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Replication and Transformation Functions of In Vitro-Generated Simian Virus 40 Large T Antigen Mutants

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We used sodium bisulfite mutagenesis to introduce point mutations within the early region of the simian virus 40 genome. Seventeen mutants which contained amino acid changes in the amino-terminal half of the large T antigen coding sequence were assayed for their ability to replicate viral DNA and to induce transformation in the established rodent cell line Rat-3. The mutants fell into four basic classes with respect to these two biological functions. Five mutants had wild-type replication and transformation activities, six were totally defective, three were replication deficient and transformation competent, and two were replication competent and transformation deficient. Within these classes were mutants which displayed intermediate phenotypes, such as four mutants which were not totally deficient in viral replication or cellular transformation but instead showed reduced large T antigen function relative to wild type. Three large T mutants displayed transforming activity that was greater than that of wild type and are called supertransforming mutants. Of the most interest are mutants differentially defective in replication and transformation activities. These results both support and extend previous findings that two important biological functions of large T antigen can be genetically separated.

Numerous biological and biochemical functions have been attributed to the large T antigen of simian virus 40 (SV40). Viral replication, which is dependent on large T antigen (42, 82), requires binding of this protein to the SV40 origin of replication (16, 18, 47, 52, 59, 75, 76). The DNA binding function of T antigen has also been correlated with its ability to autoregulate its own synthesis (18, 31, 58, 67) and to activate late viral transcription (4, 38). Both cellular DNA and RNA synthesis are stimulated by large T antigen (9, 43, 49, 70, 77), although in an apparently DNA binding-independent fashion (11). T antigen can establish and maintain transformation in both primary and established nonpermissive cells (5, 8, 28, 39, 40, 48, 61, 71, 79, 82, 83). The helper function of SV40 that allows adenovirus to productively and efficiently infect monkey cells has been attributed to T protein (22, 57, 85). Large T antigen also encodes a host range function that allows the CV-1 monkey cell line to be productively infected by SV40, although this function is not required for SV40 infection of the BSC monkey cell line (46, 64). Biochemically, large T antigen is a phosphoprotein of approximately 94,000 molecular weight. Forms of T antigen which are more phosphorylated appear to be hindered in their ability to bind DNA (56, 72). The protein has apparent ATPase activity (10, 86) which roughly correlates with its replication and transformation functions (11, 80). Separate populations of large T antigen within the infected cell have also been described. T antigen is localized both in the nucleus (84) and on the plasma membrane of cells (17). This protein may play different roles in transformation, depending on its location, as suggested by analysis of the transformation phenotypes of nuclear localization mutants (23, 41). T antigen has been seen as a monomer, dimer, tetramer, or oligomer complexed with the nonviral T antigen, p53 (3, 21, 25, 32, 50). Different oligomers of T antigen that differ in their phosphorylation states can be identified (21, 72), and biological roles for these populations are being defined (3, 25, 55, 73, 90).

Original analyses of mutant large T antigens employed *tsA* mutants which were defective in both transforming and replication functions (87). It was not known, therefore, whether distinct domains of the large T protein were responsible for these different phenotypes or whether separate subsets of T antigen existed which exhibited different functions. Deletions and point mutants have since been described which produce T antigen capable of transformation but unable to replicate SV40 DNA (26, 36, 37, 45, 46, 62, 65, 81). These data argue that different portions of T antigen are involved in different biological functions. In addition, replication activity can be correlated with the DNA binding domain of T antigen in that binding mutants are all replication negative, although not all replication mutants are defective in DNA binding.

A few mutants with changes in large T antigen sequences have been isolated which can replicate their DNA but cannot transform established rodent cell lines. Cosman and Tevethia (13) found *tsA1642* to be reduced in the ability to replicate SV40 DNA and unable to transform Brown Norwegian rat kidney (B/NRK) cells at the nonpermissive temperature. Two point mutants have been isolated which can replicate at wild-type levels but transform rat cells at less than 10% of the efficiency of wild-type large T antigen (37). The latter investigators have also identified a domain of T antigen that, when mutated, results in a replication-deficient protein that appears to transform cells better than wild-type T antigen does. This supertransforming phenotype is characterized by an increase in the number and earlier appearance of foci on a cell monolayer (37).

We generated mutants of large T antigen to further define the replication and transformation domains of SV40 large T antigen. The approach taken was to generate single base pair changes in the cloned large T coding sequence by using the single-strand-specific mutagen sodium bisulfite. This method of generating mutants has the advantage of yielding mutants

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which need not be viable. The target area was the *Nde*IB restriction fragment of SV40 (see Fig. 1), which contains sequences that have been implicated in both specific SV40 DNA binding and stimulation of cellular DNA synthesis and in which the replication and transformation functions of T antigen have been separated (37, 46, 62, 81). Also included in the *Nde*IB fragment are sequences of large T antigen in which point mutations have not yet been studied. Mutants were assayed for the ability to replicate viral DNA in the monkey cell line BSC-40 and to transform the rat fibroblast line Rat-3.

MATERIALS AND METHODS

Plasmids. The M13 derivatives mp8, mp9 (54), and mp18 (60) were propagated in JM101 as described previously (53). The plasmid pSY343 (91; from D. Clewell, University of Michigan) is an R plasmid that bears no sequence homology to either M13 or pBR322. The SV40 *Nde*IB fragment was blunt-end cloned into the unique *Eco*RI site of this vector to yield pSY343-*Nde*IB. pJYM (44) was a gift of M. Botchan. A plasmid deleted for the SV40 *Nde*IB fragment (pJYMΔ*Nde*IB) was constructed by cleaving pJYM with *Nde*I and recircularizing. All plasmids were maintained in DH1 (30). Plasmid DNAs were amplified (12) before DNA was isolated by either sodium dodecyl sulfate lysis (27) or alkaline lysis (2) followed by cesium chloride-ethidium bromide density gradient centrifugation. The double-stranded replicative form (RF) DNA of M13 bacteriophage was isolated similarly to plasmid DNA without amplification. Small preparations of plasmid or RF DNA were isolated from 1.5-ml cultures by either alkaline lysis or lysis by boiling (34). Single-stranded M13 phage DNA was isolated as described by Sanger et al. (68).

Virus and cells. SV40 strain 776 was used as the source of viral DNA for cloning and was propagated in BSC-40 cells as described previously (6, 7). Viral DNA was isolated as described by Danna and Nathans (14). The TK^- rat fibroblast line, Rat-3 (88; from J. Stringer, University of Cincinnati), was used to assay viral transformation. All cells were maintained in minimal essential medium (MEM; MA Bioproducts) supplemented with 0.2 mg of $NaHCO_3$, 0.29 mg of glutamine, 100 U of penicillin, and 100 U of streptomycin per ml and 10% fetal calf serum (FCS).

Enzyme reactions. All enzymes were from New England BioLabs, Inc., and were used as specified by the supplier. We found it necessary to use blunt-end conditions for cohesive-end *Nde*I ligations; this is probably because the 5′-TA-3′ overhang does not form a stable hybrid under cohesive-end conditions.

Sodium bisulfite mutagenesis. The sodium bisulfite mutagenesis procedure was performed essentially as previously described (89). The target single-stranded M13 DNA was passed over a hydroxylapatite column to ensure removal of any contaminating double-stranded RF DNA. Linearized RF DNA that was used to protect certain sequences from sodium bisulfite was also passed over a hydroxylapatite column to ensure removal of any single-stranded phage DNA. The linear fragment was eluted from a 1.0% agarose gel (19) followed by passage through an elutip (Schleicher & Schuell, Inc.; as directed by the manufacturer) to remove any contaminating material. We annealed a fivefold excess of the linearized protection fragment to the single-stranded target DNA by boiling the mixture in 0.05× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 3 min, incubating it at 60°C for 30 min, and then bringing the mixture to room temperature. This annealed sample was then treated with sodium bisulfite, as described by Shortle and Nathans (74), in the dark at 37°C for 7 or 10 min. Desalting was accomplished by passing the mixture over a Sephadex G-50 column, and sulfite adducts were removed by making the sample 0.1 M in Tris base and incubating at 37°C overnight. The gap was repaired by addition of Klenow enzyme and deoxynucleoside triphosphates to 6 μM, and the double-stranded, bisulfite-treated DNA was used to transfect JM101.

DNA sequencing. Dideoxy chain termination sequencing was performed as described by Sanger et al. (69) and modified by Biggin et al. (1) with the following changes. To be able to read the sequences from 300 to 500 nucleotides past the universal primer, we reduced the dideoxy nucleotide concentration in each reaction mixture by 10-fold to allow chains to be synthesized further before termination. Specifically, the final concentration of di-dATP was made 1.25 μM, di-dCTP was made 3 μM, di-dGTP was made 6 μM, and di-dTTP was made 6 μM. Gels were made up in 0.5 × TBE (1× TBE is 0.13 M Tris, 2.4 mM EDTA, 90 mM boric acid [pH 8.7]) and run until two sequential loadings of xylene cyanol dye migrated off the gel. These runs took approximately 8 h at 35 mA (1,200 to 1,500 V) with a change of the 1× running buffer after 4 h.

Shotgun cloning of sonicated DNA. To check the efficiency of protection during bisulfite mutagenesis, we shotgun cloned selected mutants (see Results) into M13 and determined their sequences as previously described (15). Five micrograms of DNA in 250 μl of 0.5 M NaCl–0.1 M Tris (pH 7.4)–1 mM EDTA was sonicated by five 5-s pulses separated by 1.5-min incubations on ice. Staggered ends were repaired by Klenow enzyme and deoxynucleoside triphosphates, and the blunt-end molecules were ligated into the *Sma*I site of mp18. After transformation of JM101, white plaques were screened by plaque hybridization with pSY343-*Nde*IB. Phage DNA from positive plaques was sequenced.

Replication assay. Replication of SV40-containing plasmid DNA was assayed essentially as described by Peden et al. (63) and Pipas et al. (65). BSC-40 cells, at 50% confluence in a 60-mm (diameter) dish, were transfected with 25 ng of uncut plasmid DNA by the DEAE-dextran transfection protocol (51). Cells were washed twice with MEM, and DNA in 0.3 ml of 0.05 M Tris (pH 7.6)–250 μg of DEAE-dextran per ml was put on cells for 30 min at room temperature. Two more washes with MEM–2% FCS followed, and cells were incubated in MEM–2% FCS for 48 h at 37°C. DNA was extracted essentially as described by Hirt (33) and modified by Pipas et al. (65).

Hirt DNA was digested with either *Dpn*I-*Bcl*I or *Mbo*I. Restricted DNAs were fractionated on a 1.0% agarose gel and analyzed by the method of Southern (78). Filters were hybridized with ^{32}P-labeled pJYM (66).

Transformation assay. Rat-3 cells were transfected with plasmid DNA by the calcium phosphate procedure (29). A precipitate of 1 μg of mutant plasmid DNA, 1 μg of pSV2neo DNA, and 8 μg of salmon sperm DNA in 0.5 ml was made and added to Rat-3 cells that were 50% confluent in a 60-mm dish with MEM-10% FCS. Fifteen to 18 h after the precipitate was added, cells were fed with MEM-10% FCS. Transfected cells were put into transformation assays 2 days following precipitate addition.

We plated one half of each dish into a new 60-mm dish with MEM-5% FCS to assay focus formation (9) at 37°C. These dishes were fed every 3 to 4 days. After 5 weeks, cells

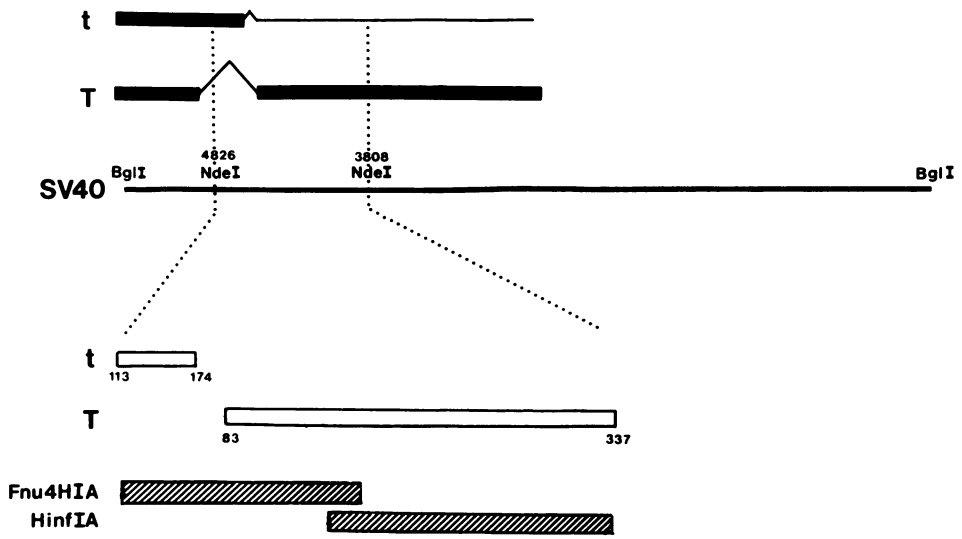

FIG. 1. Physical map locating the *Nde*IB fragment on the SV40 genome. The SV40 chromosome is linearized at the unique *Bgl*I site (5235) and drawn in the middle of the figure. The two *Nde*I sites and their nucleotide positions are indicated. Above the SV40 genome are the transcripts for small t and large T antigens, with the coding sequences (▬) indicated. Below the SV40 genome, the protein coding sequences (▭) for small t and large T antigens which lie within the *Nde*IB fragment are shown, and the corresponding amino acid numbers are given. At the bottom of the figure are the two protection fragments (▨), *Fnu*4HIA and *Hin*fIA, used during bisulfite mutagenesis.

were fixed with methanol-acetic acid (3:1 [vol/vol]) and stained with Giemsa.

For the G418 assay, 1/10 (experiments 1 and 2) or 1/20 (experiments 3 to 6) of each transfection dish was plated into a 60-mm dish containing 400 μg of G418 (GIBCO Laboratories) per ml–MEM–10% FCS. G418^r colonies were fixed and stained 10 to 12 days after plating.

RESULTS

Generation of SV40 T-antigen mutations. To generate mutations in a specific region of the SV40 genome, we used the sodium bisulfite mutagenesis procedure first described by Shortle and Nathans (74) and later modified by Folk and Hofstetter (24) and Everett and Chambon (20). The fragment of SV40 DNA that we chose to mutagenize is the *Nde*IB fragment (Fig. 1). This fragment extends from nucleotides 3808 to 4826, or map units .386 to .581, and includes the 3′-terminal 189 base pairs (bp) of small t antigen unique coding sequence, the 66-bp small t intron, the large T and small t splice acceptor site, and 764 bp of the large T antigen coding sequence. We chose this fragment of DNA because previous experiments indicated that both a DNA binding domain important for replication and a domain in which one can genetically separate the replication and transformation functions of T antigen map in this region of large T antigen.

To clone the SV40 *Nde*IB fragment into the M13 vector mp8, we first cloned this fragment into the unique *Nde*I site of pBR322. The hybrid plasmid was cleaved with *Sfa*NI, which cuts throughout pBR322 sequences but not within *Nde*IB, to yield the *Nde*IB insert flanked by 14 and 20 bp of pBR322. This fragment was then blunt-end cloned into the *Sma*I site of M13 mp8 and mp9. By cloning into both mp8 and mp9, each half of the *Nde*IB fragment is adjacent to the M13 universal sequencing primer binding site (68).

Two subclones of mp8-*Nde*IB, mp8-*Hin*fIA and mp9-*Fnu*4HIA, were made to be used as protection fragments during bisulfite mutagenesis. mp8-*Nde*IB was digested with either *Hin*fI or *Fnu*4HI to yield subfragments that span opposite halves of the *Nde*IB fragment (Fig. 1). Insertion of these subfragments into either mp8 or mp9 produced molecules that contained a deletion of *Nde*IB sequences. By annealing the single-stranded mp8-*Nde*IB target DNA with denatured, linearized mp8-*Hin*fIA RF DNA (or, conversely, mp9-*Nde*IB with mp9-*Fnu*4HIA), we protected M13 sequences essential for the propagation of the phage and half of the *Nde*IB sequences from mutagenesis by sodium bisulfite. This protection reduced the single-stranded bisulfite target area from ca. 1,000 nucleotides to ca. 500 nucleotides and allowed either exposed DNA region to be sequenced from the M13 universal sequencing primer in a single set of reactions.

Two sets of mutants were generated. The first was made by protecting mp8-*Nde*IB with the mp8-*Hin*fIA subclone. After exposure of the DNA to bisulfite for 10 min, 60 mutated phages were isolated and sequenced to locate mutations in the unprotected area, nucleotides 4826 to 4376. When the conditions for bisulfite treatment were chosen, we were aiming to make one amino acid change per DNA molecule. The 10-min incubation time used in the first experiment gave an average of 2.5 base pair changes and an average of 1.75 corresponding amino acid changes per clone (data not shown). The second set of mutants was generated by treating the mp9-*Nde*IB/mp9-*Fnu*4HIA hybrid with bisulfite. This molecule had a target area of 504 nucleotides, from 3808 to 4312. Since the average number of amino acid changes in the first experiments was approximately two, the bisulfite treatment time was reduced to 7 min. Here, 27 sequenced phage isolates generated had an average of 1 base pair and 0.5 amino acid changes (data not shown).

Sixty-one mp8-*Nde*IB and mp9-*Nde*IB clones that had nucleotide changes resulting in one or more amino acid differences were isolated from the two bisulfite mutagenesis experiments. Of these, 26 had predicted amino acid changes only in large T antigen. Since we were interested in fine mapping regions of large T antigen important in either replication of the SV40 genome or transformation, 17 mutants that had one to three amino acid substitutions which

TABLE 1. Nucleotide and amino acid changes in 17 large T antigen mutants

Mutant name	Base substitution[a]	Amino acid substitution[b]	Large T (T) or small t (t) mutation[c]
9e	G (4618) → A		t IVS
	G (4544) → A	Glu (92) → Lys	T
	G (4466) → A	Ala (118) → Thr	T
	G (4473) → A	(115)	T
11d	G (4687) → A	(159)	t
	G (4517) → A	Glu (101) → Lys	T
	G (4397) → A	Glu (141) → Lys	T
6a	G (4499) → A	Glu (107) → Lys	T
	G (4496) → A	Glu (108) → Lys	T
5f	G (4483) → A	Ser (112) → Asn	T
7c	G (4481) → A	Asp (113) → Asn	T
	G (4466) → A	Ala (118) → Thr	T
	G (4424) → A	Val (132) → Ile	T
6b	G (4472) → A	Ala (116) → Thr	T
	G (4425) → A	(131)	T
	G (4397) → A	Glu (141) → Lys	T
	G (4392) → A	(142)	T
8h	G (4822) → A	(114)	t
	G (4466) → A	Ala (118) → Thr	T
11g	G (4473) → A	(115)	T
	G (4424) → A	Val (132) → Ile	T
7f	G (4645) → A	(173)	t
	G (4637) → A		t IVS
	G (4607) → A		t IVS
	G (4421) → A	Glu (133) → Lys	T
10c	G (4410) → A	(136)	T
	G (4387) → A	Ser (144) → Asn	T
	G (4373) → A	Ala (149) → Thr	T
15-7	C (4259) → T	His (187) → Tyr	T
8-3	C (4220) → T	Pro (200) → Ser	T
4-3	C (4211) → T	His (203) → Tyr	T
3-2	C (4198) → T	Ala (207) → Val	T
5-2	C (4114) → T	Ala (235) → Val	T
7-1	C (4033) → T	Ala (262) → Val	T
13-9	C (4024) → T	Thr (265) → Ile	T

[a] The mutated base and its location are given, followed by the replacement base.

[b] The altered amino acid and its location are given, followed by the replacement amino acid. If the base pair change does not alter the amino acid, only the location of that amino acid is given.

[c] The protein affected by the altered amino acid is indicated. If the mutation is within the small t intron, t IVS (intervening sequence) is noted.

would not lead to a truncated polypeptide were selected for further study (Table 1).

Reconstruction of the SV40 genome with mutated *Nde*IB fragments. To assay the biological activities encoded by these mutant large T antigens, we reinserted the mutated *Nde*IB fragments into the SV40 genome. The cloning vector used for mutant reconstruction was pJYMΔ*Nde*IB, which contains the entire SV40 genome except for the *Nde*IB fragment. *Nde*I-digested mp8- or mp9-*Nde*IB RF DNA was shotgun cloned into the unique *Nde*I site of pJYMΔ*Nde*IB. Plasmids were screened for the proper insert by colony hybridization of recombinants with ^{32}P-pSY343-*Nde*IB, and the correct insert orientation was determined by restriction analysis. Once isolated, all pJYM mutants were examined by restriction analysis to verify that gross rearrangements did not occur during cloning (data not shown).

It is possible that, during mutagenesis, protection of SV40 sequences by annealing with protection clones may not be complete, leading to the introduction of mutations within what should be protected DNA. To gauge the extent of mutations occurring within protected sequences, we sequenced the complete *Nde*IB fragments of three pJYM mutant constructs. The *Nde*IB fragment, at 1,018 bp, is too large to sequence by dideoxy sequencing from a single primer, so the sonication cloning protocol of Deininger (15) was used. Mutant pJYM DNA was randomly sheared by sonication and blunt-end cloned into the *Sma*I site of mp18. Phage clones containing inserts that overlap *Nde*IB were identified by plaque hybridization with ^{32}P-pSY343-*Nde*IB and sequenced. Sequencing of 20 to 30 positive clones for each mutant gave enough overlapping sequence data to generate the entire DNA sequence of the mutated *Nde*IB fragment. Analysis of mutants 6b, 5f, and 4-3 confirmed the mutations identified from the original DNA sequence analysis; we found no mutations within protected DNA sequences (data not shown). (For ease of reading, we refer to the mutants by the names we assigned to them as they were isolated. Table 2 indicates the number assigned to each mutant by the numbering system of J. Pipas [personal communication]).

Replication assays of T antigen mutants. To determine the optimal kinetics for the replication assay, we transfected pJYM into BSC-40 cells and isolated low-molecular-weight DNA at 24, 48, and 72 h after transfection. Southern analysis of Hirt DNA showed the maximal amount of newly replicated DNA at 48 h after transfection (data not shown). This

TABLE 2. Mutant name comparison[a]

Mutant no.	Common name
3212	5f
3213	6a
3214	6b
3220	7c
3223	7f
3232	8h
3238	9e
3243	10c
3246	11d
3249	11g
3257	3-2
3259	4-3
3261	5-2
3264	7-1
3266	8-3
3271	13-9
3275	15-7

[a] The number assigned to each mutant is from the numbering system developed by J. Pipas (personal communication). The common name refers to the name given to each mutant at the time of isolation.

time point was used for all subsequent replication assays with the mutant clones. Use of a single time point only indicates whether newly replicated DNA is present at that time and does not identify mutants which might be capable of replicating their DNA but with different kinetics.

The mutants were transfected into BSC-40 cells, and Hirt DNA was isolated after 48 h. We cleaved the DNA with either of two enzymes that discriminate between methylated and unmethylated sequences to distinguish between input and newly replicated plasmid DNAs. The input DNA, which was generated from a Dam^+ bacterium, is methylated at G^mATC, whereas plasmid sequences replicated in BSC-40 cells are unmethylated at this site. Digestion products were run on a 1.0% agarose gel and analyzed by the method of Southern with nick-translated pJYM as a probe. In the experiment shown in Fig. 2, the DNA was digested with *Mbo*I to cleave unmethylated GATC sequences. Input DNA is seen as undigested form I and II molecules at the top of the gel, whereas newly replicated DNA exhibits a characteristic pattern of smaller bands. In a separate experiment, DNA was cleaved with *Dpn*I, which cleaves input DNA, followed by *Bcl*I to linearize any newly replicated DNA (data not shown). With these enzymes, mutants which could replicate their DNA exhibited a linear-sized DNA fragment, whereas replication-defective mutants did not. The results from these two experiments were identical, and identified 9e, 7f, and 11d as severely reduced (less than 1% of the wild type) and 6a, 10c, 15-7, 8-3, 4-3, and 3-2 as totally deficient in their ability to support viral DNA replication. Clones 5-2, 7-1, 13-9, 7c, 11g, 6b, 8h, and 5f appeared able to initiate plasmid replication from an SV40 origin at efficiencies similar to that of wild-type T antigen. It should be noted that, although 8h seems to be replication deficient, the amount of input DNA in this sample was low (see top two bands). Thus, when one

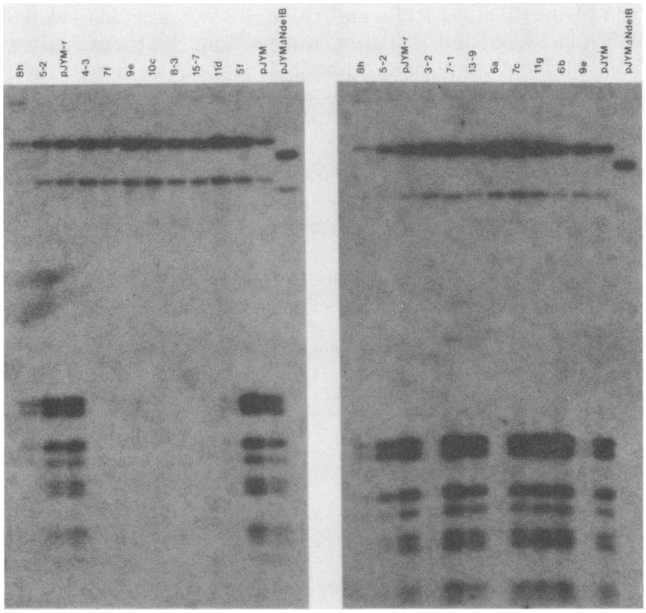

FIG. 2. Ability of large T antigen mutants to replicate in BSC-40 cells. BSC-40 cells were transfected as described in Materials and Methods. Hirt DNA was cleaved by *Mbo*I, which only cuts unmethylated, replicated DNA. pJYM is wild-type DNA. pJYM-r is a wild-type reconstruction of pJYM achieved by cloning the wild-type *Nde*IB fragment from mp8-*Nde*IB into pJYMΔ*Nde*IB.

TABLE 3. Ability of large T antigen mutants to transform Rat-3 cells as assayed by focus formation

Mutant	Expt no.[a]	No. of $G418^r$ colonies[b]	No. of foci[c]	No. of foci/ no. of wild-type foci
pJYM (wild type)	1	80, 191	15, 11	1
	2	48, 34	15, 18	1
	2	67, 55	21, 11	1
	3	56, 47	15, 32	1
	4	46, 41	15, 19	1
	5	10	16, 19	1
	6	66, 85	59, 61	1
9e	2	174, 143	1, 0	0.03
	4	75, 76	0, 0	<0.06
	6	159, 150	0, 0	<0.02
11d	1	77, 42	0, 2	0.08
	3	92, 81	1, 2	0.06
	5	54	0, 0	<0.06
6a	2	25, 47	0, 0	<0.06
	4	80, 61	0, 0	<0.06
	6	143, 163	0, 0	<0.06
5f	2	75, 82	6, 3	0.28
	4	115, 78	8, 12	0.59
	5	106	5, 10	0.43
7c	2	81, 41	37, 31	2.1
	4	67, 37	44, 37	2.4
	6	135, 122	46, 62	0.9
6b	2	114, 102	0, 0	<0.06
	4	63, 87	0, 2	0.06
	6	66, 41	1, 1	0.02
8h	1	234, 165	7, 8	0.58
	3	66, 71	32, 51	1.8
	5	149	20, 30	1.4
11g	2	9, 46	21, 25	1.4
	4	73, 48	54, 51	3.1
	6	35, 19	38, 21	0.49
7f	1	172, 164	1, 2	0.12
	3	127, 144	0, 0	<0.04
	5	58	0, 0	<0.06
10c	1	129, 196	36, 30	2.5
	3	97, 104	39, 47	1.8
	5	44	21, 24	1.3
15-7	1	161, 133	0, 0	<0.08
	3	135, 147	0, 0	<0.04
	5	62	0, 0	<0.06
8-3	1	134, 90	0, 0	<0.08
	3	114, 136	0, 0	<0.04
	5	36	0, 0	<0.06
4-3	1	92, 149	38, 48	3.3
	3	85, 82	95, 123	4.6
	5	72	52, 70	3.5
3-2	2	148, 83	71, 51	3.8
	4	90, 74	57, 84	4.2
	6	118, 78	69, 73	1.2
5-2	3	81, 83	24, 28	1.1

Continued

TABLE 3—*Continued*

Mutant	Expt no.[a]	No. of G418[r] colonies[b]	No. of foci[c]	No. of foci/ no. of wild-type foci
5-2	5	4	12, 19	0.89
	6	139, 141	20, 33	0.44
7-1	2	109, 75	92, 82	5.4
	4	35, 83	44, 69	3.3
	6	123, 120	63, 58	1.0
13-9	2	75, 109	21, 19	1.2
	4	65, 40	38, 47	2.5
	6	66, 54	37, 51	0.73
pJYM-r[d]	1	143, 147	26, 12	1.5
	3	74, 67	71, 44	2.5
	5	66	31, 19	1.4
	6	85, 36	76, 33	0.91
Neo[e]	1	109, 41	1, 0	0.04
	2	46, 65	0, 0	<0.06
	3	14, 13	0, 0	<0.04
	4	6, 3	0, 0	<0.06
	5	61	0, 0	<0.06
	6	69, 59	0, 0	<0.02
No DNA[f]	1	0, 0	0, 0	<0.08
	2	0, 0	0, 0	<0.06
	3	0, 0	0, 0	<0.04
	4	0, 0	0, 0	<0.06
	5	0, 0	0, 0	<0.06
	6	0, 0	0, 0	<0.02

[a] Each experiment consisted of transfecting subconfluent Rat-3 monolayers with mutant pJYM and pSV2neo DNA by calcium phosphate precipitation. Cells were trypsinized and put into G418 and focus assays 2 days after transfection.

[b] The number of G418[r] colonies given is the number per 0.1 ml of transfected cells. Two dishes per experiment were usually used, and the values for all of the dishes used are listed.

[c] Foci were stained 5 weeks after transfection. Two dishes per experiment were usually used, and the values for all of the dishes used are listed.

[d] pJYM-r is a wild-type reconstructed plasmid.

[e] Only pSV2neo and salmon sperm DNAs were transfected.

[f] Only calcium phosphate without DNA was transfected.

corrects for the input, it is clear that 8h actually replicated at wild-type levels.

Transformation assays of large T antigen mutants. The pJYM mutant constructs were also used to assay for the ability to transform an established nonpermissive cell line, Rat-3. Plasmid DNA was introduced into these cells by calcium phosphate transfection as described in Materials and Methods. pSV2neo DNA was added to ensure that DNA was both getting into the cells and being expressed. The average number of G418[r] colonies observed suggests that ca. 0.5% of cells initially present were successfully transfected per microgram of pSV2neo DNA. A total of six transfections were performed, and each mutant was assayed three times. After 48 h, the cells were split into focus and G418 assays (Table 3). Although the number of G418[r] colonies varied, their presence suggested that DNA was able to get into the cells in every transfection dish. This was especially important for mutant DNAs that did not give rise to any transformed foci. Mutants 9e, 11d, 6a, 6b, 7f, 15-7, and 8-3 were all deficient in producing transformed foci, whereas mutants 7c, 8h, 11g, 10c, 5-2, and 13-9 seemed to have wild-type transforming ability. Three mutants, 4-3, 3-2, and 7-1, appeared to be able to transform Rat-3 cells slightly better than wild type did. Foci induced by these mutants appeared earlier, looked more vigorous (data not shown), and were more abundant than those induced by wild type (Table 3). These mutants were also better than wild type at producing colonies in soft agar (data not shown). Mutant 5f consistently produced fewer (Table 3) and less dense foci than did wild type and so was designated as having a transformation-reduced phenotype. The pJYM clone reconstructed from a wild-type *Nde*IB fragment produced foci comparable in number to those produced by the original wild-type pJYM, as expected. Transfections which did not contain SV40 DNA did not produce transformed foci.

DISCUSSION

We chose the *Nde*IB fragment of SV40, which encodes a segment of SV40 large T antigen, as a target for mutagenesis. This region of the SV40 chromosome contains both small t unique and large T antigen coding sequences in addition to a portion of the large T and small t introns. The large T sequences within this region are especially interesting, since previous mutations here altered the DNA-binding activity of the protein. Mutants were identified which separate the replication and transformation phenotypes of T antigen, a separation which had not been detected earlier with *tsA* mutants. Also contained in this region of DNA are sequences which encode amino acids 225 to 337, where point mutants had yet to be described.

Seventeen large T antigen point mutants were assayed for two functions, the ability to support DNA replication from an SV40 origin of replication and the ability to transform established nonpermissive cells. A summary of the biological phenotypes of these seventeen mutants is diagrammed in Fig. 3. Three of the mutants which maintain a wild-type phenotype, 7c, 8h, and 11g, alter amino acids 113, 118, and 132 and are clustered in the region of T antigen thought to be important for origin binding and replication. One would have to argue that these mutations do not affect the previously identified functional domain. The other two replication- and transformation-competent mutants, 5-2 and 13-9, have changes in amino acids 235 and 265, respectively. These mutations are within a region of T antigen where point mutations have not been previously identified. Further analysis of mutations in this region is needed before any function(s) can be definitively assigned. The second group of mutants, defective in both replication and transformation (6a, 15-7, and 8-3), are all within the portion of T antigen where replication-negative, transformation-positive mutants have been isolated. Their identification suggests that either their altered amino acids are important in maintaining overall protein stability or the replication and transformation functions of T antigen at least partially overlap on this portion of the linear genetic map. The one replication-negative, transformation-positive mutant, 10c, lies in a similar region of T antigen (amino acids 144 and 149) as do other mutants with the same phenotype.

Mutants 6b and 5f make up a fourth group that are replication positive and transformation negative (R^+ T^-). Mutant 6b, which is fully defective in transformation, has the same glutamate to lysine substitution at position 141 as does mutant 11d but also has an amino acid change at 101. Since 11d is replication reduced and transformation defective (Fig. 3) and 6b is R^+ T^-, one can tentatively conclude that amino acid 141 is important for transformation. Mutant 5f is capable of replication but reduced in its ability to transform. Its amino acid change at position 112 is within the region of the

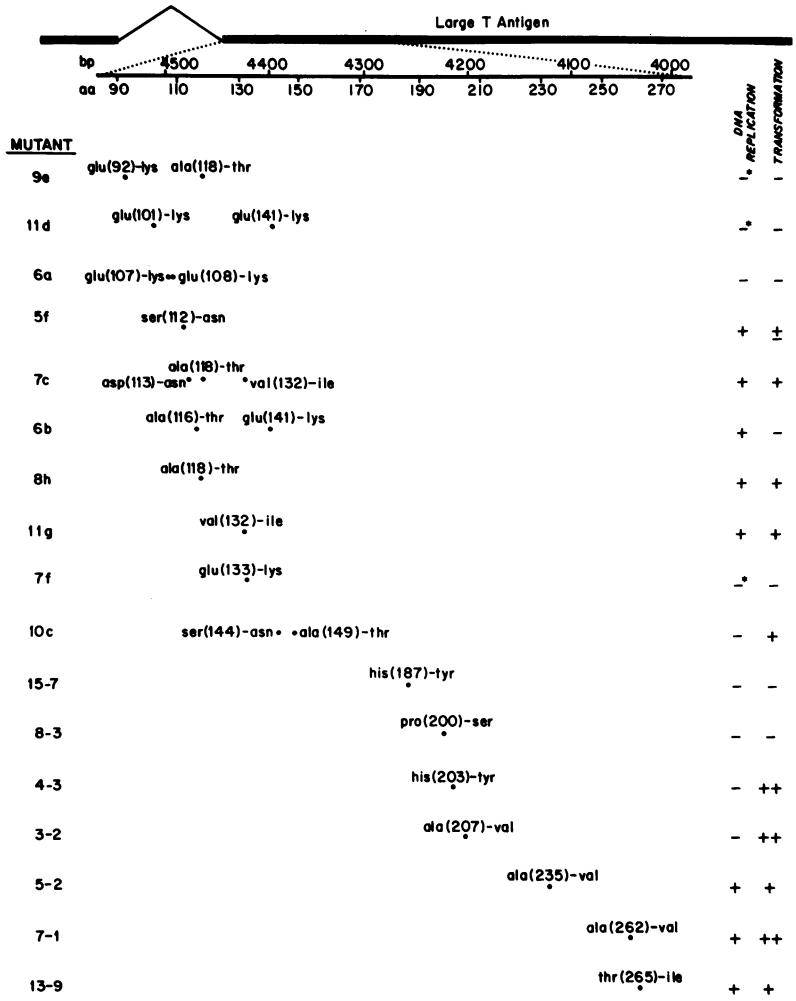

FIG. 3. Summary of large T antigen mutants. The *Nde*I region of large T antigen where bisulfite mutations were induced is expanded, and amino acid (aa) and base positions (bp) are marked. The predicted amino acid change(s) for each mutant is diagrammed. Base changes which do not result in an amino acid substitution are not shown. A summary of the ability of each mutant to replicate in BSC-40 cells and to transform Rat-3 cells is also given. Symbols: +, the mutant performed as wild type; −, the mutant was defective in the tested phenotype; ++, the mutant performed better than wild type; ±, the mutant was reduced in its ability to perform the tested phenotype; −*, DNA replication of the mutant was <1% of that of wild type.

other R^+ T^- mutants described in this report and by others (37).

Our analysis also defines two subclasses of mutants. One subclass is deficient in replication but has a supertransforming phenotype similar to that first described by Kalderon and Smith (37). The amino acids altered in mutants 4-3 (His[203]-Tyr) and 3-2 (Ala[207]-Val) are somewhat removed from the domain of amino acids 144 to 156 defined by those authors as responsible for supertransformation. It is interesting that Manos and Gluzman (46) have isolated a point mutant, T22, which contains an amino acid change at the same position as that in 4-3. The mutation in T22 alters histidine residue 203 to glutamic acid instead of tyrosine. T22 is defective in DNA binding and replication but can transform primary mouse cells. It is not known whether T22 also exhibits a supertransforming phenotype, as it was not analyzed for ability to transform established cell lines. Mutant 7-1 also exhibits the supertransforming phenotype but, unlike the other supertransforming mutants, 7-1 is replication competent. The lesion in 7-1, at amino acid 262, is in the same region of T antigen as mutants 5-2 and 13-9, which have wild-type phenotypes. Another subclass displaying an intermediate phenotype is composed of 9e, 11d, and 7f. These mutants are severely reduced in their ability to replicate and are transformation deficient. The lesion most likely responsible for this phenotype in 9e is the glutamate to lysine change at position 92, since the second alteration (Ala[118]-Thr) is also present in 8h but does not affect its function. Mutations at positions 101 and 141 in 11d and 133 in 7f are all within the replication domain of T antigen.

We sequenced three bisulfite-induced mutants, 6b, 5f, and 4-3, throughout the *Nde*IB region of their genomes to ensure that mutations did not occur in the protected region during bisulfite treatment. Both 6b and 5f belong to the R^+ T^- class of T-antigen mutants, which has very few members. In fact, very few mutations that destroy transforming function do not also destroy replication function. There are a number of possible explanations for this. The replication function of T antigen may require a very precise conformation of T protein to bind DNA, and even changes outside the binding site

might destroy the ability of the protein to bind and therefore replicate viral DNA. The transformation function, on the other hand, may be more tolerant to conformational changes. Alternatively, the DNA-binding domain of T antigen might be larger than that required for transformation and therefore more likely to be mutated. This would explain the prevalence of replication-negative, transformation-positive ($R^- T^+$) mutants. Regardless of the explanation, it is certain from the existence of $R^+ T^-$ and $R^- T^+$ mutants that the replication and transformation functions of T antigen are indeed encoded by at least partially separate domains. Mutant 4-3 is interesting, since it is one of the mutants obtained that can transform Rat-3 cells better than wild-type pJYM. This supertransforming phenotype may be a result of an alteration of T antigen that allows for better interaction between T antigen and any cellular factor(s) important in eliciting the transformed phenotype.

As a crude approximation of protein secondary structure, we examined the effect of amino acid changes on protein hydropathy. By the method of Hopp and Woods (35), which predicts protein antigenic sites, the effect on regional hydrophilicity for each amino acid substitution was calculated. This analysis indicated that most of the mutations which produced an altered phenotype are located in hydrophilic regions of the protein (data not shown). Changes to a more hydrophobic or hydrophilic nature in a region did not correlate with retention or loss of a phenotype, however. Further structural definition of these mutants must wait for the three-dimensional structure of large T antigen to be solved.

The analysis of the 17 mutants generated here, together with published reports of other large T antigen mutants, shows that one can indeed separate the replication and transformation functions of large T antigen. Although domains of T antigen can be defined, it is difficult to put a box around the amino acids responsible for any one function. Any attempt at drawing boxes indicates that functions are not contiguous but are indeed overlapping. This is not surprising, since, whereas the coding sequence is linear, the protein is a three-dimensional molecule. Also, some mutations may simply alter protein stability or gross protein conformation rather than directly altering the functional active site. The definition of functional domains of large T antigen does not preclude the possibility that different populations of T antigen (i.e., monomers versus oligomers or modified versus unmodified) exist that have separate biological functions. For example, mutations such as those we have introduced may affect the ability of one or more of these populations to exist. Indeed, recent results suggest that physically different populations of T antigen which have different activities do exist (55, 90). It is likely the case that both different intrinsic active sites and subpopulations of the protein allow SV40 large T antigen to carry out its variety of functions.

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