2 cell lines. Rat 2 cells transformed by each DNA were isolated from dense foci and were cloned by limited dilution. JCV-transformed Rat 2 cell lines could not be established from the few foci induced by pMad1-TC DNA in these experiments. Although some foci contained cells expressing T protein,

FIG. 1. Transformation efficiency of regulatory region hybrid polyomavirus genomes in Rat 2 cells. Rat 2 cells (8×10^5) were transfected with 1 μ g of each recombinant DNA and were maintained at confluence until focus formation was complete; at that time, the cells were fixed and stained with hematoxylin. Data obtained from these transformation experiments are presented graphically to facilitate comparison of the effects of JCV, BKV, and SV40 regulatory and coding sequences on cellular transformation. Relative transforming efficiencies of the three parental DNAs (A) are shown. Changes in transforming efficiency following replacement of the Mad 1 early coding (B) or regulatory (C) region with that of BKV-9 or SV40 are illustrated. Cells transfected with the control pM-1(Δ Nco) and calf thymus DNAs formed less than one focus per plate after >60 days; transfection with pdl892 DNA [used to construct pM-1(SV40)] induced 687 foci per plate after 10 days. Letters at the top of the figure indicate from which DNA (M, Mad 1; B, BKV-9; S, SV40) the regulatory region (RR) and T-antigen-coding region (T) were derived. Numbers indicated on the histograms represent the average number of foci per plate induced by each DNA (three experiments, three plates per experiment). Days refer to the time at which foci were first detected on the plates. The length of this latency period was inversely proportional to the number of foci that later appeared on the plates.

these cells did not compete successfully with untransformed Rat 2 cells and were lost after being passaged.

The growth properties of the Rat 2 transformants were characterized to examine whether differences existed among the various cell lines. The abilities of each cell line to grow to high saturation density in medium supplemented with 10 or 1% FBS and to form colonies in soft agarose were determined. Results (Table 1) indicate that significant differences were not apparent between cell lines transformed by the different viral DNAs. Once transformation was established, all cloned cells had a fully transformed phenotype; they reached high saturation densities and exhibited anchorage-independent growth.

Construction of viral genomes encoding chimeric T antigens. Studies using regulatory region hybrid polyomavirus genomes indicated that the early coding sequences of JCV

TABLE 1. Characterization of Rat 2 cells transformed by regulatory region hybrid polyomavirus genomes

Cell line ^a	Saturation density ^b (no. of cells/ cm ² , 10 ⁵)		Colony formation in soft agar ^c (%)
	10% FBS	1% FBS	
Rat 2	4	2	0.02
CSV40-R2 no. 1	10	7	0.7
CSV40-R2 no. 4	10	10	1.6
CBKV-R2 no. 2	15	6	3.8
CBKV-R2 no. 3	13	3	3.5
CM1(SV40)-R2 no. 1	14	4	0.4
CM1(SV40)-R2 no. 3	8	5	0.9
CSV40(M1)-R2 no. 4	11	6	2.5
CSV40(M1)-R2 no. 6	7	6	1.8
CM1(BKV)-R2 no. 1	11	4	0.3
CM1(BKV)-R2 no. 3	9	7	1.1
CBKV(M1)-R2 no. 1	10	7	0.2
CBKV(M1)-R2 no. 2	10	4	0.3

^a Two cloned (C) transformed cell lines, isolated independently following transfection of Rat 2 (R2) cells with the recombinant DNAs shown in Fig. 1, were characterized. Mad 1 transformants had not yet been isolated at this time (see Table 2).

^b For each cell line, 12 plates (60 mm) were each seeded with 10⁴ cells. The cells were grown in medium supplemented with 10 or 1% FBS, and the numbers of cells on duplicate plates were determined after 10, 17, and 28 days or 14, 22, and 36 days, respectively. The greatest number of cells per square centimeter was taken as the saturation density, as explained in Materials and Methods.

^c For each cell line, three 60-mm plates were each seeded with 10⁵ cells in medium containing 0.25% agarose. The cells were fed once a week, and after 3 weeks, colonies with a diameter of >0.1 mm were counted (see Materials and Methods for details). The numbers in this column represent the percent cells which developed into colonies having a diameter of >0.1 mm.

contributed to the restricted transforming behavior of this virus. To investigate further the influence of these viral sequences, chimeric DNAs were constructed by exchanging sequences encoding the carboxy regions of the JCV and BKV T proteins. The restriction enzyme *Pst*I cleaves each of the JCV and BKV parental and regulatory region hybrid plasmids two times, once within the viral early coding sequences and once within the pBR322 sequences. The T-antigen chimeras were produced by exchanging the small *Pst*I fragment between pMad1-TC and pBKV-9 to yield pMRMBT and pBRBMT (Fig. 2A) and between pM-1(BKV) and pBKV(M-1) to generate pBRMBT and pMRBMT (Fig. 2B). These manipulations resulted in four new hybrid DNAs in which the carboxy regions of T antigen (amino acids 438 to 688 for JCV) and VP1 (amino acids 86 to 354 for JCV) of one viral genome had been replaced with the corresponding sequences of the second genome. Because *Pst*I and *Eco*RI (the exchange point in the late region; Fig. 2) cleaved each viral DNA at the same positions, the proper reading frames for the T and VP1 proteins were preserved, and there was no insertion or deletion of coding information.

Transformation of Rat 2 cells by T-antigen hybrid viral DNAs. The availability of parental and regulatory region-T-antigen hybrid DNAs made it possible to investigate the transforming activities of viral genomes having different combinations of regulatory sequences and amino and carboxy regions of T antigen. Rat 2 cells (8 × 10⁵ cells per 60-mm plate) were transfected with 1 μg of plasmid DNA,

FIG. 2. Construction of T-antigen hybrid polyomavirus genomes. The pMad1-TC and pBKV-9 recombinant DNAs were digested with *Pst*I to yield two restriction fragments for each DNA. The smaller *Pst*I fragment from each digest was exchanged to generate the T-antigen hybrid genomes pMRMBT and pBRBMT (A). This same construction scheme was carried out with the regulatory region hybrids pM-1(BKV) and pBKV(M-1) as the source of the *Pst*I fragments to generate the T-antigen hybrids pBRMBT and pMRBMT (B). Viral (—) and pBR322 (■) sequences are shown. Locations of the regulatory regions (RR) and the coding sequences for the early (T, t) and late (VP1, VP2, VP3) proteins (→) are indicated. The sequences exchanged (□ or ▨) during these constructions included those coding for the carboxy ends of the T and VP1 proteins.

maintained at confluence until focus formation was complete, and fixed and stained to allow enumeration of dense foci (Fig. 3). The parental and regulatory region hybrid DNAs shown in Fig. 3 have been renamed by using the T-antigen hybrid-naming scheme to facilitate comparisons between constructs. Pairwise comparisons are made of the transformation efficiencies of DNAs in which sequences have been exchanged at only one of the three possible locations: the regulatory region (Fig. 3A), the amino region of T antigen (Fig. 3B), or the carboxy region of T antigen (Fig. 3C). Those DNAs expressing the amino portion of the BKV-9 T protein by using BKV-9 regulatory signals demonstrated the greatest transforming potential. Replacement of the BKV-9 regulatory signals or BKV-9 amino-terminal sequences encoding T protein, or both, with the corresponding JCV sequences resulted in a reduction of transforming efficiency of 6-, 14-, or >100-fold, respectively. The exchange of BKV-9 and JCV sequences encoding the carboxy

FIG. 3. Transformation efficiency of T-antigen hybrid polyomavirus genomes in Rat 2 cells. Data obtained from transformation experiments are presented graphically to facilitate the comparisons of transformation efficiencies of parental and hybrid JCV and BKV genomes as described in the legend to Fig. 1. Changes in transforming efficiency following the replacement of the Mad 1 regulatory region (RR) (A), the amino portion of T antigen (B), or the carboxy portion of T antigen (C) with the corresponding regions of the BKV-9 genome are illustrated. The underlined letter in the name of each recombinant indicates the exchanged sequences being compared. By using the scheme followed for the T-antigen hybrid constructs to allow direct comparisons of the various DNAs, the names of the four parental and regulatory region hybrid DNAs were changed as follows: pBKV-9 to pBRBBT, pM-1(BKV) to pBRMMT, pBKV(M-1) to pMRBBT, and pMad1-TC to pMRMMT. Cells transfected with calf thymus DNA formed one focus per plate after >60 days; cells transfected with pM-1(Δ 98) and pBRBBT formed two foci per plate after 51 and 47 days, respectively. Numbers indicated on the histograms represent the average number of foci per plate induced by each DNA (three experiments, four plates per experiment). Days refer to the time at which foci were first detected on the plates. The length of this latency period was inversely proportional to the number of foci that later appeared on the plates.

region of T protein had little effect on transforming activity. In agreement with our findings for regulatory region hybrid DNAs, the results implicate more specifically two regions of the JCV genome which are responsible for the restricted transforming behavior of the virus in vitro: the sequence encoding the amino portion of T protein and, to a lesser extent, the regulatory region. The control DNAs pM-1(Δ 98), a JCV deletion mutant containing only one copy of the 98-nucleotide-pair tandem repeat, and pBRBBT, a BKV-9 deletion mutant lacking the carboxy-terminal sequences of T antigen [the poly(A) signal is retained], transformed Rat 2 cells slightly, but reproducibly, better than did the prototype JCV plasmid (pMad1-TC). Cell lines transformed by these two control plasmids were also easier to establish than JCV transformants.

Two cell lines transformed independently by each of the parental and hybrid DNAs were isolated and cloned from these experiments (only one line each of CM1-R2 and CMRMBT-R2 could be established). Each cell line was tested for the ability to form colonies in soft agarose. While most transformants exhibited anchorage-independent growth, differences in colony-forming ability were often greater between a pair of cell lines transformed by the same DNA than between cell lines transformed by different DNAs (Table 2). Therefore, as in Table 1, no correlation was established between the extent of anchorage independence of a cell line and the viral DNA that initiated the transformation event.

Viral proteins expressed by transformed cell lines. To further characterize the transformed cell lines listed in Tables 1 and 2, viral proteins expressed in each transformant were analyzed. Cells (1×10^6) were labeled with [35 S]methionine, and cellular extracts were prepared 16 h

later. Proteins were immunoprecipitated with antisera obtained from hamsters bearing SV40- or JCV-induced tumors, were electrophoresed on SDS-polyacrylamide gels, and were visualized by fluorography. The viral early and cellular p53 proteins expressed in Rat 2 cells transformed by parental and regulatory region hybrid DNAs are shown in Fig. 4, and the proteins immunoprecipitated from extracts of Rat 2 cells transformed by parental and regulatory region-T-antigen hybrid DNAs are shown in Fig. 5. To improve the resolution of the bands representing large T and small t proteins, two polyacrylamide gels were used: 10% polyacrylamide for T (Fig. 4A and 5A) and 15% polyacrylamide for t (Fig. 4B and 5B). Similar immunoprecipitation patterns were observed when the second of a pair of cloned cell lines representing the same recombinant DNA was tested (data not shown).

Large T proteins were expressed in all transformed cell lines, although only degradation products of T could be detected in CBRBBT-R2 cells (Fig. 5). This cell line is expected to produce a truncated, potentially unstable BKV-9 T antigen. The T protein from each Rat 2 transformant migrated as a doublet band (Fig. 4A and 5A). The SV40 T protein had an apparent molecular mass of 90,000 daltons (90 kDa), and, as predicted from sequence data (9), the JCV and BKV-9 T antigens migrated as slightly smaller proteins (88 to 90 kDa). The electrophoretic mobilities of the different T proteins were a function of the carboxy-terminal sequences of the proteins (e.g., the T antigens present in the cells transformed by pMad1-TC, pM-1(SV40), pM-1(BKV), pBRBMT, and pMRBMT all had JCV carboxy-terminal sequences and all migrated at the same position on the gels).

Small t proteins were expressed in every transformant. The SV40, JCV, and BKV-9 small t antigens had apparent molecular masses of 20, 21, and 21 kDa, respectively.

TABLE 2. Anchorage independence of Rat 2 cells transformed by T-antigen hybrid polyomavirus genomes^a

Cell line ^b	Colony formation in soft agarose ^c (%)
Rat 2	<0.02 ^d
CM1Δ98-R2 no. 11	3.5
CM1Δ98-R2 no. 12	<0.02 ^e
CM1-R2 no. 12	1.3
CMRMBT-R2 no. 11	0.02
CMRBMT-R2 no. 11	0.1
CMRBMT-R2 no. 12	0.5
CM1(BKV)-R2 no. 12	0.4
CM1(BKV)-R2 no. 13	0.3
CBKV-R2 no. 11	0.5
CBKV-R2 no. 13	0.2
CBRMBT-R2 no. 11	2.1
CBRMBT-R2 no. 12	0.6
CBRBMT-R2 no. 11	0.4
CBRBMT-R2 no. 12	0.8
CBKV(M1)-R2 no. 12	0.4
CBKV(M1)-R2 no. 13	0.6

^a For each cell line, three 60-mm plates were each seeded with 10⁵ cells in medium containing 0.25% agarose. The cells were fed once a week, and after 3 weeks, colonies with a diameter of >0.1 mm were counted (see Materials and Methods for details).

^b Two cloned (C) transformed cell lines, isolated independently following transfection of Rat 2 (R2) cells with the recombinant DNAs shown in Fig. 3, were characterized. For pMRMT and pMRMBT, only one transformed cell line could be isolated.

^c Percentage of cells developing into colonies with diameters of >0.1 mm.

^d Only single cells could be detected.

^e Contained a few colonies with diameters of <0.1 mm.

Autoradiograms of the SDS-15% polyacrylamide gels revealed additional bands at positions slightly below those representing small t (Fig. 4B and 5B). The band profiles were identical for cells transformed by recombinant DNAs having the same early coding region, thus indicating that these peptides represented breakdown products of T (T'). The T' peptides were more prominent in extracts of cells transformed by JCV or BKV-9 than in those transformed by SV40 early-region constructs.

The cellular phosphoprotein p53 was detected in all cell extracts in quantities that generally correlated with the level of T-antigen expression (Fig. 4 and 5), indicating that p53 forms stable complexes with the large T antigens of all three polyomaviruses. Substituting the monoclonal antibody PAb 122, which recognizes p53, for the anti-T hamster serum used in these immunoprecipitation experiments suggested that nearly all of the p53 present in the cell extracts was associated with T antigen and that only a subpopulation of this viral protein was bound to p53 (Fig. 5 and 6).

DISCUSSION

The focus of our laboratory is to understand the molecular basis for the unique biology of the human polyomavirus JCV. The objective of this study was to identify those regions of the JCV genome which contribute to the restricted

FIG. 4. Expression of early viral proteins in Rat 2 cells transformed by regulatory region hybrid polyomavirus genomes. Proteins extracted from cloned Rat 2 transformants were immunoprecipitated with anti-SV40 T hamster serum and analyzed by SDS-polyacrylamide gel electrophoresis (5). Two gels, containing different polyacrylamide concentrations, were used to run each set of immunoprecipitated extracts. The 10% polyacrylamide gel (A) permitted a better distinction of the large T proteins, and the 15% polyacrylamide gel (B) allowed us to detect the small-t- and large-T-related (T') proteins. This two-gel approach proved superior to the use of gradient gels for resolving the various proteins. Protein molecular weight markers were visualized by Coomassie brilliant blue staining and ³⁵S-labeled viral proteins were visualized by fluorography. Locations of large T, p53, small t, and T' proteins are indicated. K, Molecular mass in thousands.

transforming behavior of this virus. Our approach has involved the construction of hybrid viral genomes in which the regulatory sequences and portions of the viral T-antigen-coding region of JCV were replaced with the corresponding sequences of BKV and SV40. The transforming capabilities of the parental and chimeric DNAs were then tested in nonpermissive rat cells in vitro.

The exchange of genetic information between pairs of the three viruses was accomplished in such a way as to minimize

FIG. 5. Expression of early viral proteins in Rat 2 cells transformed by T-antigen hybrid polyomavirus genomes. Proteins extracted from cloned Rat 2 transformants were immunoprecipitated with anti-SV40 T hamster serum and were analyzed by SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 4. Immunoprecipitated cell extracts run on 10% (A) and 15% (B) polyacrylamide gels are shown. CSV40-R2, which expresses both early SV40 proteins, and CBR Δ T-R2, which expresses a truncated BKV large T protein and a full-length small t protein, were included as controls. K, Molecular mass in thousands.

deleterious alterations to viral regulatory signals and proteins; intact regulatory regions were exchanged, and the joining of sequences encoding amino or carboxy portions of two different T proteins did not result in the insertion or deletion of any amino acids. The choice of the hybrid over a mutagenesis approach was based on the fact that the efficiency of transformation by wild-type JCV is already so low that mutating the virus would have little additional effect. On the other hand, the replacement of JCV sequences with more active BKV or SV40 sequences might elevate transforming potential and thereby point to specific regions of the JCV genome responsible for the poor transforming activity of the virus.

Rat 2 cells proved to be a useful line for our transformation studies; they maintain their contact-inhibited nature even 2 months after reaching confluence, and they fail to grow in semisolid medium. Furthermore, Rat 2 cells are transformed efficiently by SV40 and BKV-9 DNAs and inefficiently by JCV DNA. These cells allowed us to quantitate the transforming efficiencies of the parental and chimeric viral genomes.

The results described in this paper indicate that two regions of the JCV genome contribute to the restricted transforming behavior of this virus in vitro: the regulatory region and the region encoding the amino-terminal portion of the large T protein. Alterations of either of these regions are known to affect the transforming activities of the polyomaviruses. Some rearrangements of the regulatory sequences of the SV40 and BKV genomes have been shown to increase oncogenic potential (39, 51, 54). For example, removal of one or two of the three tandem repeats representing the BKV enhancer results in a several-fold enhancement of transforming capability (13, 53), while replacement of the SV40 enhancer with the long terminal repeat of Harvey murine sarcoma virus increases the transforming efficiency of this virus in NIH 3T3 cells (15). In regard to the BKV enhancer results, it is important to point out that one of our constructs, pM-1(Δ 98), in which one copy of the 98-nucleotide-pair repeats has been deleted, transformed better than the intact pMad1-TC DNA; the efficiency was slightly higher (Fig. 3) and the transformants were easier to isolate. It is not known at present whether this increase in transforming activity occurs by a process analogous to that operating in BKV or whether it is due to the fact that, in JCV, this deletion removes a second TATA box that might interfere with T-antigen expression (9).

Although the large T protein of BKV has not been examined in detail, a large number of studies have focused on the corresponding protein of SV40. These studies indicate that alterations of sequences encoding the amino portion of the T antigen, as well as of carboxy-terminal sequences, can elevate or depress the transforming activity of SV40 (for review, see reference 28). In many of these experiments, it has been difficult to determine whether the mutations directly or indirectly affected a transformation domain of the T protein. For example, Tevethia (44) concluded that the carboxy region was important, either because this portion of T antigen contained a separate transformation-enhancing function or because it was involved in maintaining a functional structure of T protein. Since our T-antigen hybrid constructs encoded chimeric T proteins that demonstrated transforming activities equivalent to or greater than that of the intact JCV protein, we believe that the structural integrity of the chimeric proteins has been maintained and that changes in transformation efficiency reflect the exchange of one or more transformation domains. Chimeras having BKV amino-terminal sequences of T protein transformed more efficiently than those containing the corresponding JCV sequences. Recently, this region of the SV40 T antigen was shown to contain a binding domain (amino acids 105 to 120) (6) for the retinoblastoma susceptibility gene product. Mutations to these sequences reduced transforming activity. The JCV and BKV T proteins differ here from the SV40 protein in six and five residues, respectively; it is not known whether these two proteins interact with the retinoblastoma susceptibility gene product *in vivo*. While the exchange of amino-terminal sequences of the JCV and BKV T proteins altered transforming efficiency significantly, the exchange of carboxy-terminal sequences had little effect. This latter

finding suggests that either the carboxy region lacks a transformation-enhancing function or this portion of the JCV and BKV T antigens has an equivalent activity. Recent studies with JCV-SV40 T-antigen hybrids support the latter possibility and indicate that this portion of the JCV and BKV T antigens is less active than the same region of the SV40 protein (S. Haggerty, D. Walker, and R. J. Frisque, submitted for publication).

While many small alterations have been introduced into the T-antigen-coding region, a number of large insertions and deletions have also been produced in the A gene. Sompayrac and Danna (40) investigated the SV40 mutant *F8dl*, which encodes a truncated T protein (40% wild-type length), and found that it transformed Fisher rat F111 and mouse BALB/3T3 cells with wild-type efficiency. The same mutant transformed C3H10T1/2 mouse cells with only 1% of the efficiency of wild-type SV40. It was suggested that transformation of some cells might require increased levels of the truncated protein. The transformation efficiency of one of our control BKV plasmids, pBRBΔT (nucleotides 2897 to 3447 deleted), was 1% of that of the BKV-9 plasmid (Fig. 3). As with *F8dl* transformants, cells transformed by pBRBΔT expressed T protein and an unstable truncated T protein (Fig. 5). In these examples, it is possible that decreased transforming activity resulted both from the absence of the carboxy-transforming domain and from the production of an unstable T protein (26, 40, 45).

The ability of polyomavirus transformants to grow under anchorage-independent conditions is a stringent transformation parameter. Although we detected differences in the efficiencies with which chimeric DNAs induced transformation, we were unable to demonstrate consistent phenotypic differences in the cells once the transformation event was established. Our inability to detect such variations might be the result of our selection procedure—cells were isolated on the basis of a transformation parameter (dense focus formation)—or the result of examining too few cell lines (two clones for each set of chimera-specific transformants) to see a consistent pattern. An alternative explanation is that the various T antigens may differ in their initiation, but not maintenance, functions for transformation, and, therefore, once cells were transformed they would exhibit similar phenotypes.

The analysis of viral proteins present in extracts of transformed cells by immunoprecipitation and electrophoresis on SDS-polyacrylamide gels revealed that the T antigens of the Rat 2 transformants migrated as doublet bands. Similar findings have been reported for some cells transformed by SV40 (36) and may involve the tandem integration of early viral coding sequences (18). The T proteins of all three parental viral DNAs migrated more slowly than predicted from sequence data (Fig. 4A and 5A), with SV40 T appearing to be the largest (~90 kDa) and JCV T being the smallest (~88 kDa). The aberrant mobilities have been shown to be a function of the carboxy-terminal sequences of T antigen (2). As expected, all hybrid T proteins migrated with the same mobility as the wild-type T protein sharing the same carboxy terminus (Fig. 5A).

These studies demonstrated that the expression of T antigen varied greatly in the cells transformed by the different DNAs. Large amounts of this protein were produced in SV40 transformants, intermediate levels were produced in BKV-9 transformants, and low amounts were produced in the JCV-transformed lines. Cells transformed by hybrid constructs containing a JCV regulatory region expressed reduced levels of T protein relative to those transformed by

constructs containing the regulatory sequences of SV40 or BKV-9 (Fig. 4A and 5A). It is important to note that the patterns of T protein (as well as t and p53 protein; see below) expression were consistent for a second cell line transformed by the same DNA (data not shown; only one line transformed by either pMad1-TC or pMRMBT DNA was available for these experiments).

On the basis of our analysis of the immunoprecipitated proteins on the SDS-polyacrylamide gels, the SV40 T protein was more stable than those of JCV and BKV. Viral proteins (T') migrating slightly faster (~17 kDa) than the small t protein were detected in all cell lines transformed by JCV and BKV wild-type or hybrid T antigens (Fig. 4B and 5B). Identification of these peptides as degradation products of T protein was supported by the observation that the T'-band patterns were identical for those cells transformed by constructs expressing the same T protein (regardless of the regulatory sequences present). In the cells transformed by the control plasmid pBRBΔT, the truncated T protein was not detected and only the T' peptides were observed. Similar patterns have been reported for cells transformed by SV40-truncated T antigens (26, 40).

The large T antigens of all three polyomaviruses formed stable complexes in Rat 2 cells with the cellular phosphoprotein p53. Earlier reports suggested that little or no p53 was present in JCV-transformed hamster (10) or monkey (21) cells, respectively. The amounts of coprecipitated p53 in all cell lines correlated with the levels of T protein expressed (Fig. 4A and 5A). Incubation of the transformed cell extracts with either anti-T hamster serum or the monoclonal antibody PAb 122 resulted in the immunoprecipitation of nearly equal amounts of p53, indicating that most, if not all, of the cellular phosphoprotein was in complex with T antigen (Fig. 6). In these same experiments, less T antigen was brought down with PAb 122 than with anti-T serum, suggesting that only a subpopulation of the viral T antigen was bound to p53. Determination of the amount of radioactivity present in each protein band indicated that this subpopulation represented a smaller percentage of the total JCV T-antigen species (~10%) compared with those of SV40 and BKV (~30 to 50%) (Fig. 6). These findings, coupled with the suggestion that the T-antigen-p53 interaction involves mid-region to carboxy-terminal sequences of the viral protein (27, 43, 45), would explain the observation that the hybrid T antigens containing a JCV carboxy terminus (amino acids 438 to 688) bound proportionally less p53 than those with a BKV carboxy terminus (Fig. 5 and 6).

These studies are the first to detect a JCV small t protein; several other reports failed to identify this protein, leading to speculation that its absence might explain the poor transforming ability of JCV (10, 22). Although the predicted molecular mass of the t protein for each of the three viruses is about 20 kDa, the proteins from the two human viruses appeared to be slightly larger (~21 kDa) than their SV40 counterpart on our gels (Fig. 4B and 5B). To confirm the identification of the JCV small t protein, immunoprecipitates of extracts of BHK-21 cells transformed by the JCV small t mutant K-1 (23) have been analyzed, and the band identified as small t has been shown to be absent in these cells (data not shown).

The aim of this investigation was to determine the regions of the JCV genome which contribute to the restricted transforming activity of this virus. By using parental and chimeric DNAs composed of JCV, BKV, and SV40 sequences, it was found that both the regulatory and early coding regions of JCV were responsible for this behavior. The study was

FIG. 6. Expression of p53 in Rat 2 cells transformed by parental and hybrid polyomavirus genomes. Proteins extracted from cloned Rat 2 transformants were immunoprecipitated with p53-specific monoclonal antibody PAb 122 (11) and were analyzed on SDS-10% polyacrylamide gels. The ratios of T antigen to p53 precipitated with anti-T serum or with PAb 122 were directly compared on the same gel for cell lines transformed by pSV40, pMad1-TC, and pBKV-9. Proteins precipitated from these transformed cell extracts by using normal hamster serum (N) are also shown. K, Molecular mass in thousands.

extended to an analysis of the viral proteins expressed in Rat 2 cell transformants. Cell lines transformed by the parental DNAs expressed different amounts of T protein, with the SV40 transformants expressing the most T antigen and JCV transformants expressing the least. All three T antigens formed stable complexes with the cellular phosphoprotein p53, although the T-antigen subpopulation that participated in the interaction with p53 represented a smaller percentage of the total T protein in JCV transformants than in SV40 and BKV transformants. For the first time, a JCV small t protein was detected in transformed cells. Small peptides that migrated ahead of the t protein on the SDS-polyacrylamide gels were identified as large T degradation products, indicating that the large T antigens of JCV and BKV were less stable than that of SV40. The low transforming activity of JCV relative to those of the other polyomaviruses may result from a combination of factors, including reduced expression of the early proteins from less active, or perhaps more cell-specific, promoter-enhancer elements, decreased stability of the transforming protein, reduced stabilization of p53, and differences in another, as yet unidentified, transformation domain within the amino portion of the T protein.

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