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JC Virus-Simian Virus 40 Genomes Containing Heterologous Regulatory Signals and Chimeric Early Regions: Identification of Regions Restricting Transformation by JC Virus

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The papovavirus JC virus (JCV) is highly oncogenic in experimental animals but, unlike simian virus 40 (SV40), is severely restricted in its ability to transform cells in culture. We exploited the close genetic relatedness of these two viruses to delimit region(s) of the T protein which can restrict transforming activity. Novel chimeric genomes were produced by exchanging various segments of the JCV and SV40 T-protein-coding regions. These DNA constructs specified early proteins with in-frame substitutions of analogous amino acid sequences. A second set of genomes was prepared which, in addition to chimeric early proteins, contained substituted regulatory regions. The transformation efficiencies of these chimeric genomes were intermediate between those of SV40 and JCV, with the source of T protein exerting a greater effect than that of the regulatory region. The ability of certain constructs to induce efficient transformation required the presence of an SV40 regulatory region or specific sequences within the SV40 early coding region. Cloned cell lines prepared from representative transformants were characterized; the ability to form colonies in soft agarose was investigated, and the presence of viral T and cellular p53 proteins was determined. The various T proteins differed in amount, stability, and the ability to form stable complexes with p53.

Infection by JC virus (JCV) is prevalent in the human population (25, 47) and under certain conditions may lead to the fatal brain disease progressive multifocal leukoencephalopathy (68). Evidence indicates that the virus may persist in human kidneys (12, 17, 28) and mononuclear bone marrow cells (29). JCV is highly oncogenic in hamsters and rats (46, 48, 69) and is the only human virus known to cause solid tumors in primates (37, 53). Although biologically active in vivo, JCV exhibits a very restricted behavior in vitro; it transforms cells inefficiently and multiplies in a limited number of cell types (67).

Early immunological and physical studies indicate that JCV and simian virus 40 (SV40) are closely related; the proteins strongly cross-react antigenically (68) and the genomes contain many structural features in common (23, 42). DNA sequencing confirms a high degree of sequence homology (ca. 70% overall) and a similar genetic organization of the two viral genomes (23).

Alterations to the large (T) and small (t) tumor antigens and the regulatory region are known to affect the efficiency of transformation by the polyomaviruses. The expression of T protein is necessary and sufficient for the initiation and maintenance of cellular transformation in vitro and for the induction of tumors in vivo (64). The SV40 T antigen also plays several roles in viral DNA replication and transcription (36). Many of these activities have been assigned to discrete domains within this protein (see Fig. 1; for a review, see reference 20); however, transformation-related functions have been more difficult to localize. The N-terminal half of T antigen has been shown to immortalize rat embryo cells (3, 10, 16, 59) and to have significant transforming activity (14, 16, 49, 58–60). The region encompassing amino acids 105 to 114 is important for the induction of focus formation and growth in soft agar by established cell lines (11, 30) and has recently been suggested to be part of a binding domain for the retinoblastoma susceptibility gene product (RB) (19). This region is conserved among the transforming proteins of certain tumor viruses and a cellular oncogene (21); chimeras produced by the exchange of this region between the SV40 and adenovirus E1A proteins retain transforming activity (44). Other studies have suggested that the transforming activity of T antigen may be related to its ability to bind and stabilize the growth-regulating cellular phosphoprotein p53 (41, 45, 52); the domain for this function was localized to amino acids 271 to 708 (34, 43). More-specific activities of T antigen required for its oncogenic behavior are not yet defined but may be related to the ability of T antigen to stimulate the metabolism of the host cell (64).

Small t antigen is functionally distinct from T antigen and is required for the expression of some aspects of the transformed phenotype (64). Its effect is seen only in certain transformation assays; for example, quiescent rat cells transfected with SV40 small t antigen deletion mutants form dense foci but are unable to grow in soft agar (7, 54, 56). A recent report indicates that the expression of t antigen in mouse cells enhances anchorage-independent growth when the concentration of T antigen is low (5).

The noncoding regulatory region contains the viral promoter-enhancer elements and the origin of DNA replication. Alterations in this region are known to affect the transformation efficiency of the polyomaviruses (27, 32, 57, 70, 71).

Transformation by the polyomaviruses provides a model system in which to study oncogenic behavior. The long-range goal of our research is to use the restricted transforming ability of JCV as a tool to resolve the molecular events involved in the transformation process. This article presents the initial steps in this effort by identifying the general regions of the JCV early coding sequences which reduce its

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transformation potential. Eight chimeric genomes were constructed in which noncoding regulatory sequences and various segments of the JCV early coding region were replaced with the corresponding segments of SV40 DNA. Since SV40 and JCV are similar in genomic organization and exhibit extensive DNA and amino acid homology (23; see Fig. 2), the chimeric genomes can be considered polyomavirus variants. Analysis of these variants has led to the identification of sequences which contribute to a more active transformation phenotype.

MATERIALS AND METHODS

Rat 2 cells. The Rat 2 cell line is derived from Rat 1, a rat fibroblastoid 3T3-like cell line (65). These cells have been recloned to maintain a flat untransformed morphology and were routinely passed 1:20 at subconfluence. The cell line was propagated in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (99 U/ml), and streptomycin (73 U/ml) and was incubated at 37°C in a humidified 10% CO_2 atmosphere.

Recombinant plasmids. All recombinant plasmids used in this study contained full-length viral genomes joined to pBR322 at the unique *Eco*RI sites. A descriptive nomenclature has been used for the four parental plasmids used to create the T-antigen chimeras. The name pMad1-TC indicates that this recombinant plasmid contains viral DNA from the prototype JCV(Mad-1) strain (22) which had been propagated in tissue culture. pSV40 has a similar construction in which the DNA from the SV40 small-plaque strain 776 was used. The parental regulatory region hybrid plasmids pM-1(SV40) and pSV40(M-1) contain the coding sequences of one virus joined to the regulatory region of the other virus (regulatory region source in parentheses; for construction details, see reference 13). The pM-1(SV40) recombinant was recloned into full-length pBR322 (the original hybrid construct contained a deletion within pBR322 that spanned the *Cla*I site) (13) at the start of these studies to allow construction of some T-antigen chimeras.

Chimeric genome construction. The construction schemes for eight chimeric JCV-SV40 genomes are diagrammed (see Fig. 3). Recombinant parental plasmids were digested with *Cla*I and either *Nsi*I or *Bst*XI to generate the fragments (2 to 7 kilobases) that were to be exchanged between the viruses. When partial *Nsi*I digestions were required, a population of linear, full-length *Nsi*I-generated fragments was isolated from a preparative gel prior to complete digestion with *Cla*I. The appropriate fragments were resolved and extracted from agarose gels by a modification of the collapsing-gel DNA elution procedure (55). Solutions containing the DNA fragments (75 to 200 μl) were dialyzed on small nitrocellulose disks (0.025 μm; VSWP 02500, Millipore Corp.) against TE buffer to remove salts and contaminating nucleotides prior to ligation (38).

Recombinant DNAs containing chimeric genomes were extensively analyzed by restriction enzyme digestion to ensure the presence of the appropriate number and size of DNA fragments. After identification of the appropriate clones, large-scale preparations of the recombinant plasmids were made (6). To confirm that the correct sequences were present at the junction boundaries, fragments encompassing each of these sites were end labeled with ^{32}P and were sequenced by the chemical cleavage procedure of Maxam and Gilbert (39). The labeled reaction products were resolved on 8 and 12% polyacrylamide gels containing 8 M urea. Autoradiography proceeded for 12 h to 2.5 days at −70°C.

Rat 2 transformation assay. The focus formation assay was a modification of the procedure described by Todaro and Green (63). Briefly, each of five 60-mm dishes per DNA sample was seeded with 4×10^5 cells; at the time of transfection (12 to 16 h later), the cells were 40 to 60% confluent. The calcium phosphate-DNA mixture (72), containing 1 μg of sample DNA and 9 μg of carrier (calf thymus) DNA per 0.5-ml aliquot, was added to each dish. Five hours later the medium was removed from the plates, the cells were rinsed once with DMEM without serum, and the medium (DMEM plus 10% fetal bovine serum) was replaced. When cells were nearly confluent, the serum concentration was reduced to 5%. The cells were refed every 4 days. Every other day, plates were monitored by reflected light for regions containing refractile areas. The presence of dense foci in these areas was then confirmed by phase-contrast microscopy, and the day on which foci were first seen for each sample was noted. When foci were large but not coalescing, the cell sheets on four dishes were fixed with 3.5% formaldehyde in phosphate-buffered saline (PBS) and were stained with hematoxylin. On each day that transformed cells were fixed, cells on a mock-transfected (calf thymus DNA only) dish and an untransfected dish were also fixed to enable the assessment of spontaneously arising foci. Cells growing on cover slips in the fifth dish were removed at various times after transfection. The cells were fixed in methanol-acetone (1:1) and were stained for the presence of T-antigen-containing nuclei by the indirect immunofluorescence method.

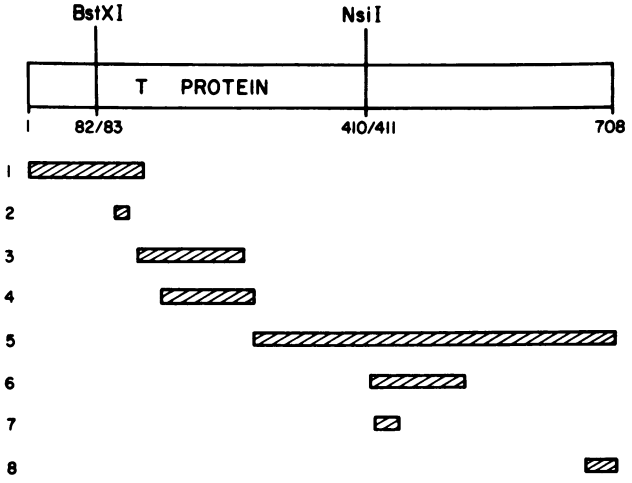
Cloning of transformed cell lines. Cell lines were prepared from the foci which developed in the Rat 2 transformation assay. Prior to formaldehyde fixation, a representative focus in each 60-mm dish was identified, scored with a Pasteur pipette, aspirated from the monolayer, and expanded into a mass culture. At subconfluence, the cells were harvested and 20 to 100 cells were added to each of six 100-mm dishes. Single cells were allowed to grow into colonies for at least 2 weeks. Isolated colonies (only one from each original dish) which exhibited transformed phenotypes were selected and established as cell lines. The presence of T antigen was verified by indirect immunofluorescence.

Fluorescent antibody staining. DNA-transfected Rat 2 cells growing on cover slips were rinsed in PBS, air dried, and fixed in acetone-methanol (1:1) at room temperature for 90 s. Cover slips were overlaid with serum derived from a hamster bearing an SV40-induced tumor and were incubated for 45 min at 37°C. The cells were then rinsed in PBS. After an additional incubation with fluorescein-conjugated goat anti-hamster immunoglobulin G, the cells were extensively rinsed in PBS, followed by one rinse with double-distilled water. The cells were observed with a Diaphot-TMD microscope (Nikon) with a TMD-EF epifluorescence attachment.

Immunoprecipitation of proteins in transformed cells. Transformed Rat 2 cells were seeded at a density of 2×10^5 to 7×10^5 cells per 35-mm dish. Approximately 12 to 18 h later, the cells were rinsed with warm PBS and were metabolically labeled in 2 ml of DMEM containing 10% of the normal concentration of methionine supplemented with 5% fetal bovine serum and 50 μCi of [^{35}S]methionine (1,000 Ci/mmol). After incubation for 12 to 16 h, the cells were rinsed in STE, suspended in 0.5 ml of RIPA buffer, and solubilized as described by Coussens et al. (18). The lysate was clarified, and the supernatant was preabsorbed with protein A-containing *Staphylococcus aureus*. The specific antibody used for the formation of the immune complexes was derived from hamsters bearing SV40-induced tumors

(anti-T) or cell culture supernatants (monoclonal anti-p53 antibody PAb 122) (26). Protein A-containing *S. aureus* was suspended in RIPA buffer (18) containing 1 mg of bovine serum albumin per ml and was then used to precipitate the immune complexes as described elsewhere (18). The bacteria bound to the immune complexes were washed once with 1.0 M NaCl–10 mM Tris hydrochloride (pH 7.2)–0.5% Triton X-100 and twice with RIPA buffer; all solutions contained aprotinin and phenylmethylsulfonyl fluoride. The samples were suspended in volumes of electrophoresis sample buffer sufficient to normalize the trichloroacetic acid-precipitable radioactivity. The immune complexes were solubilized by being boiled for 2 min in the presence of sodium dodecyl sulfate and β-mercaptoethanol, and the mixtures were centrifuged at 12,000 × *g* for 2 min. Samples containing equivalent total counts were electrophoresed through discontinuous 10 and 15% sodium dodecyl sulfate-polyacrylamide gels (33). The gels were dried and subjected to fluorography for 4 to 36 days. Equal-area regions of two similar dried gels, which contained the T and p53 protein bands, were rehydrated and then solubilized in 30% hydrogen peroxide for 5 h at 100°C. The radioactive mixture was redissolved in 500 μl of distilled water and was counted in an LS 5801 liquid scintillation counter (Beckman Instruments, Inc.). Control values, obtained from similar-area regions directly above the p53 bands, were subtracted from the average T and p53 values prior to the calculation of the T/p53 ratios. Fluorographs were also scanned with an UltroScan XL laser densitometer (LKB Instruments, Inc.), and the ratios of the amounts of T and p53 proteins were compared.

Anchorage-independent growth assay. Cells representing each cell line (1×10^5) were suspended in DMEM containing 0.25% agarose and were layered onto 0.5% agarose medium in each of three 60-mm dishes. Cells were fed once a week with 3 ml of 0.25% agarose medium. DMEM was supplemented with 10% fetal bovine serum throughout the study. After 3 weeks, 25 fields from each of the three dishes were randomly examined, and colonies with diameters larger than 0.1 mm were counted. The percentage of plated cells which were able to grow suspended in soft agarose was calculated.



	DOMAIN	AMINO ACIDS
1	Immortalization	1-137
2	Retinoblastoma Gene Product Binding	105-120
3	Specific Origin Binding	131-259
4	Stimulation of Cell DNA Synthesis	160-272
5	p53 Binding	271-708
6	ATP Binding	413-528
7	ATP'ase Consensus Sequence	418-447
8	Adenovirus Helper/Host Range Effect	674-708

FIG. 1. Approximate boundaries of functional domains of the SV40 T protein. The approximate locations are indicated, as previously reported (immortalization [3]; RB binding [19, 21]; specific origin binding [2, 50]; stimulation of cell DNA synthesis [61]; p53 binding [34, 43]; ATP binding [8, 9, 15]; ATPase consensus sequence [1]; and adenovirus helper-host range effect [66]). The restriction endonucleases *Bst*XI and *Nsi*I were used to create the JCV-SV40 chimeric T antigens described in the text. Positions of the cleavage sites for these enzymes relative to the T-antigen domains are shown.

RESULTS

Construction and verification of recombinant genomes. To determine the regions of JCV T antigen which contribute to restricted transformation, recombinant genomes encoding chimeric T antigens were constructed by exchanging corresponding segments of SV40 and JCV DNA. The locations of restriction enzyme cleavage sites used as exchange points for these switches are indicated in Fig. 1 relative to functional domains proposed for SV40 T antigen (for a review, see reference 20). Because the cleavages resulted in switches between analogous amino acids, the continuity and reading frame of the early coding region were conserved, allowing intact chimeric viral proteins to be expressed.

Figure 2 represents a polyomavirus genome cloned into pBR322 and serves as a key for details of the chimeric genome construction scheme shown in Fig. 3. The parental genomes have already been described (13). In general, the exchanged DNA fragments contained plasmid and viral sequences and were produced by digestion with *Cla*I and either *Bst*XI or *Nsi*I. Prior to the construction of each chimeric genome, it was necessary that both parental recombinant genomes have an unaltered *Cla*I site and exhibit the same orientation of plasmid to viral sequences. The orientation of the plasmid in the parental pSV40 clone (13) was reversed prior to construction of the hybrids to align it with the orientation of pMad1-TC. The original pM-1(SV40) recombinant contained a deletion in the pBR322 vector which had removed the *Cla*I site (13); therefore, M-1(SV40) was recloned into full-length pBR322.

To assess the contribution of the regulatory sequences to the restricted transforming activity of JCV, the construction scheme was repeated with the hybrid parental genomes, pSV40(M-1) and pM-1(SV40), which contain heterologous regulatory regions (the source of the regulatory region sequences is indicated in parentheses; Fig. 3) (13). The first 10 amino acids of the early (T- and t-) coding regions are the same in JCV and SV40 (23); therefore, the early protein sequences were unaltered by these regulatory region substitutions (which included the first 29 base pairs of the early protein-coding sequences). The compositions of the functional sequences within each of the recombinant genomes are summarized in Table 1.

The chimeric genomes have been given designations following the format $pX_1RX_2X_3T$-E. This is a descriptive nomenclature in which the viral source of the regulatory region, N-terminal, and C-terminal sequences of T antigen are indicated, as well as the restriction enzyme used to

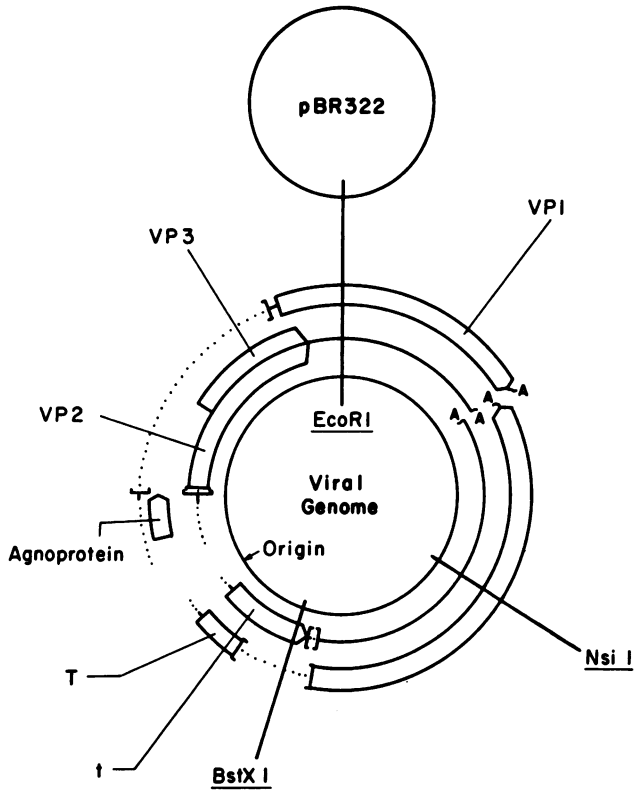


FIG. 2. Locations of restriction enzyme junction sites within a representative recombinant polyomavirus genome. A JCV or SV40 genome is shown ligated to pBR322 sequences at the *Eco*RI site; the circles representing the two DNAs are drawn to scale. The early (T and t) and late (VP1, -2, and -3) viral proteins and the agnoprotein are depicted, and the transcriptional directions of the coding regions are indicated (▭). A, Poly(A) tail of the viral mRNAs. Regulatory sequences within the noncoding region include the origin of viral DNA replication (Origin). The restriction enzymes used in the chimera construction divided the T-antigen-coding region into three parts: the amino portion extends from the N terminus to the *Bst*XI site (amino acids 1 to 82 for SV40 and 1 to 81 for JCV), the central region includes sequences between the *Bst*XI and *Nsi*I sites (amino acids 83 to 410 for SV40 and 82 to 411 for JCV), and the carboxy sequences extend from the *Nsi*I site to the C terminus (amino acids 411 to 708 for SV40 and 412 to 688 for JCV).

create the sequence switch within T antigen. X_1 to X_3, when replaced by either S or M, identify the source of the viral DNA as SV40 or JCV(Mad-1), respectively. X_1 designates the parental DNA that donated the regulatory region (R), whereas X_2 and X_3 indicate the viral DNA source for the N-terminal or C-terminal T-antigen sequences (T), respectively. The name of the restriction enzyme (E) used in creating the junction between the X_2 and X_3 regions of T antigen is placed after the hyphen. To facilitate comparisons, we renamed the parental plasmids pSV40, pMad1-TC, pSV40(M-1), and pM-1(SV40), designating them pSRSST, pMRMMT, pMRSST, and pSRMMT, respectively.

Extensive restriction enzyme analysis demonstrated that each of the chimeric genomes was constructed correctly. Restriction fragments encompassing each of the junctions within the chimeric T-antigen-coding regions were sequenced by the procedure of Maxam and Gilbert (39) to confirm the predicted sequence.

Focus formation on Rat 2 cells. The transforming activities of the recombinant DNAs were assessed by their ability to produce dense focal areas of growth on monolayers of Rat 2 cells. This cell line is highly transfectable and maintains a monolayer for extended periods of time (65). Furthermore, since SV40 and JCV DNAs differ to a large extent in their ability to transform Rat 2 cells (~1,000 foci per μg versus <1 focus per μg), the ability to discern intermediate levels of transforming activity was enhanced.

One microgram of each of the four parental and eight T-antigen chimeric genomes was transfected into subconfluent cultures of Rat 2 cells. The progression of T-antigen expression was monitored by indirect immunofluorescence at 2, 10, and 21 days posttransfection. All viral DNAs produced T protein in Rat 2 cells; however, this expression was transient, and the number of cells containing T antigen did not correlate with the number of dense foci counted at the end of the assay. At 2 days posttransfection, patches of T-antigen-expressing cells were seen, probably representing cells which had undergone division while the cultures were subconfluent. At 21 days, the number of T-antigen-expressing cells had decreased greatly but they were still present in all samples, including those in which foci were never detected. Over the course of the experiment, the number of cells producing T antigen decreased with time and then increased in a focal manner in dishes in which transformed foci developed. For samples which had started producing dense foci, 100% of the cells within a focus expressed T antigen.

Mock (calf thymus DNA)-transfected and untransfected control dishes (12 of each) were included in the experiments to determine the background values for spontaneously arising foci in this system; these cells maintained monolayers until the end of the assay (64 days). A set of sample dishes was fixed and stained (along with an untransfected dish and a mock-transfected control dish) when the foci had become large enough to enumerate. Often foci which had not been discernible by eye were obvious after the monolayers were stained; this was particularly true for the smaller spontaneously arising foci.

The days the foci were first seen and the average number of foci induced by each DNA are shown in Table 2. To facilitate analysis of the data, the DNA samples have been divided into two groups on the basis of the source of the regulatory region; genomes in lines 1 to 6 contain the SV40 regulatory region, and those in lines 7 to 12 contain the JCV regulatory region. Within each group, the samples have been arranged according to the identity of the chimeric T protein.

The time for foci development and the final number of foci per plate, two indicators of transformation efficiency, correlated well. Constructs containing the SV40 regulatory region were more active than those encoding the same proteins joined to the regulatory region of JCV. Transforming efficiencies also varied greatly, depending on the composition of the T-protein-coding region. The stronger SV40 regulatory signals induced a large number of transformants when T-antigen chimeras were composed of SV40 sequences from the central (lines 2 and 4) or carboxy (lines 3 and 4) regions. However, when under the control of the weaker JCV signals, only the intact SV40 T antigen (line 7) and the chimeric T antigen composed of SV40 amino and central region sequences (line 8) demonstrated significant transforming behavior. These are the only two DNAs within this group that specify a complete SV40 small t antigen. The low transforming efficiencies for constructs in lines 9, 10, and 11 were not the result of limiting amounts of DNA (data not shown).

Isolation and characterization of virally transformed Rat 2

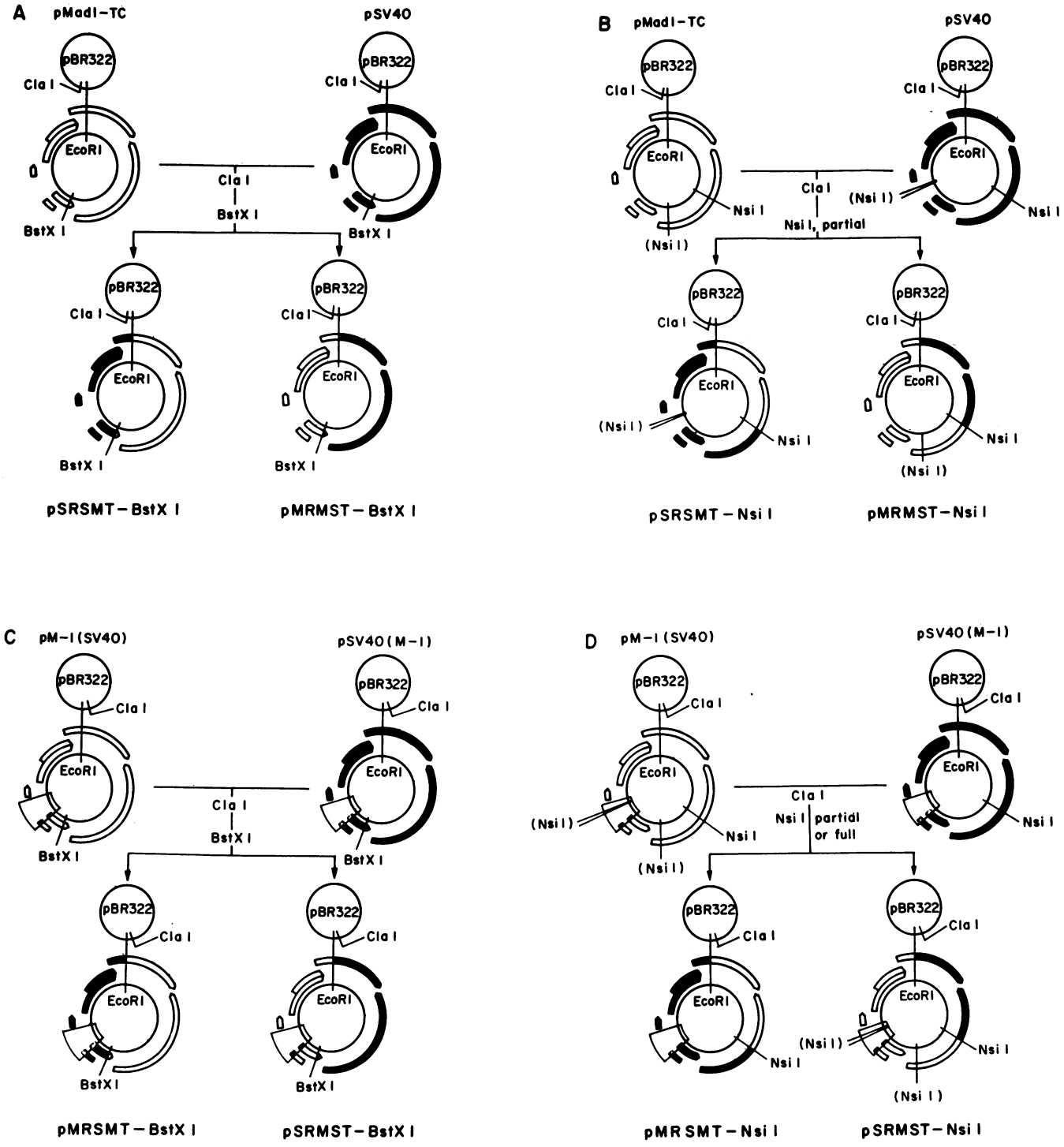


FIG. 3. Construction of T-antigen chimeric genomes. The parental genomes pMad1-TC and pSV40 (A and B) or pM-1(SV40) and pSV40(M-1) (C and D) were digested with *Cla*I and *Bst*XI (A and C) or *Cla*I and *Nsi*I (B and D), and the appropriate fragments were recombined and ligated. Parentheses indicate restriction enzyme sites which were left uncut by performing partial digests. Genomic diagrams are based on Fig. 2. The pBR322 and viral DNAs are drawn to scale and are joined at the unique *Eco*RI site. Coding regions of SV40 (▬) and JCV (▭) are shown. Heterologous regulatory regions in the pM-1(SV40) and pSV40(M-1) DNAs are indicated by open wedges. Although the first 10 amino acids of the T and t proteins were exchanged in the construction of the two regulatory region hybrids, these amino acids are identical in the JCV and SV40 proteins (13).

TABLE 1. DNA source for JCV-SV40 chimeric genomes[a]

DNA	Regulatory region	Source (amino acids)[b] of antigen:			
		Large T		Small t	
		N terminus	C terminus	N terminus	C terminus
pSRSMT-BstXI	SV40	SV40 (1–82)	JCV (82–688/end)	SV40 (1–133)	JCV (132–172/end)
pMRMST-BstXI	JCV	JCV (1–81)	SV40 (83–708/end)	JCV (1–131)	SV40 (134–174/end)
pSRSMT-NsiI	SV40	SV40 (1–410)	JCV (412–688/end)	SV40	SV40
pMRMST-NsiI	JCV	JCV (1–411)	SV40 (411–708/end)	JCV	JCV
pMRSMT-BstXI	JCV	SV40 (1–82)	JCV (82–688/end)	SV40 (1–133)	JCV (132–172/end)
pSRMST-BstXI	SV40 (*dl*892)[c]	JCV (1–81)	SV40 (83–708/end)	JCV (1–131)	SV40 (134–174/end)
pMRSMT-NsiI	JCV	SV40 (1–410)	JCV (412–688/end)	SV40	SV40
pSRMST-NsiI	SV40 (*dl*892)	JCV (1–411)	SV40 (411–708/end)	JCV	JCV

[a] Since a functional late region of the viral genome is not involved in transformation, the compositions of these sequences are not included in this table.
[b] The specific amino acids taken from each viral DNA source.
[c] *dl*892 is a viable deletion mutant of SV40. The elimination of an *Nco*I site in the regulatory region by the 19-base-pair deletion facilitated construction of pM-1(SV-40) (13).

cell lines. The chimeric T-protein constructs demonstrated distinct capacities to transform Rat 2 cells. To investigate the transformed phenotypes of these cells, lines were derived from individual foci. At least three T-antigen-positive cell lines were prepared from cells transfected with each of the genomes except pMad1-TC and pMRMST-NsiI. A few small foci were apparent on these sample dishes but usually only after the cells were fixed and stained. One cell line was prepared from a focus of cells growing on a pMRMST-NsiI-transfected monolayer; however, T protein could not be detected in these cells by indirect immunofluorescence. This line was included in all experiments as a spontaneous (T-antigen-negative) transformant.

The ability of papovavirus-transformed cells to grow suspended in a semisolid medium is a stringent criterion for transformation and is the best in vitro correlate of tumorigenicity (24, 51). To determine whether there was a correlation between this parameter and particular chimeric constructs, two independent clones transformed by each DNA were tested for the ability to grow while suspended in soft agarose (Table 3). Although the various constructs exhibited differences in their ability to transform cells, nearly all of the resulting cell lines demonstrated some degree of anchorage-independent growth over the controls. The ability of each cloned cell line to form colonies in the semisolid medium varied greatly; the anchorage independence values for a paired set of cloned cell lines often differed to a greater extent than those for cell lines transformed by different DNAs.

Immunoprecipitation of T antigen. There is less T antigen

TABLE 2. Transformation of Rat 2 cells by JCV-SV40 regulatory-early region chimeric DNAs

DNA[a]	Foci[b]	
	First day observed	No./plate
1. pSRSST [pSV40]	7	1,032
2. pSRSMT-NsiI	10	316
3. pSRMST-NsiI	12	166
4. pSRMST-BstXI	10	229
5. pSRSMT-BstXI	15	19
6. pSRMMT-[pM-1(SV40)]	22	41
7. pMRSST [pSV40(M-1)]	9	207
8. pMRSMT-NsiI	21	38
9. pMRMST-NsiI	—[c]	1
10. pMRMST-BstXI	26	3
11. pMRSMT-BstXI	33	2
12. pMRMMT-[pMad1-TC]	—	<1
13. Control[d]	—	1

[a] The name enclosed in brackets is the original name given to the recombinant DNA (13).
[b] Numbers are the average of three experiments (two experiments for DNAs in lines 9 to 12), with four to five plates per experiment.
[c] —, Foci were not evident until plates were fixed and stained at 64 days.
[d] At day 64.

TABLE 3. Anchorage-independent growth of transformed Rat 2 cells

Cell line[a]	% Cells able to grow in agarose[b]
1. pSRSST-c	1.6
2. pSRSMT-NsiI-a	11.3
pSRSMT-NsiI-c	0.1
3. pSRMST-NsiI-a	1.7
pSRMST-NsiI-c	6.1
4. pSRMST-BstXI-a	2.3
pSRMST-BstXI-c	2.1
5. pSRSMT-BstXI-b	<0.1
pSRSMT-BstXI-c	1.6
6. pSRMMT-a	5.2
7. pMRSST-d	1.1
8. pMRSMT-NsiI-c	2.7
pMRSMT-NsiI-d	0.6
9. pMRMST-NsiI-b[c]	1.8
10. pMRMST-BstXI-a	4.8
pMRMST-BstXI-b	1.1
11. pMRSMT-BstXI-a	3.0
pMRSMT-BstXI-b	1.5

[a] The names of the recombinant DNAs used to transform the Rat 2 cells are indicated, followed by a letter to distinguish independent clones. The nomenclature is described in Materials and Methods. The line numbers correlate with those used in Table 2.
[b] Cells from each cell line (1×10^5) were plated in triplicate onto 60-mm dishes. After 3 weeks, the number of colonies per dish with diameters greater than 0.1 mm were calculated from a sampling of 25 fields. Less than 0.1% of normal Rat 2 cells were able to grow suspended in soft agarose. These control cells had been transfected with calf thymus DNA and maintained as a monolayer for 33 days prior to trypsinization and expansion.
[c] Spontaneous transformant. T-antigen-positive transformants were not obtained after transfection of Rat 2 cells with pMRMST-NsiI. This T-antigen-negative line was derived from a focus on Rat 2 cells which had been transfected with pMRMST-NsiI. The expanded focus of cells was then carried through the cloning procedure.

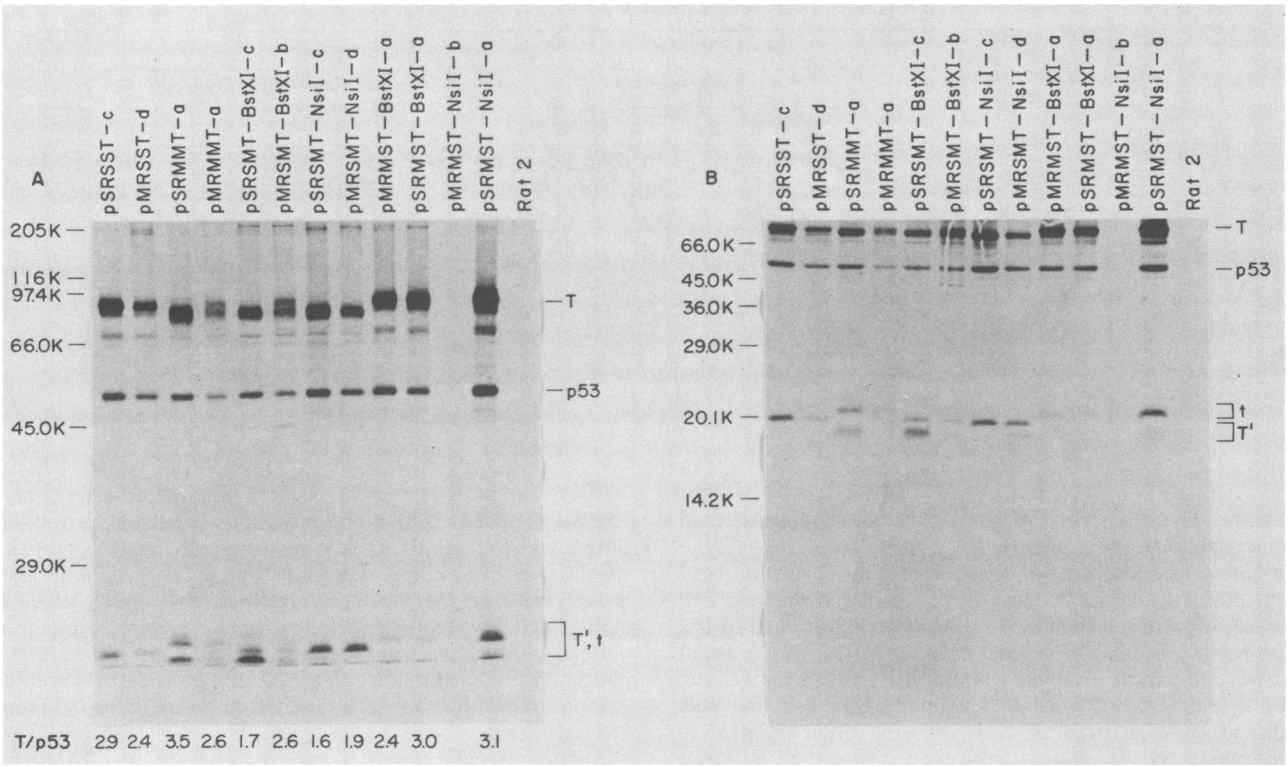


FIG. 4. Anti-T serum immunoprecipitation of proteins from JCV-SV40-transformed cells. Radiolabeled proteins from chimera-transformed cell lines were incubated with polyclonal antiserum directed against early viral proteins. The immune complexes were precipitated with *S. aureus* protein A, and samples were electrophoresed through 10% (A) and 15% (B) discontinuous polyacrylamide gels. The identity of the recombinant DNA used to transform each cell line is indicated above each lane; small letters designate the cell line clone. These same clonal designations are used in Table 3. To reduce the intensity of the T band in the pSRSST-c lane, only 62% of the total counts in this sample were loaded on the gel. T-antigen-positive foci were not recovered from the pMRMST-NsiI-transfected dishes; however, one T-antigen-negative focus was expanded and cloned as a spontaneous transformant (pMRMST-NsiI-b). Lane pMRMMT-a represents a single pMad1-TC Rat 2 transformant isolated in an earlier study (6a) and was included here for comparison. The T/p53 ratios were calculated from the average solubilized counts within equal-area regions from two similar dried gels, as described in Materials and Methods. These values were also compared with those obtained from densitometer tracings of the fluorograph. The positions of large T antigen (T), p53, small t antigen (t), large-T-antigen breakdown products (T'), and molecular weight marker proteins (shown in thousands [K]) are indicated. Resolution of t and T' is shown in panel B. Lanes Rat 2, Control.

in JCV-transformed cells than in SV40-transformed cells (4), in part due to less active promoter-enhancer signals and to a lower stability of the protein (6a, 31). An increase in the amount of T protein of JCV may be conferred through elevated expression from the regulatory sequences of SV40 or by substitution with stabilizing regions of SV40 T antigen. To determine whether the expected sizes of T antigen were present and whether the transformation efficiency correlated with the amount and stability of this protein, T antigen was immunoprecipitated with polyclonal anti-T antibody (see Materials and Methods). Extracts from two cell clones representing each chimera-specific cell line were immunoprecipitated with amounts of polyclonal anti-T-antigen antibody known to be saturating for both SV40 and JCV proteins under the conditions employed. The relative intensities and patterns of bands representing immunoprecipitated proteins for a clonal pair of cell lines were similar; the results from one representative clone of each line is shown in Fig. 4. p53 and T antigen are visualized best on the 10% gel (Fig. 4A); the T-antigen breakdown products (T') and small t antigen are more easily distinguished on the 15% gel (Fig. 4B). The lanes in both panels are paired according to the T-antigen construct. The negative-control cells (lanes Rat 2) had been transfected with calf thymus DNA and maintained as a monolayer for 33 days. Equivalent amounts of radiolabeled cell protein were loaded in each lane; however, the intensities of bands representing cellular p53 and the T and t proteins varied among samples. Full-length T antigen was produced in all viral transformants and migrated as a doublet. T antigen was not detected in the spontaneous transformant (lane pMRMST-NsiI-b) or in the Rat 2 control cells. Transformed cell lines expressing the same T-protein construct (but different regulatory regions) expressed T antigens which migrated similarly. Depending on the viral source of the sequences within the carboxy portion of T, the chimeric T antigens migrated with apparent molecular weights of 90,000 (SV40) or 88,000 (JCV). The migration pattern of large-T-antigen breakdown products, easily detected on the 15% gel (Fig. 4B, T'), was similar for chimeras with identical chimeric T proteins. T' products were not seen in cells transformed by pSRSST, pMRSST, pMRMST-BstXI, and pSRMST-BstXI, suggesting that sequences encoded by the second exon of the T-antigen gene are responsible for the instability of the JCV protein.

Small t protein is seen in all lanes; the JCV and SV40 t proteins migrate with apparent molecular weights of 22,300 and 20,800, respectively. The sizes predicted by the DNA sequence are M_ws 20,200 (172 amino acids, JCV) and 20,400

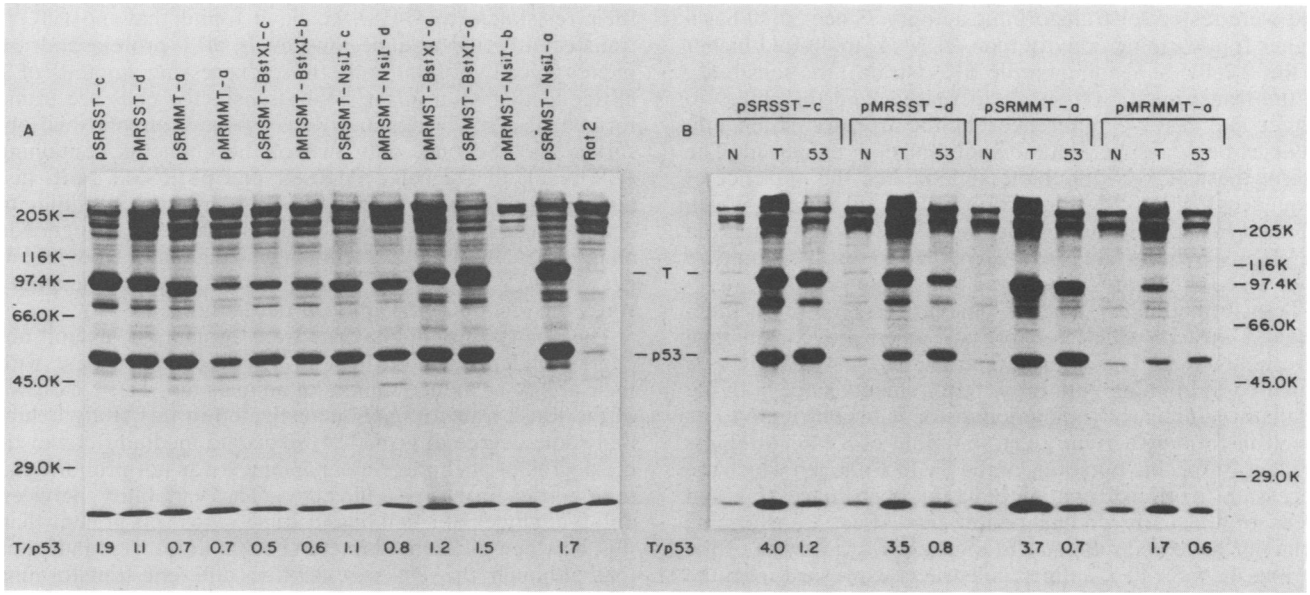


FIG. 5. Anti-p53 monoclonal antibody immunoprecipitation of proteins from JCV-SV40-transformed cells. (A) Radiolabeled proteins from chimera-transformed cell lines were incubated with the anti-p53 monoclonal antibody PAb122, and the immune complexes were precipitated with *S. aureus* protein A. The samples were electrophoresed through 10% discontinuous polyacrylamide gels. The identity of the recombinant chimera used to transform each cell line is indicated above each lane; small letters designate the cell line clone. Lane Rat 2, Control. (B) Proteins precipitated with PAb122 (lanes 53) were compared with those precipitated by either normal hamster serum (lanes N) or polyclonal anti-T serum (lanes T). The labeling of these fluorographs and the calculation of the T/p53 ratios are described in the legend to Fig. 4.

(174 amino acids, SV40), indicating that the mobility to the JCV t protein (Fig. 4B) is retarded. The chimeric small t proteins migrate coincident with those of the parental t proteins from which the majority (N-terminal 131 or 133 amino acids) of the coding sequences were derived (cf. lanes pSRMST-BstXI-a and pMRMST-BstXI-a with pMRMMT-a and lanes pSRSMT-BstXI-c and pMRSMT-BstXI-b with pSRSST-c; Table 1). This indicates that the first 131 amino acids of the JCV small t protein are responsible for the apparent size differences between the JCV and SV40 small t proteins.

p53 was coprecipitated in all virally transformed cell lines. The amount of p53 coprecipitated with anti-T serum correlated with the amount of T antigen present in each cell line, indicating the formation of a stable complex between p53 and each of the T-antigen species. Densitometer tracings of the fluorograph indicated that the ratios of the T to p53 bands were within a narrow range (1.6 to 3.5) for all transformed cell lines.

Immunoprecipitation of p53. Studies suggest that the cell cycle regulatory phosphoprotein p53 (40, 52) may be stabilized through its binding to the SV40 T antigen (35, 45), thereby contributing to the transforming activity of this virus. The immunoprecipitation results with anti-T serum shown in Fig. 4 indicated that the amount of p53 coprecipitated from the different transformed cell extracts was elevated above that found in T-antigen-negative cells and depended on the T construct with which it interacted. To verify the identity of the p53 band and to investigate further the stable interaction between p53 and each T-antigen construct, immunoprecipitation experiments were repeated with PAb122. This anti-p53 monoclonal antibody specifically recognizes the C terminus of p53 and is known to coprecipitate bound T antigen (62).

Two cloned cell lines representing each of the DNA-specific transformants were tested, and the results for both lines were similar. A fluorograph showing the proteins immunoprecipitated from one of these sets of cell lines is shown in Fig. 5A. Figure 5B shows a comparison of the amount of p53 immunoprecipitated with anti-T and anti-p53 antibodies and indicates that most p53 was in complex with T antigen in these cells. Very low levels of p53 were detected in the spontaneously transformed (pMRMST-NsiI-b) and untransformed (Rat 2) cell lines. Higher levels of p53 were immunoprecipitated from the transformed cell extracts, and the ratios of the T to p53 bands ranged from 0.5 to 2.0. The lowest ratios were found consistently for the cell lines transformed by constructs containing the second exon of the JCV T-antigen gene: pMRMMT-a, pSRMMT-a, pMRSMT-BstXI-b, and pSRSMT-BstXI-c (Fig. 5).

DISCUSSION

JCV and SV40 are closely related viruses that display distinct lytic and oncogenic properties in vitro. We have taken advantage of the low transforming activity of JCV to investigate the regions of the polyomavirus genome that contribute to an efficient transforming capability. To utilize this approach, JCV-SV40 chimeric genomes were constructed in which the regulatory and early coding sequences were exchanged. The precise exchanges left the regulatory sequences intact and the reading frames of the large and small T proteins uninterrupted. An important consequence of these manipulations was that functional domains of T antigen could be analyzed in a biologically relevant context. Because the replacement of SV40 for corresponding JCV sequences yielded genomes with higher transforming activities, we believe that the structural integrity of the chimeric proteins was maintained and that changes in transforming efficiency reflect the exchange of a transformation domain(s) of T antigen.

In a recent study (6a), hybrid genomes were constructed with the human polyomaviruses JCV and BK virus (BKV)

and were tested for transforming activity. Since SV40 has a higher transforming activity than BKV (5- to 40-fold higher in Rat 2 cells, depending on the BKV strain), the sensitivity of the transformation assay is increased, and the ability to detect and resolve differences in the transformation efficiencies between the recombinant DNAs is enhanced. The use of these JCV-SV40 chimeras indicated the presence of sequences within the carboxy third of T antigen which affected the transforming potential of SV40 and which the JCV-BKV hybrids had previously failed to resolve; compare lines 1 and 2, 3 and 6, and 7 and 8 in Table 2.

Transformation by the polyomaviruses requires a functional T protein which is expressed at or above a minimal threshold level. The data in Table 2 suggest that JCV is impaired in fulfilling both these requirements since both the regulatory and early coding regions of JCV contribute to its low transforming activity relative to that of SV40. Attempts to identify specific functions of the SV40 T antigen which are necessary for transformation have not been successful, and areas of the early coding region that affect transforming behavior have been difficult to localize. Our analysis of the various JCV-SV40 T-antigen constructs expressed from the SV40 promoter-enhancer signals indicates that there are distinct regions within both the central and carboxy regions of the T protein that are important for transformation. Replacement of either of these two regions of the JCV T antigen with those of SV40 significantly increased the transformation efficiency of the construct. It is likely that the interaction of T antigen with specific cellular proteins triggers the events leading to a transformed phenotype; recent results suggest two possible candidates, the cellular protein p53 (41, 45, 52) and the retinoblastoma susceptibility gene product (RB; 19). The binding domains for p53 and RB are specified by sequences within the second exon of the SV40 T-antigen gene (Fig. 1). Because the RB-binding region is believed to involve a small region of the SV40 T antigen (amino acids 105 to 120) (19), it is possible to compare this sequence in SV40, JCV, and BKV. Such a comparison indicates that JCV and BKV differ from SV40 at 6 and 5 residues, respectively. It is not known at this time whether the JCV and BKV proteins form a complex with RB in vivo.

To induce transformation, a functional T protein must be expressed. Recently it has been suggested that the SV40 small t antigen enhances transformation when the concentration of T protein is low (5). This implies that a threshold level of T protein is required to cause transformation; when the levels of T are limiting, small t protein would be necessary for transformation. The data of this study support these suggestions. First, our constructs which contained JCV regulatory sequences in place of those of SV40 transformed less efficiently. It is known that the JCV regulatory signals are less active than those of SV40 (31; F. White III and R. J. Frisque, unpublished observations). One would predict that fewer cells would express levels of T antigen that surpassed the threshold requirement for transformation when that T antigen was driven by the JCV signals. While lower levels of T antigen are usually detected in cells transformed with constructs containing a JCV regulatory region (Fig. 4), a strict correlation between low levels of T antigen and the presence of JCV regulatory signals is not always found. This is not unexpected, since in the experiments reported here, cells expressing T antigen were selected on the basis of a transformation parameter and thus should contain at least the threshold amount of T antigen. Using a cotransfection technique with SV40 or JCV DNA plus plasmid pSV2-neo and selecting cells on the basis of their resistance to G418, we have found that most JCV-transfected cells produce low levels of T protein and are phenotypically normal; only those expressing amounts of T closer to that seen in the SV40-transfected cells are transformed (P. Trowbridge and R. J. Frisque, unpublished observations). Second, only two of the chimeras containing JCV regulatory signals, pMRSST and pMRSMT-NsiI, displayed transforming activities above background. Significantly, these DNAs represent the only chimeras that encode an intact SV40 small t protein, suggesting that the SV40, but not the JCV, t antigen can enhance transforming ability when the amounts of T antigen are limiting.

The ability of polyomavirus transformants to exhibit anchorage-independent growth in vitro correlates best with their ability to induce tumors in animals (24, 51). Nearly all of the Rat 2 transformants investigated in this study exhibited some degree of growth in semisolid medium; however, the degree of anchorage independence was not proportional to the transformation efficiency. The variability between different clones of a cell line in some cases was greater than that between different chimera-specific lines. This indicates that although the DNAs exhibited different transforming efficiencies, once the transformation event was established the cells had the capacity to express a fully transformed phenotype.

Precipitation of extracts of transformed cells with polyclonal anti-T serum revealed large T (migrating as a doublet) and small t antigens, large T antigen breakdown products (T'), and the cellular phosphoprotein p53. T' has also been seen previously in cells transformed by unstable truncated SV40 and intact JCV T antigens (6a, 41, 58). Instability of the JCV T antigen, which appears to be a function of that part of the protein encoded by sequences downstream of the early mRNA acceptor splice site, may contribute to the lower amounts of this protein detected in the JCV versus SV40 transformants (4).

Depending on the viral source of the C-terminal segment of T antigen, the apparent molecular weight of the chimeric T antigens was either that of the JCV protein (88,000; all constructs designated XRXMT) or the SV40 protein (90,000; all constructs designated XRXST) (Fig. 4A). The difference in molecular weight reflects the deletion of a number of amino acids at the extreme carboxy end of the JCV T antigen. Mobility differences were also observed for small t antigen (Fig. 4B) and appeared to correlate with the source of the amino portion of the protein (N terminus to *Bst*XI site). Since there is a difference in length of only two amino acids within this portion of t antigen (131 and 133 amino acids for JCV and SV40, respectively) and because the SV40 protein has the correct size on the basis of nucleotide sequence, the aberrant migration of JCV t protein may be the result of the different proline contents of the two proteins (nine versus six for JCV and SV40, respectively).

Immunoprecipitation of the transformed cell extracts with anti-p53 serum indicated that small but reproducible differences could be detected in the amounts of the various T proteins bound to p53 (Fig. 5A, T/p53 ratios). Most of the p53 was in complex with T antigen (Fig. 5B; cf. anti-T serum versus anti-p53 antibody), and only a subset of T antigen formed a complex with p53 (Fig. 5A) (34). Less T antigen complexed to p53 in the cells transformed by constructs containing the central and carboxy regions of the JCV protein. Since these regions of T are involved in the binding and stabilization of p53, this indicates that this domain of the JCV and SV40 T antigens differs in p53-binding activity.

The construction and characterization of JCV-SV40 chi-

meric DNAs has revealed several regions of the JCV genome which contribute to the restricted transforming behavior of this virus and suggest specific sequences upon which to focus a mutagenic approach. The chimeric T antigens will also serve as valuable reagents for investigating lytic activities and immunological features of this multifunctional protein.

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