

Local mutagenesis: A method for generating viral mutants with base substitutions in preselected regions of the viral genome*

(simian virus 40/*in vitro* mutagenesis/restriction endonucleases/DNA replication)

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ABSTRACT DNA from simian virus 40 (SV40) was prepared for local mutagenesis by nicking the molecule at a specific site with a restriction endonuclease that recognizes one site in SV40 DNA and then extending the nick enzymatically to expose a short, single-stranded segment of DNA. The "gapped" DNA was treated with a single-strand-specific mutagen, sodium bisulfite, which converts cytosine to uracil. After mutagenesis, the gap was repaired with DNA polymerase, generating molecules resistant to the restriction enzyme used to make the initial nick. From cells infected with DNA thus modified, SV40 mutants were isolated that had enzyme-resistant genomes. In some cases, precise positions of G·C to A·T transitions could be inferred from the patterns of susceptibility of mutant DNA to other restriction endonucleases whose recognition sequences were altered by the mutagenesis procedure. One of the restriction endonuclease sites mutagenized (*Bgl* I) maps at the origin of SV40 DNA replication and near sequences corresponding to the 5' ends of viral mRNAs. Many of the resulting *Bgl* I-resistant mutants yielded small plaques, suggesting partial defectiveness in DNA replication or transcription.

Genetic analysis of viruses depends on the isolation and characterization of mutants with specific physiological defects. Generally, viral mutants have been isolated by treating the virus or virus-infected cells with mutagens and selecting desired mutant phenotypes from the pool of randomly mutagenized virus. With the advent of site-specific restriction endonucleases, enzymes that cleave duplex DNA at particular nucleotide sequences, it has been possible to construct mutants with deletions at preselected enzyme cleavage sites in a viral genome (1-3). In the case of simian virus 40 (SV40), to which these methods have been applied most extensively, a collection of deletion mutants constructed *in vitro* has been useful for identifying viral genes and their products (4-6).

We wished to extend the functional map of the SV40 genome by isolating and characterizing point mutants in those regions of the DNA whose function is not yet well characterized. Such mutants could be particularly useful in dissecting early viral functions and in identifying regulatory signals involved in replication and transcription of viral DNA or in the processing and translation of viral RNA. In this report we describe a method for producing base substitutions at predetermined restriction sites in the viral genome. Two sites were chosen for the initial experiments, both outside known structural genes. In each case, local point mutants were generated with high efficiency.

MATERIALS AND METHODS

SV40 form I DNA was prepared from BSC-1 cells infected with SV40 strain 776, as described (7), and purified by electropho-

resis (8). Restriction endonucleases were from commercial sources, except *Bgl* I and *Alu* I which were prepared by published procedures (9, 10). Electrophoresis of DNA in agarose or polyacrylamide gels was carried out as described (8). Singly nicked SV40 DNA was prepared from form I DNA by incubating DNA (100 µg/ml) with *Bgl* I (100 units/ml) or *Hpa* II (80 units/ml) in the presence of ethidium bromide (11) (120 µg/ml for *Bgl* I, 20 µg/ml for *Hpa* II) at 25° for 2 hr; 80-90% of the DNA was converted to singly nicked form II. To obtain randomly nicked form II DNA, ethidium bromide (100 µg/ml) was used with 150 ng of pancreatic DNase I per ml.

DNA Polymerase Reactions. All experiments in which DNA polymerase I from *Micrococcus luteus* (Miles Laboratory) was used were carried out at 11° in 70 mM Tris-Cl, pH 8.0/7 mM MgCl₂/7 mM 2-mercaptoethanol with approximately 1 unit of enzyme and 1 µg of DNA per 10 µl of reaction mixture. When the enzyme was used as an exonuclease, 0.6 mM dTTP was present (12). To repair DNA, all four deoxynucleoside triphosphates were added to a concentration of 0.4 mM. After all enzymatic steps, the DNA was extracted with phenol and dialyzed against 15 mM NaCl/1.5 mM Na citrate. The size of local gaps in DNA was determined with ³²P-labeled SV40 DNA nicked and gapped at the *Hpa* II site and subsequently digested with *Kpn* I and *Hpa* I to isolate a 200-base-pair fragment containing the gap. This fragment (the electrophoretic mobility of which was less than that of the corresponding ungapped fragment) was then denatured with glyoxal and the single strands were subjected to electrophoresis to determine their length relative to standards (13).

Sodium Bisulfite Reactions. A solution of 4 M bisulfite (pH 6.0) was prepared immediately prior to use by dissolving 156 mg of NaHSO₃ and 64 mg of Na₂SO₃ in 0.43 ml of deionized water (14). The final incubation mixture consisted of 3 volumes of this solution, 1 volume of DNA solution (approximately 50 µg/ml in 15 mM NaCl/1.5 mM sodium citrate, pH 7.0), and 0.04 volume of 50 mM hydroquinone (14), with all components at 0° when mixed. After paraffin oil was layered above the solution, the tube was incubated in the dark at 37° for the specified time. To terminate the reaction, the incubation mixture was dialyzed against the following sequence of buffers: (i) 1000 volumes of 5 mM potassium phosphate, pH 6.8/0.5 mM hydroquinone at 0° for 2 hr; (ii) repeat of (i); (iii) 1000 volumes of 5 mM potassium phosphate, pH 6.8 at 0° for 4 hr; (iv) 1000 volumes of 0.2 M Tris-HCl, pH 9.2/50 mM NaCl/2 mM EDTA at 37° for 16-24 hr; (v) 1000 volumes of 2 mM Tris-HCl, pH 8.0/2 mM NaCl/0.2 mM EDTA at 4° for 6-12 hr. The water used to prepare the first four dialysis buffers was degassed by

Abbreviations: SV40, simian virus 40; *Hpa* II^r and *Bgl* I^r, *Hpa* II- and *Bgl* I-resistant mutants.

* This is publication no. 20 in a series on the genome of simian virus 40. Publication no. 19 is ref. 4.

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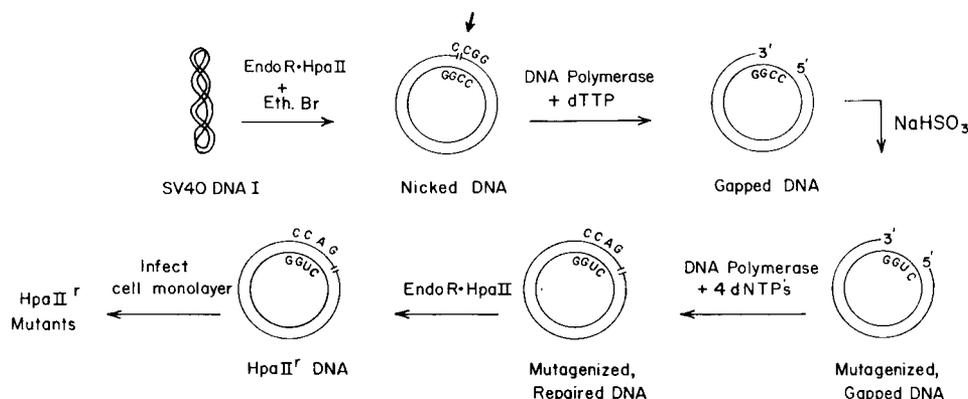


FIG. 1. Steps used to generate SV40 mutants with base substitutions at a preselected site in viral DNA. *Hpa* II^r indicates resistance to the *Hpa* II restriction endonuclease.

vigorous boiling prior to use. To quantitate the level of deamination, linear SV40 DNA that had been digested to approximately 40% with exonuclease III was carried through the bisulfite incubation and used as a template-primer with DNA polymerase. The ratio of [α -³²P]dATP to [³H]dGTP incorporation was determined with both bisulfite-treated and untreated template-primer by assay of radioactivity made acid insoluble on PEI-cellulose. From these results, the percentage conversion of cytosine to uracil in the bisulfite-treated template DNA was calculated.

RESULTS

Outline of the Method. A diagram of the method used to generate SV40 mutants with local base substitutions is shown in Fig. 1. Covalently closed duplex circles of viral DNA (form I) were nicked in one strand in the presence of ethidium bromide with a restriction endonuclease, such as *Hpa* II or *Bgl* I, that recognizes one site in SV40 DNA. The nick was then converted into a small gap by limited exonuclease action of DNA polymerase I in the presence of a single deoxynucleoside triphosphate. It should be noted that only one strand is nicked and gapped in any given molecule and that different nucleotides are exposed in each DNA strand. The gapped molecules were treated with the single-strand-specific mutagen sodium bisulfite, which deaminates cytosine to uracil, and the gaps were then filled in by incubating the DNA with DNA polymerase I in the presence of all four deoxynucleoside triphosphates. To enrich the mutagenized population of molecules, the same restriction enzyme used to nick the DNA at the beginning of the procedure was used to linearize those molecules whose restriction sites escaped mutagenesis. After isolation of the resistant circular DNA, individual mutants were cloned by infecting cell monolayers with the restriction enzyme-resistant DNA to form viral plaques. Isolated plaques were then surveyed for the presence of mutants with enzyme-resistant viral DNA. Thus, the phenotype defining the mutant class in these prototype experiments is resistance of viral DNA to cleavage by a restriction enzyme that recognizes only one site in SV40 DNA.

Effect of Sodium Bisulfite on Single-Stranded Template DNA. In preliminary experiments we determined the effect of bisulfite treatment of single-stranded SV40 DNA on its template activity with DNA polymerase I of *M. luteus*. For this purpose, SV40 DNA was linearized with *Hpa* II, digested with *Escherichia coli* exonuclease III to give 40% acid solubilization of DNA nucleotides, treated with bisulfite, and "repaired" with polymerase in the presence of [α -³²P]dATP, [³H]dGTP, and unlabeled dTTP and dCTP to estimate the percentage of cytosine residues deaminated. After bisulfite treatment sufficient

to deaminate approximately 14% of the cytosine residues in single-stranded DNA (1 M bisulfite, 16 hr, 37°, pH 6.0), repair was essentially the same as that of unmodified DNA and was complete as judged by electron microscopic examination and restriction enzyme analysis. Similarly, after about 58% conversion of cytosine to uracil residues (3 M bisulfite, 8 hr, 37°, pH 6.0), repair was 84% as efficient as the control. We conclude that, even after extensive deamination of single-stranded DNA by bisulfite, the DNA serves as an efficient template for *M. luteus* polymerase, resulting in replacement of many G-C base pairs by A-U pairs. A similar conclusion was reached by Kai *et al.* (14).

Effect of Bisulfite on Duplex DNA. Bisulfite has been reported to act on cytosine residues only in single-stranded polynucleotides; by chemical analysis no detectable reaction with duplex polynucleotides was observed (16). To assay its activity on duplex DNA in a more sensitive way, we measured the effect of bisulfite on the specific infectivity of randomly nicked SV40 DNA. (This relaxed form was used because of the single-stranded regions in supercoiled DNA.) As shown in Table 1, there was no decrease in specific infectivity even after 16 hr of incubation with 3 M sodium bisulfite. However, the interpretation of the results is complicated by the consistent enhancing effect of incubation in 3 M NaCl. Taking the NaCl-incubated DNA as control, there may be some decrease in specific infectivity after prolonged exposure to bisulfite. On the basis of these results we conclude that, even under conditions that result in over 50% deamination of cytosine residues in single-stranded DNA, there is little or no lethal effect of bisulfite on relaxed duplex circles of SV40 DNA. The experiments clearly do not exclude the possibility that nonlethal base substitutions have occurred (see *Discussion*).

Table 1. Specific infectivity of SV40 DNA II after incubation with sodium bisulfite

Conditions	pfu/ng DNA*	
	Exp. 1	Exp. 2
No incubation	28 ± 9	45 ± 19
3 M NaCl, 4 hr	59 ± 8	—
3 M NaCl, 16 hr	—	105 ± 25
3 M NaHSO ₃ , 4 hr [†]	43 ± 14	60 ± 17
3 M NaHSO ₃ , 8 hr	29 ± 5	40 ± 6
3 M NaHSO ₃ , 16 hr	—	36 ± 6

* Results are expressed as mean plaque-forming units (pfu) ± SD from six dishes (Exp. 1) or four dishes (Exp. 2).

[†] Four-, 8-, and 16-hr incubations with bisulfite produce 30%, 58%, and >60% deamination of cytosine residues, respectively, in single-stranded DNA.

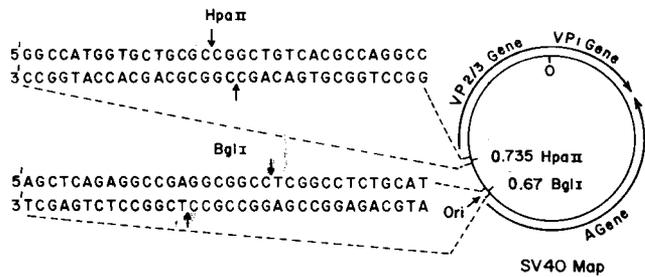


FIG. 2. Physical map of the SV40 genome indicating location of known genes, the direction of transcription, the origin of DNA replication (Ori), and cleavage sites for *Bgl* I and *Hpa* II. Numbers refer to map units. A gene codes for SV40 T antigen; VP1 is the major capsid protein; VP2 and VP3 are minor capsid proteins. Sequences about the *Bgl* I and *Hpa* II sites, shown at the left, are from ref. 15. The *Bgl* I cleavage site was reported to us by B. S. Zain and R. J. Roberts (personal communication).

Mutagenesis around Restriction Endonuclease Sites. To prepare SV40 DNA for mutagenesis at small local gaps, form I DNA was nicked with *Hpa* II or *Bgl* I (Fig. 1). Each of these enzymes cuts SV40 DNA at a unique site outside of known genes, and each site contains cytosine residues in the recognition sequence (Fig. 2). The single-strand breaks were then extended by means of the 3' and 5' exonuclease activities of DNA polymerase I of *M. luteus* (17). To estimate the distribution of gap sizes, [³²P]DNA was carried through the gapping procedure (starting with *Hpa* II-nicked molecules), and the length distribution of single-stranded fragments derived from gapped molecules was determined by gel electrophoresis. From the mobility of the resulting single-strand fragments relative to standards, we estimate that, after a 30-min incubation at 11° with DNA polymerase in the presence of dTTP, the predomi-

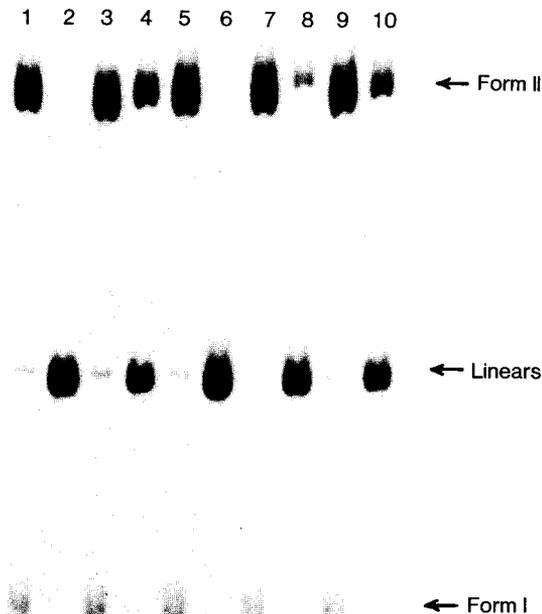


FIG. 3. Changes in sensitivity of SV40 DNA to *Bgl* I during the course of the mutagenesis procedure, as monitored by gel electrophoresis. Lanes: 1 and 2, DNA nicked with *Bgl* I; 3 and 4, after the gapping reaction; 5 and 6, after repair of the gap (no mutagenesis); 7 and 8, after repair of gapped molecules that had been treated with 3 M bisulfite for 1 hr; 9 and 10, after repair of gapped molecules that had been treated with 3 M bisulfite for 4 hr. For each pair, the even-numbered sample was cleaved with *Bgl* I prior to electrophoresis. Note the presence of *Bgl* I-resistant form II in lanes 8 and 10.

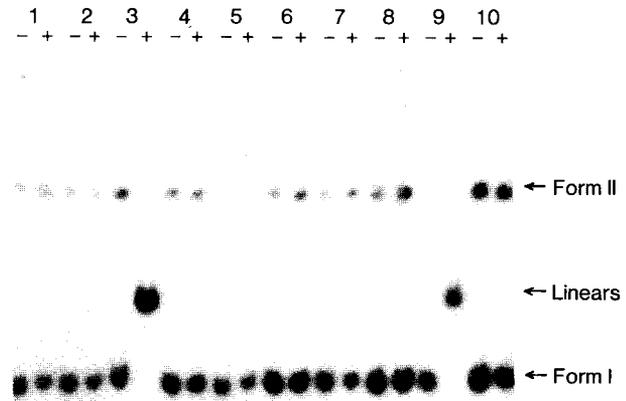


FIG. 4. Survey of plaques for the presence of *Hpa* II^r mutants. ³²P-Labeled viral DNA extracted from cells infected by a single plaque suspension was subjected to electrophoresis before (–) and after (+) digestion with *Hpa* II. All but two of the isolates (lanes 3 and 9) had *Hpa* II^r DNA. Unlabeled wild-type DNA present in each sample was linearized completely, as shown by staining the gel with ethidium bromide. Similar autoradiograms were obtained with DNA from *Bgl* I^r mutants treated with *Bgl* I.

nant gap was about 5 nucleotides long in the 5' → 3' direction.

After the gapping reaction, the DNA was treated with sodium bisulfite under conditions estimated to deaminate either 8% or 30% of the cytosine residues in single-stranded DNA, and the gap was then repaired. In Fig. 3 are the results of monitoring the DNA at each step of the above procedure for sensitivity to linearization by *Bgl* I, the enzyme used to produce nicked molecules in the experiment illustrated. Nicked DNA remained sensitive to the enzyme; after gapping, much of the DNA became resistant; and after repair of nonmutagenized gapped molecules, the enzyme site was restored. However, after repair of *mutagenized* gapped molecules, a variable fraction of the originally resistant gapped DNA remained resistant, the amount depending on the bisulfite reaction time. From these results we infer that some of the molecules have undergone at least one G·C → A·U change in the *Bgl* I recognition site. Similar results were obtained with *Hpa* II-nicked SV40 DNA.

Isolation of Restriction Endonuclease-Resistant Mutants. After purification of mutagenized and repaired resistant DNA by gel electrophoresis, this DNA was used to infect BSC-1 cell monolayers in order to isolate individual plaques (18). (We later found that *in vitro* repair of the gap was not essential.) In these initial experiments, designed to evaluate the method, we simply sought mutants with altered enzyme sites and made no attempt to isolate defective mutants in the population by complementation (8). Thirty-five plaques from the *Hpa* II series and 23 plaques from the *Bgl* I series were selected randomly and were surveyed for the presence of *Hpa* II-resistant (*Hpa* II^r) or *Bgl* I-resistant (*Bgl* I^r) mutants, respectively, by analyzing ³²P-labeled viral DNA extracted from cells infected by a single plaque suspension for enzyme resistance (Fig. 4). Of 35 "*Hpa* II plaques" tested, 21 yielded *Hpa* II^r DNA; 19 of 23 "*Bgl* I plaques" yielded *Bgl* I^r DNA. It should be noted that many of the *Bgl* I^r and *Hpa* II^r mutants produced plaques at 37° that were smaller than wild-type plaques, suggesting that local base substitution caused a partial defect in viral function (see *Discussion*). That the generation of resistant mutants was dependent on treatment with bisulfite was shown by repeating the entire procedure with *Hpa* II-nicked molecules but incubating the gapped DNA in 3 M NaCl (at pH 6.0) instead of sodium bisulfite. In contrast to the results with bisulfite-treated DNA,



FIG. 5. Inferred sequence changes in *Hpa* II^r mutants, based on the results shown in Fig. 6 and those described in the text. G·C → A·T changes are noted in bold print. [Restriction endonuclease sites are from ref. 20 and R. J. Roberts (personal communication).]

no detectable *Hpa* II-resistant viral DNA was generated in cells infected with unrepaired, gapped DNA or after repair with polymerase. Our general conclusion is that the mutagenesis procedure generated local mutants with high efficiency.

Analysis of DNA from Restriction Endonuclease-Resistant Mutants. In order to determine whether the mutants generated by the above procedure actually contained point mutations rather than small deletions, we analyzed several mutant DNAs by restriction enzyme cleavage, to look for changes in electrophoretic mobility of small fragments containing the mutational site. The *Bgl* I site is within *Alu* fragment D (270 base pairs) and the *Hpa* II site is within fragment *Hae* III-0 (30 base pairs) (19). In all of the 15 *Bgl* I^r DNAs analyzed, the mobility of *Alu*-D was identical to that of *Alu*-D from wild-type virus. Similarly, all of the 19 *Hpa* II^r DNAs analyzed yielded an *Hae* III-0 fragment with normal mobility. Therefore, by this criterion there is no evidence for deletion at either enzyme site. A more stringent test for base substitution could be readily applied in the case of *Hpa* II^r mutants because changes of G·C to A·T pairs within the *Hpa* II site could eliminate the overlapping *Hha* I site or possibly generate new *Alu* I and *Pvu* II sites (Fig. 5). We therefore screened 21 *Hpa* II^r DNAs for *Hha* I, *Pvu* II, and *Alu* I sites near the original *Hpa* II site. Mutant DNAs fell into three classes. One class (3 of 21 mutants) gained new *Alu* I and *Pvu* II sites within the *Alu* fragment R adjacent to the original *Hpa* II site, and none of the mutant DNAs had lost the *Hha* I site (Fig. 6). A second class (three mutants) lost the *Hha* I site but did not gain an *Alu* or a *Pvu* site. The third class (15 of 21) retained the *Hha* I site without gaining an *Alu* or a *Pvu* site. As shown in Fig. 5, each of the "phenotypes" can be explained by conversion of a single G·C pair within the *Hpa* II site to an A·T pair.

DISCUSSION

Site-specific cleavage and nucleotide sequence analysis of SV40 DNA coupled with physiological studies have provided a physical and functional map of the viral genome (19, 21, 22). With the map in hand, it is possible to select functionally interesting sites for perturbation in order to determine the effect of such alterations on biological activities of the viral genome.

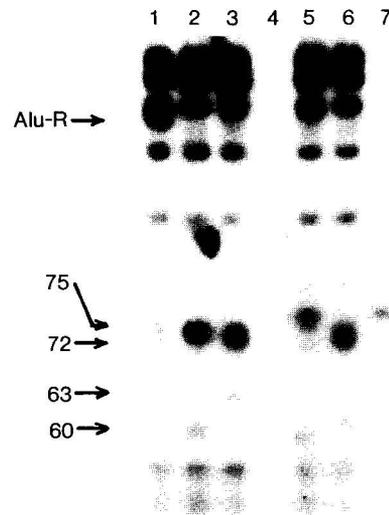


FIG. 6. *Alu* I and *Pvu* II digests of DNA from a class 1 *Hpa* II^r mutant and from wild-type SV40. Only the relevant part of the pattern is shown. The numbers on the left refer to fragment lengths in nucleotide pairs. Lanes: 1-4, wild-type DNA; 5-7, mutant DNA; 1 and 5, *Alu* I alone; 2, *Alu* I + *Hpa* II; 3 and 6, *Alu* I + *Hha* I; 4 and 7, *Pvu* II alone. With wild-type DNA, *Alu*-R is cleaved by *Hpa* II (lane 2) and *Hha* I (lane 3), yielding the expected smaller fragment pairs (75 and 60, or 72 and 63 nucleotide pairs, respectively); *Pvu* II yielded no small fragments (lane 4). With mutant DNA, *Alu* I alone (lane 5) yielded no *Alu*-R, but two new fragments nearly identical in size to those present in *Alu* I + *Hpa* II digests of wild-type DNA appeared; the *Hha* I site was still present (lane 6); and a new *Pvu* II fragment corresponding to the larger new *Alu* I fragment was apparent (lane 7). These results localize the new *Pvu* II and *Alu* I sites to within a few base pairs of the *Hpa* II and *Hha* I sites.

The method described in this communication represents an example of this general approach. Localized targets, created by exposing short single-stranded regions of viral DNA at specific restriction endonuclease sites, have been mutagenized with high efficiency by a single-strand-specific mutagen. We chose sodium bisulfite as the mutagenizing agent because of its specific deamination of cytosine to uracil in single-stranded polynucleotides (23). The resulting uracil-containing DNA served as an effective template for DNA polymerase *in vitro*, yielding A·U pairs in place of G·C pairs. When the mutagenized restriction enzyme-resistant DNA was used to infect cell monolayers, most of the resulting viral clones were mutants identifiable by resistance of their DNAs to the restriction enzyme used to construct the mutant DNA. In some cases, new restriction sites were created within the mutagenized region. From the restriction patterns we could infer that specific G·C pairs were replaced by A·T pairs.

For the local mutagenesis method to be generally useful, duplex regions of DNA must not suffer irreversible base changes. Our data indicate that, even under conditions that result in deamination of more than half of the cytosine residues in single-stranded DNA, there is little or no lethal effect of bisulfite on SV40 form II DNA. Nor were silent base alterations extensive, because all of 36 cloned *Hpa* II^r or *Bgl* I^r mutant DNAs cleaved with *Alu* I, an enzyme that recognizes 32 ^{AGCT}/_{TCGA} sites in SV40 DNA (2.9% of all G·C pairs), yielded a digestion pattern indistinguishable from that of unmutagenized DNA (or had the additional site noted in Fig. 5). Similar results were obtained with 19 cloned *Hpa* II^r DNAs cleaved with *Hae* III, which recognizes 18 different ^{GGCC}/_{CCGG} sites in SV40 DNA (3.3% of G·C pairs). More direct measurements by Lindahl *et al.* (24)

on bisulfite-treated form I PM2 DNA indicated that an average of 0.6 cytosine residue per molecule of 6×10^6 daltons was converted to uracil after incubation with 1.5 M bisulfite for 5 hr at 37°. Presumably, the rate of deamination would be even lower with relaxed circular DNA. In addition to this protective effect of duplex structure, cells contain an enzymatic system that excises uracil-containing segments of DNA (25). Were bisulfite to deaminate cytosine residues in duplex regions, such correction systems should restore the original G-C base pairs preferentially.

Several extensions and applications of the local mutagenesis method come to mind. One can vary the size and position of the single-strand gap in a number of ways—for example, by using different exonucleolytic enzymes and incubation conditions, by nick translation prior to gap generation, or by constructing gapped molecules with appropriate single strands of DNA. Furthermore, by using multicut restriction enzymes or even pancreatic DNase to nick SV40 DNA in the presence of ethidium bromide, one can produce base substitutions at many different sites in a population of molecules, from which mutants of interest could be isolated. To select nonviable, transcomplementable mutants, either in genes or in regulatory signals, deletion mutants of SV40 can be used as helpers to form “complementation plaques” (7). All of these procedures should also be applicable to other cloned, propagatable DNA molecules.

Other methods for producing base substitutions at preselected sites of viral genomes have been reported. Weissmann and his colleagues (26) described a site-directed mutagenesis procedure that uses coliphage Q β replicase to incorporate nucleotide analogs at specific sites in Q β RNA, and Borrias *et al.* (27) isolated temperature-sensitive mutants of coliphage ϕ X174 after transfection with a partial heteroduplex consisting of single-stranded phage DNA and a mutagenized fragment. With suitable modifications, each of these approaches might have more general applicability.

For the initial experiments reported in this communication, our search for mutants was limited to those that were viable and resistant to a one-cut restriction enzyme. We chose two sites in the SV40 genome that appear to lie outside structural genes and for which no point mutants were available (28). The *Bgl* site is particularly interesting because it is at or near the origin of viral DNA replication and perhaps near promoter sites for both early and late transcription (see Fig. 2). One might therefore hope to isolate point mutants with alterations within regulatory sequences. Because some of our *Bgl* I^r mutants produced normal plaques, it is clear that certain G-C pairs within the *Bgl* I site are not required for viral DNA replication or transcription. However, other *Bgl* I^r mutants produced small plaques and, in a third class, plaque formation was sensitive to high and low temperatures, a phenotype suggestive of altered protein-DNA interaction. Preliminary experiments with the temperature-dependent mutants indicate that they are defective in viral DNA replication. Detailed molecular and biological characterization of these and other novel mutant classes generated by

local mutagenesis should help extend the functional analysis of SV40.

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