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Construction of a Specific Amber Codon in the Simian Virus 40 T-Antigen Gene by Site-Directed Mutagenesis

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The site-directed bisulfite mutagenesis technique has been used to construct a specific mutation, *am*404, at nucleotide position 3124 in the simian virus 40 genome. The mutation was contained within a *Pst*I restriction site (map position 0.27) and prevented cleavage by *Pst*I at that position. Nucleotide sequence analysis of the mutagenized region indicated that only a single base pair change had occurred: a guanosine·cytosine → adenine·thymine transition. Comparison of the nucleotide sequence of *am*404 with the known DNA sequence of simian virus 40 indicated that the mutation in *am*404 resulted in the conversion of a glutamine codon to an amber codon. *am*404 could not replicate autonomously when transfected into monkey cells (BSC-40) but did replicate when it was cotransfected with the late deletion helper virus *dl*1007. On the basis of its position in the T-antigen, gene *am*404 should produce a T-antigen 24% shorter than the wild-type protein.

Simian virus 40 (SV40) T-antigen is a multifunctional protein which is involved in SV40 replication, regulation of its own synthesis, and transformation (review in reference 9). By analogy with other multifunctional proteins, it may be possible to delineate the functional regions of T-antigen by isolating appropriate nonsense mutations in the T-antigen gene. Nonsense mutations have been difficult to isolate in most mammalian genes because of the lack of appropriate suppressor strains (3). The T-antigen gene of SV40, however, is unusual because both the complete DNA sequence and the reading frame for T-antigen are known (6, 8). In view of this, it should be possible to use the recently developed site-directed in vitro mutagenesis techniques (17, 21, 22) to construct specific nonsense mutations in various locations within the T-antigen gene. Inspection of the T-antigen sequence has revealed a number of codons which can be altered by the sodium bisulfite mutagenesis technique developed by Shortle and Nathans (21). This report describes the construction of one of these mutations, namely, an amber codon at nucleotide position 3124 (map position 0.27) in the SV40 genome. (The nucleotide numbering system proposed by Reddy et al. [18] is used in this communication.)

SV40 small-plaque variety 776 was obtained from the laboratory of Daniel Nathans and propagated as described previously (14). Form (fm) I SV40 DNA was isolated (14) and treated with *Pst*I in the presence of ethidium bromide (16) to produce a partial digestion mixture which included singly-nicked fm II circles (Fig. 1). The fm II SV40 circles consisted of molecules which were nicked at one of four locations on the SV40 genome, namely, on either the sense or nonsense strand at each of the two SV40 *Pst*I sites (map positions 0.27 and 0.04). Figure 1 illustrates the sequence of events at the nick which would ultimately yield an amber codon. The unfractionated *Pst*I digest mixture was treated with T4 bacteriophage DNA polymerase in the presence of dTTP (5) to expose a three-nucleotide, single-stranded gap. One of these nucleotides was a C residue (nucleotide position 3124) which was now susceptible to in vitro deamination with sodium bisulfite. The C residue is part of a CAG glutamine codon in the sense strand of the T-antigen gene. Mutagenesis of the cytosine residue to a uracil created the amber codon UAG. It also altered the recognition sequence for *Pst*I so that this site was no longer susceptible to cleavage by the restriction enzyme. Because procaryotic cells contain uracil *N*-glycosidases (11), the mutagenized fm II molecules were then repaired with T4 polymerase to insure that both strands of the genome contained the mutation. The mutagenized and repaired molecules were then digested to completion with *Pst*I. Molecules which had been successfully mutagenized in vitro were cleaved only once (at the nonmutagenized *Pst*I site) and were recovered as full-length linear SV40 molecules by agarose gel electrophoresis. Because this protocol created a mixture of four different mutations (two at each *Pst*I site), the mutagenized *Pst*I linear molecules

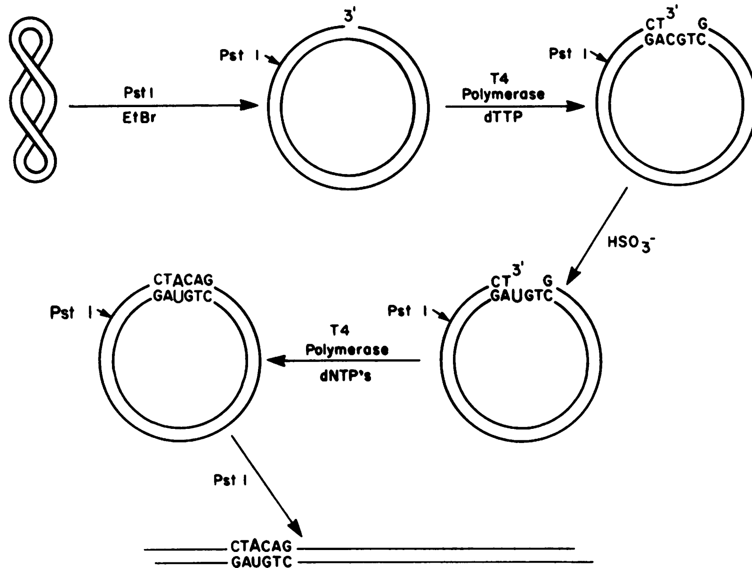

FIG. 1. *Construction of an amber codon in the SV40 T-antigen gene at map position 0.27. SV40 fm I DNA was converted to fm II relaxed circular DNA by nicking with PstI in a reaction mixture which contained in 2 ml: 20 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 50 mM $(NH_4)_4SO_4$, 50 μg of ethidium bromide per ml, 70 μg of SV40 fm I DNA per ml, and 50 U of PstI. The reaction mixture was protected against light and incubated at room temperature for 4 h. It was then phenol extracted and dialyzed against 1 mM Tris-hydrochloride (pH 7.5). T4 polymerase was used to create a three-nucleotide gap essentially as described by Englund (5), except that 1.6 mM dTTP was used. The gapped DNA molecules were then subjected to bisulfite mutagenesis as described by Shortle and Nathans (21), converting the non-base paired cytosine to a uracil. T4 DNA polymerase in the presence of equimolar amounts of all four dNTP's was used to repair the single-stranded region of the molecule (15). The DNA was then cleaved by PstI at the single intact PstI site, generating a fm III linear molecule to be cloned into the PstI site of pBR322 (2).*

were cloned into the bacterial vector pBR322 (2) to facilitate further screening. (The cloning procedure is described in the legend to Fig. 2.)

The pBR322/SV40 clones were screened by *Pst*I-*Hpa*I digestion (Fig. 2) for the absence of a *Pst*I site at SV40 map position 0.27 (see also Fig. 3A). Clones which were missing this *Pst*I site could be identified by the presence of an intact SV40 *Hpa*I-C fragment and the absence of the *Pst*I cleavage products of the *Hpa*I-C fragment (Fig. 3A). The digestion pattern of one of these clones, pDR404, is shown in Fig. 2, lane e. The cleavage products of the *Hpa*I-C fragment consisted of two fragments which could not be separated on 1.4% agarose gels and comigrated as the smallest band in wild-type digests (Fig. 2, lanes c and f). Figure 2, lane f illustrates the *Pst*I-*Hpa*I digestion pattern of a clone which apparently contained wild-type SV40 DNA. Presumably, the SV40 sequences in this clone either escaped mutagenesis or were repaired during replication in bacteria. Figure 2, lane d illustrates the restriction pattern of a clone which is missing the *Pst*I site at map position 0.04. Of 26 clones which were screened in this fashion, 11 were missing the SV40 *Pst*I site at map position 0.27, 4 were missing the SV40 *Pst*I site at map position 0.04, 1 contained only the SV40 *Pst*I-A fragment, 1 contained a small deletion at position 0.27 (approx 100 base pairs), and 2 were clones whose structures were not immediately clear. The remaining contained wild-type SV40 DNA as mentioned above.

The apparent preference for mutations at map position 0.27 as opposed to 0.04 (11 versus 4) is not fortuitous, but rather reflects the preferential cutting by *Pst*I at position 0.27 under conditions of partial digestion (Rawlins, unpublished observation). We have no biochemical explanation for this preference.

Three of the clones which were missing a *Pst*I site at position 0.27 (pDR404, pDR206, and pDR307) were chosen for further study. In principle these mutants were expected to consist of two types of mutations, one at position 3124 and the other at position 3125 (Fig. 3B). A guanosine·cytosine → adenine·thymine transition at position 3125 would cause no change in the amino acid composition of T-antigen. To determine which clones contained the amber codon, they

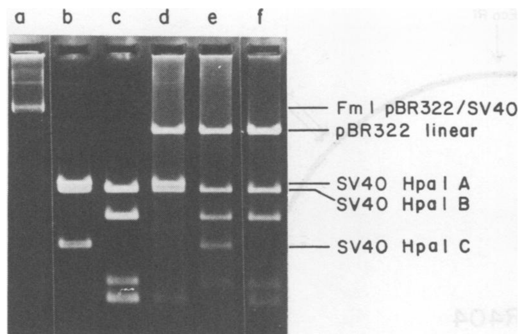

FIG. 2. *Restriction analysis of cloned SV40 PstI mutants. Fm III mutagenized SV40 molecules with PstI ends (see Fig. 1) were extracted from 1.4% agarose gels by an elution buffer containing 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% sodium dodecyl sulfate, and 0.1 mM EDTA. The mutant SV40 linear DNA (0.25 μg) and PstI-restricted pBR322 (0.25 μg) were ligated with T4 ligase by following the protocol of the enzyme supplier (Bethesda Research Laboratories) with a ligation efficiency of approximately 80%. The ligation mixture was phenol extracted, dialyzed, and concentrated to 20 μl. Ten microliters of the ligation mixture was transfected into Escherichia coli HB101 as follows: HB101 was grown to 110 Klett units in L broth; the cells were centrifuged and then resuspended in 1 ml of 30 mM $CaCl_2$. After the cells were chilled for 10 min at 0°C, the DNA was added, and the cells and DNA were chilled an additional 15 min. The transfection mixture was heat shocked at 42°C for 2 min and then diluted with 5 ml of L broth. Clones were selected by plating on selective media as described by Bolivar et al. (2) and screened for those showing tetracycline resistance and ampicillin sensitivity. DNA was extracted from selected clones by the alkaline method of Birnboim and Doly (1) and restricted with PstI and HpaI restriction endonucleases (enzymes and conditions from Bethesda Research Laboratories). The digests were analyzed by electrophoresis in 1.4% agarose gels which were stained with ethidium bromide and photographed (20). Lane a, Unrestricted pBR322/SV40 DNA; lane b, HpaI digest of wild-type SV40 DNA; lane c, HpaI-PstI digest of wild-type SV40 DNA; lane d, HpaI-PstI digest of a pBR322/SV40 clone mutated in the PstI site at map position 0.04 of SV40; lane e, HpaI-PstI digest of a pBR322/SV40 clone mutated in the PstI site at map position 0.27 of SV40; lane f, HpaI-PstI digest of a nonmutant pBR322/SV40 clone.*

were tested for the ability to replicate autonomously in monkey cells.

To do this, it was necessary to excise the SV40 sequences from the respective pBR322 clones. SV40 molecules which contain pBR322 insertions in the SV40 late region replicate poorly when transfected into monkey cells (Samulski and Muzyczka, unpublished observation; Pipas, Peden, and Nathans, personal communication). (Apparently some or all of the pBR322 sequences inhibit SV40 replication in a *cis*-acting manner. The reason for this inhibition is not known.) The SV40 sequences were excised by *Pst*I digestion and then ligated to form covalently closed circles and transfected into monkey cells. Infected cells were continuously labeled in vivo with $^{32}P_i$ between 24 and 72 h after infection and harvested at 72 h. The SV40 DNA from two clones, pDR404 (Fig. 4, lanes d and e) and pDR307 (Fig. 4, lane c), could not replicate autonomously. They could, however, replicate when an appropriate helper DNA, *dl*1007 (19), was cotransfected (Fig. 4, lanes f, g, and h). *dl*1007 is an SV40 late deletion (0.83 to 0.15 map units) which contains an intact T-antigen gene and can replicate autonomously when transfected into monkey cells (Fig. 4, lane b). The third clone, pDR206, was phenotypically wild type (data not shown). Thus, presumably both types of mutation (positions 3125 and 3124) were represented in our clones. This is consistent with the reading frame for T-antigen which was inferred by Reddy et al. (18) and Fiers et al. (6). More recently this reading frame has been confirmed by Denhardt and Crawford (4) by peptide analysis of T-antigen proteins obtained from cells infected with the viable deletions *dl*1263 and *dl*1265.

To verify that the replication-negative phenotype of the *Pst*I mutants was due to the expected guanosine·cytosine → adenine·thymine transition (rather than other types of DNA modification), the mutagenized region of pDR404 was sequenced by the method of Maxam and Gilbert (12). The *Hae*III-A fragment of SV40 (Fig. 3A), which included the mutagenized *Pst*I site, was isolated from pDR404 by RPC-5 chromatography as described by Larsen et al. (10). The *Hae*III-A fragment was ^{32}P labeled by treatment with polynucleotide kinase and then digested with *Hin*dIII to obtain a singly 5′-labeled subfragment which contained the *Pst*I region. Figure 5 illustrates that the nucleotide sequence of pDR404 was identical to the wild-type sequence, except for nucleotide position 3124 which contained the expected transition.

Several investigators (7, 21) have previously pointed out the specificity of bisulfite mutagenesis for single-stranded C residues. The DNA sequence analysis of *am*404 (Fig. 5 and 3B) dramatically illustrates this specificity. Neither the C residue at position 3127, which is the terminal 3′ base pair adjacent to the mutagenized gap, nor the C residues at positions 3119 through 3122 were affected by this protocol in

Wild type SV40 GGA TTT TGT GAC GTC CGG TCT AAA 5'
CCT AAA ACA CTG CAG GCC AGA TTT 3'

Mutant SV40 GGA TTT TGT GAC ATC CGG TCT AAA 5'
CCT AAA ACA CTG TAG GCC AGA TTT 3'

FIG. 3. *(A) Map of the pBR322/SV40 clone, pDR404. The heavily shaded region of the circular map represents the portion of the genome contributed by pBR322. The "X" within the HpaI-C fragment (represented by the boxed area) indicates the location of the mutagenized base at nucleotide position 3124 in the PstI site. The two HaeIII sites which bound the SV40 HaeIII-A fragment used for sequencing are also indicated. (B) Nucleotide sequence around position 3124 in wild-type SV40 DNA and in the mutant SV40 DNA of pDR404. Cleavage sites for PstI and HaeIII are as indicated. The wild-type sequence is from Fiers et al. (6). The reading frame for large T antigen is indicated by the spacing between the nucleotides. The base change in the mutant is indicated by an asterisk.*

this mutant.

Temperature-sensitive mutants, deletion mutants, and Ad-SV40 hybrids have been useful tools for the study of the functional properties of SV40 T-antigen (9). However, each of these mutants or hybrids has inherent limitations in the amount of genetic and biochemical information it can provide. We have constructed an amber codon in the T-antigen gene of SV40 by in vitro mutagenesis of the *Pst*I site at map position 0.27. Nonsense mutants may be particularly useful since they are generally tight mutants which do not lead to reading frame shifts or the addition of novel sequences of amino acids. Future work with *am*404 and other nonsense mutants should help evaluate the biological role of various portions of the T-antigen molecule.

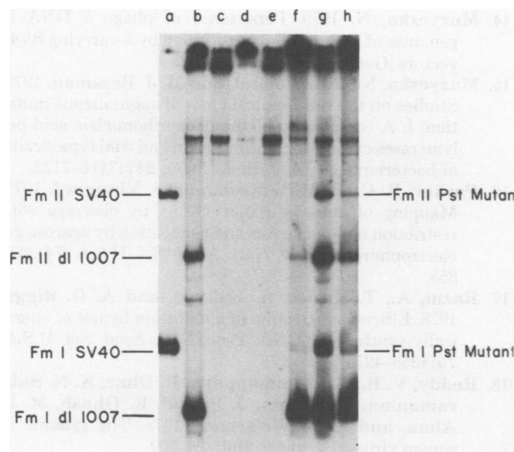

FIG. 4. *DNA transfection of BSC-40 monkey cells. DNAs from pDR307 and pDR404 were cleaved with PstI to produce linear pBR322 and linear SV40 DNA molecules. The samples were heat inactivated at 68°C for 15 min (to denature the restriction enzyme) and then ligated with T4 ligase. Analysis by agarose gel electrophoresis showed that at least 75% of the pBR322 and SV40 linear molecules had been converted to circular forms. DNA from each ligation mixture was transfected into subconfluent BSC-40 monkey cells in a 10-cm dish by the DEAE-dextran technique (13). Products of viral DNA replication were labeled in vivo by adding $^{32}P_i$ at 24 h posttransfection. The cells were harvested at 72 h, and the DNA was extracted by the Hirt procedure (8), phenol extracted, and ethanol precipitated. The DNA was then electrophoresed on a 1.4% agarose gel and autoradiographed for 20 h. Each lane represents the total ^{32}P-labeled DNA isolated from one 10-cm dish which had been transfected with: lane a, 0.1 μg of wild-type SV40 DNA; lane b, 0.1 μg of dl1007 DNA; lane c, 0.025 μg of pDR307 ligation mixture; lane d, 0.1 μg of pDR404 ligation mixture; lane e, 0.05 μg of pDR404 ligation mixture; lane f, 0.025 μg of pDR307 ligation mixture plus 0.1 μg of dl1007; lane g, 0.1 μg of pDR404 ligation mixture plus 0.1 μg of dl1007; lane h, 0.05 μg of pDR404 ligation mixture plus 0.1 μg of dl1007.*

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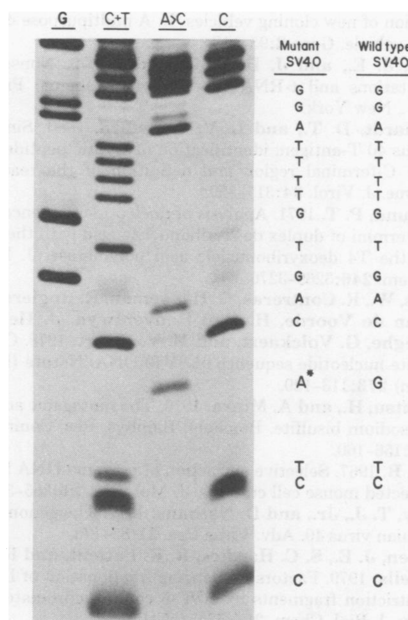

FIG. 5. *Nucleotide sequence analysis of the mutagenized DNA segment of pDR404. Purified pDR404 DNA was restricted with HaeIII (Bethesda Research Laboratories), and the SV40 HaeIII-A fragment (map position 0.27 to 0.59) was separated from other fragments by high-pressure liquid chromatography on an RPC-5 column (0.9 by 20 cm). Elution of the column was essentially as described by Larsen et al. (10). The column was eluted with a 500-ml KCl gradient (0.55 M to 0.75 M) which contained 10 mM Tris-hydrochloride (pH 7.0) at a flow rate of 1 ml/min. Elution was at room temperature, and 5-ml fractions were collected. Fractions which contained the SV40 HaeIII-A fragment were identified by agarose gel electrophoresis, concentrated by rotaevaporation, and dialyzed against 10 mM Tris-hydrochloride (pH 7.0). The fragment was then 5'-terminally labeled and cleaved with HindIII (Bethesda Research Laboratories), which cuts once within the HaeIII-A fragment. The HindIII cleavage fragment which contained the mutated PstI site was chemically sequenced according to the procedure of Maxam and Gilbert (12). The reaction products were fractionated on 20% polyacrylamide gel (3 mm by 20 cm by 40 cm) containing 7 M urea and Tris-borate buffer (pH 8.3). Nucleotide specificities are as indicated. The gel was autoradiographed for 12 h at −70°C. The site of the guanosine → adenine (G → A) transition is indicated by an asterisk. The wild-type SV40 sequence is from Fiers et al. (6).*

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