Construction of a Viable Simian Virus 40 Variant That Carries a Poly[d(GT) · d(CA)] Insertion

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A 90-base-pair tract of a simple sequence composed of alternating guanosine and thymidine nucleotide residues (poly[d(GT) · d(CA)]) was inserted into the simian virus 40 genome at nucleotide 2666 (0.17 map units). The poly[d(GT) · d(CA)] insertion was stably maintained in the viral genome, but the variant virus grew more slowly than simian virus 40.

Tracts of a simple sequence composed of alternating guanosine and thymidine nucleotide residues, poly-[d(GT) · d(CA)], are abundantly dispersed throughout the genomes of eucaryotic organisms (7-9, 13, 14, 17, 19, 21, 25, 26, 30, 33). The typical mammalian genome contains on the order of 10⁶ copies of (GT)₉₅ (8, 9).

G-T repeats are functionally uncharacterized. Sligh et al. (29) have postulated that poly(GT) sequences in the human γ globin genes served as a hotspot for gene conversion between the two nonallelic copies of the gene. Other workers have also presented evidence consistent with a role for poly(GT) in gene conversion (7) and in general homologous recombination (31, 32).

Whatever their function may be, G-T repeats are structurally interesting because they adopt a left-handed Z-DNA form when under sufficient torsional strain (10, 23). There is as yet no unequivocal evidence that Z-DNA exists in vivo. Anti-Z-DNA antibodies have been reported to bind to specific regions of Drosophila melanogaster polytene chromosomes (1, 22) and to mammalian metaphase chromosomes (34), but the validity of these results has been questioned (11). However, the existence of Z-DNA in vivo is suggested by the isolation of proteins that preferentially bind Z-DNA (24). In addition, a recent report suggests that the DNA in interwound intermediates of homologous recombination reactions may be in the Z form (15).

The virus described in this report was constructed to provide a biologically active, amplifiable substrate with which to explore the possible role of poly(GT) in recombination, chromatin structure, and DNA conformation.

Previous work by Shenk et al. (27), Fitzgerald and Shenk (6), and Brockman et al. (2, 3) suggested that simian virus 40 (SV40) might accommodate a poly(GT) insertion at the 2666 HpaI site (all nucleotide numbers and genomic map positions are based on those in reference 4). A stretch of poly(GT) was inserted at the 2666 HpaI site by manipulation of plasmid pHA, which contained a copy of SV40 HindIII fragment A (Fig. 1). A poly(GT) segment was inserted into pHA by blunt-end ligation of HpaI-linearized pHA and a poly(GT) DNA fragment purchased from Boehringer Mannheim Biochemicals. The sequence of the poly(GT) segment was as follows:

CGATG (CA)₃ CTA (TC)₁ TAA (CT)₁ TAA (TG)₄₅ CATCG

pHAGT DNA was digested to completion with endo-R-BamHI and end-labeled by treatment with the Klenow fragment of Escherichia coli DNA polymerase I in the presence of radioactive dGTP. Digestion of the end-labeled DNA with endo-R-HindIII produced a 939-base-pair fragment which was purified by electrophoresis through an agarose gel. The sequence of the first 280 base pairs from the labeled end was determined by the method of Maxam and Gilbert (18). The 135-base-pair DNA fragment contained a 90-base-pair run of alternating guanosine and thymidine (GT)₄₅. In addition, the insert contained a second poly(GT) sequence 10 base pairs in length and in the inverse orientation relative to the (GT)₄₅ run. Between the two poly(GT) tracts were two tracts of alternating cytosine and thymidine residues interspersed with three nondescript triplets.

The protocol for introduction of poly(GT) into SV40 is shown in Fig. 2. SV40 fragment PstB bearing poly(GT) was purified from plasmid pHAGT and ligated to gel-purified SV40 fragment PstA. The entire ligation reaction mixture was transfected into CVI cells by the DEAE-dextran method described by Lai (16). Transfected CVI monolayers were overlaid with agar and incubated for 13 days, at which time plaques were visualized by staining with neutral red.

A total of 30 plaques of various sizes were picked and used to inoculate fresh plates of CVI cells. All 30 plaques produced obvious cytopathology within 13 days postinoculation; however, the cytopathology was atypical of SV40 in that the cells were not heavily vacuolated. Hirt (12) DNA was prepared and analyzed by cleavage with MboI followed by electrophoresis through either 1.4% agarose or 5% polyacrylamide.

MboI fragments separated by agarose gel electrophoresis were transferred to nitrocellulose paper and probed for homology to the poly(GT) insert by hybridization to radioactive poly(GT) prepared by nick translation. All of the DNA samples produced the SV40 MboI fragment distribution and contained a small fragment (~400 base pairs) that hybridized to the poly(GT) probe (data not shown).

MboI fragments to be separated by electrophoresis through polyacrylamide were first end-labeled by treatment with the Klenow fragment of Escherichia coli DNA polymerase I. All 30 plaque isolates produced the distribution of end-labeled fragments exemplified by the seven plaque isolates shown in Fig. 3. Note that the 237-base-pair fragment produced by MboI cleavage of SV40 DNA (MboI fragment G) (4) was absent from the Hirt DNA samples derived from plaques produced by DNA reconstructed with fragment PstB that contained a poly(GT) insertion (SVGT). All of the SVGT viral genomes instead contained a new MboI fragment that migrated just ahead of 384-base-pair MboI fragment F. The
expected size for MboI fragment G that harbors the whole 135-base-pair insertion put into pHA would be 372 base pairs.

Compared with SV40, SVGT was somewhat impaired in its ability to replicate. SVGT plaques were significantly smaller, averaging 1.7 mm in size compared with 3.1 mm for SV40 plaques (13 days postinfection). Consistent with the smaller plaque size, the rate of SVGT growth, as determined by monitoring plaque titer at times postinfection, was retarded compared with that of SV40 (data not shown). Although SVGT grew more slowly than SV40, the yield of virus per infected cell was about the same. When monolayers of CVI cells were infected at a multiplicity of infection of 2.0, SV40-infected cultures deteriorated more rapidly than SVGT-infected cultures, but the yield of virus in cultures infected with SVGT was the same as that in cultures infected with SV40. Since the virus burst size of SVGT-infected cells was unattenuated, it is likely that the SVGT replication cycle is longer than that of SV40. Comparison of the growth kinetics of SV40 and SVGT indicates that although SV40 produces 100 progeny in 3 days, it takes 4.5 days for SVGT to increase 100-fold (data not shown).

The stability of the poly(GT) insertion in SVGT was assessed by analyzing the viral DNA present in cultures serially infected with SVGT. Cells (10^6) were infected with 10^3 PFU of SVGT. The culture was incubated for 5 days, and the culture medium was removed and saved. Half the infected-cell monolayer was scraped off the plate, mixed with 1 ml of medium, and extracted with CHCl_3 to recover cell-associated virus. Cell-associated virus was mixed with the medium that had been recovered from the infected culture. This mixture was diluted 10-, 100-, and 1,000-fold and used to infect fresh cultures of cells. The second-round cultures were incubated for 5 days and then processed in the same manner to start the third passage, etc. The infected cells that were not scraped off the plate were extracted by the method of Hirt (12) to prepare viral DNA. SVGT passaged four times over the course of 20 days contained no detectable DNA from which poly(GT) had been deleted (data not shown).

SVGT should be valuable in addressing a number of questions that pertain to the structure and function of G-T repeats. G-T repeats have been shown to adopt a left-handed Z conformation in supercoiled plasmids (10, 23). Because of this predilection to left-handedness, G-T repeats are a good place to look for Z-DNA in the mammalian cell. One way to do this would be to analyze SVGT chromatin by nuclease attack. Since in vitro reconstitution experiments have indicated that Z-DNA does not form nucleosomes (20), it might be expected that if poly(GT) were in the Z form, it would be hypersensitive to nuclease cleavage. A more direct way to use SVGT to look for Z-DNA would be to use chemicals that cross-link DNA to probe for left-handedness in the viral DNA in SVGT-infected cells (28).

It has been suggested that G-T repeats may be important in recombination, but the evidence linking poly(GT) se-
quences to recombination is entirely circumstantial (7, 29, 31, 32). The stability of the poly(GT) in SVGT is not what might be expected of a hyperrecombinagenic element, but it is not yet clear whether the maintenance of poly(GT) in SVGT is more apparent than real. SVGT grows more slowly than SV40, and it might be expected that the internal homology in the poly(GT) tract would present intramolecular homologous recombination as a means by which the majority of the putative debilitating insertion could be removed from the viral genome. However, deleted SVGT genomes did not emerge upon serial passage. This could mean that the deletion of most of the poly(GT) did not confer sufficient selective advantage to allow deleted virus to outgrow SVGT. Alternatively, deletion via homologous recombination may be quite rare. It is not possible to distinguish between these alternatives at this point. Experiments designed to measure the frequency of recombination between G-T repeats are under way.

The apparent stability of poly(GT) in the SV40 genome contrasts with that found for an internally repetitive insertion that is not a purine-pyrimidine repeat. In an experiment like that illustrated in Fig. 2, an insertion of eight tandemly arrayed octamers of the sequence C-T-C-T-A-G-A-G was found to be very unstable. All of the viruses produced by transfection of CVI cells with DNA containing a (C-T-C-T-A-G-A-G)6 insertion lost five or more copies of the C-T-C-T-A-G-A-G repeat. These results suggest that part of the (C-T-C-T-A-G-A-G)6 insertion was deleted via intramolecular homologous recombination between one, two, or three copies of the C-T-C-T-A-G-A-G sequence. Compared with C-T-C-T-A-G-A-G insertions, poly(GT) is surprisingly well tolerated by SV40. It remains to be seen whether this tolerance reflects some intrinsic difference in the structure of poly(GT) DNA.

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LITERATURE CITED

