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The A + T-Rich Sequence of the Simian Virus 40 Origin Is Essential for Replication and Is Involved in Bending of the Viral DNA

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The origin-promoter region of simian virus 40 contains a 17-base-pair sequence composed exclusively of adenine (A) and thymine (T). We constructed a linker replacement mutant in which this stretch of A's and T's was reduced to 11 base pairs. While not affecting the level of early gene transcription, this mutation reduced the accumulation of viral DNA in COS cells at least 10^4 fold. In addition, a restriction fragment containing the wild-type A + T-rich region migrated in nondenaturing polyacrylamide gels with an anomalous mobility characteristic of bent DNA; however, the corresponding fragment from the mutant migrated less anomalously. Therefore, bending of the DNA in this region may play a role in some step in viral DNA replication.

One of the interesting features of the promoter-origin region of simian virus 40 (SV40) is a 17-base-pair region containing exclusively adenines (A's) and thymines (T's) (Fig. 1A, nucleotide residues 15 through 31) (3). This 17-base-pair region includes eight consecutive A's (nucleotide residues 21 through 28) and contains the TATA box (nucleotide residues 15 through 21) that determines the major initiation site for early gene transcription (1, 9, 10). In addition, deletion analysis has indicated that it constitutes one of the boundaries of the *cis*-acting region required for the initiation of viral DNA replication (2, 6, 7, 20)—i.e., the core region of the replication origin (Fig. 1A). Only 2 base pairs of this 17-base-pair region, nucleotide residues 30 and 31 (Fig. 1B), have been reported to be expendable for viral DNA replication (6, 20).

However, previous experiments have not definitively shown the necessity of the A + T-rich region (A-T region) for replication: either the assays used were not very sensitive (e.g., mutant plaque morphology) (6) or the constructs used in the studies lacked the 72- and 21-base-pair repeat regions as well as the A-T region of the SV40 genome (2, 7, 20) (Fig. 1A). Since the 72- and 21-base-pair repeat regions also play roles in replication (2, 5, 7, 13, 16, 17), it was possible that the A-T region appeared to be essential only because these other two regions were absent.

To examine the necessity of the A-T region for viral DNA replication, we wanted a mutant altered only in this region. To accomplish this, we constructed the linker replacement mutant XSA (Fig. 1B) by recombination of mutants X-39 and S-321 as described by Fromm and Berg (7). This substitution mutation resulted in the alteration of 6 base pairs within and directly adjacent to the A-T region, so that the stretch of consecutive A · T base pairs was reduced from 17 to 11 while all the other sequences from the wild type were retained. An identical substitution mutation in which the 72- and 21-base-pair repeat regions were also missing had previously been shown to reduce greatly or eliminate viral DNA replication (7).

We then examined the ability of XSA DNA to replicate in COS cells, monkey cells which constitutively express the viral proteins required for SV40 viral DNA replication (10). After excision of the viral sequences from the cloning vector and ligation into monomer circles, COS cells were transfected in parallel with mutant XSA, wild-type SV40 DNA as a positive control, and mutant S-352 as a replication-negative control. Mutant S-352 (Fig. 1C) is replication negative because it lacks half of the core origin region sequences considered essential for viral DNA replication as well as the 21- and 72-base-pair repeat regions (7; see Fig. 1C for structure). At 24, 42, and 66 h after transfection, viral DNA was isolated from the transfected cells by the procedure of Hirt (14) and purified by incubation with proteinase K (500 μg/ml) and extraction with phenol-chloroform-isoamyl alcohol (50:25:1). Large RNA was removed by precipitation at 0°C for 15 min in 2.5 M ammonium acetate (200 μl/100-mm dish of cells).

The DNA samples were examined for resistance to cleavage with the restriction endonuclease *Dpn*I to determine whether they contained any viral DNA that had replicated in monkey cells (13). Since the DNA used for transfection had been grown and, thus, methylated in *dam*$^+$ bacteria, only those DNA molecules that had undergone at least one cycle of replication in monkey cells would be resistant to cleavage by this enzyme. The samples were also treated with the restriction endonuclease *Acc*I, which cleaves SV40 DNA at a single site, so that any replicated viral DNA would appear as unit-length linear DNA (form III) rather than as a mixture of supercoiled and relaxed circular DNAs. After treatment with *Dpn*I and *Acc*I, half of each sample was treated additionally with *Mbo*I. This enzyme has the same recognition sequence as *Dpn*I but only cleaves DNA that is not methylated. Therefore, cleavage by *Mbo*I showed that the DNA not cleaved by *Dpn*I was truly unmethylated (i.e., had replicated) and did not simply result from incomplete digestion with *Dpn*I.

Figure 2 shows an autoradiogram of one of the Southern blots used to quantify by densitometric analysis the relative amounts of replicated viral DNA present in each sample. The amount of replicated viral DNA present in the cells transfected with the wild type increased fivefold between 24 and 42 h posttransfection and remained constant between 42 and 66 h (data not shown). The amount of replicated viral DNA present in the cells transfected with mutant XSA also remained constant between 42 and 66 h (Fig. 2, lanes 3 and 5), but the amount of replicated viral DNA was only 10^{-4} times the amount in the cells transfected with the wild type. Two other independent experiments also indicated that the amount of replicated viral DNA present in XSA-transfected

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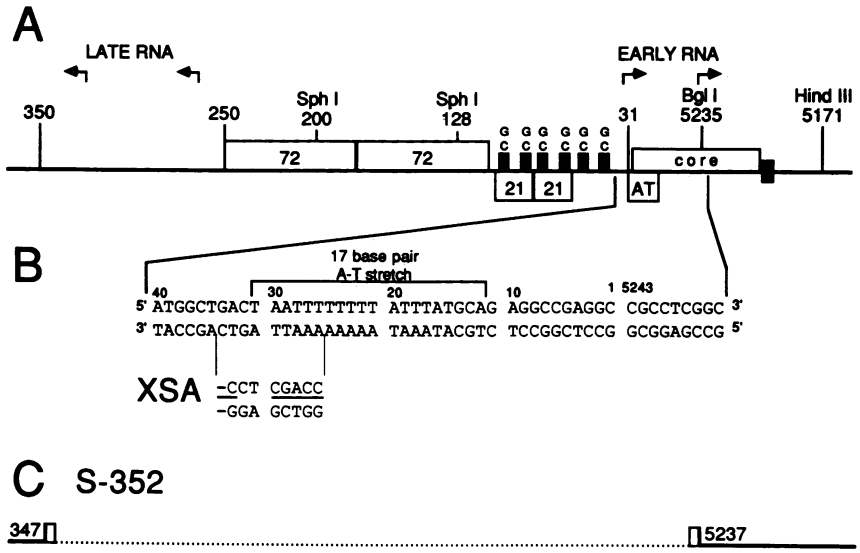

FIG. 1. Map of the promoter-origin region of the SV40 genome and structures of the mutants discussed in this study. (A) Schematic of the promoter-origin region. Nucleotides are numbered by the system of Buchman et al. (3). The major 5′ ends for early and late mRNAs (3) are indicated by arrows. Major landmarks are (i) the 72-base-pair tandem direct repeat, (ii) the 21-base-pair tandem direct repeat, (iii) the GGGCGG boxes, (iv) the 17-base-pair A-T region, and (v) the core region of the origin of viral DNA replication. A previously identified region of bent DNA (19) is indicated by the hatched box. (B) The first line is the sequence of the wild-type 17-base-pair A-T region and the surrounding base pairs. The second line is the sequence of the substitution (i.e., recombined *Xho*I and *Sal*I linker DNAs replacing 9 base pairs of the wild-type sequence) in mutant XSA; altered base pairs are marked by heavy lines. (C) Mutation in mutant S-352. The dotted line represents the deleted sequence; the numbers adjacent to the dotted line indicate the nucleotides adjacent to the deletion end points. The open boxes at the deletion endpoints indicate that the mutation contains an insertion of GGTCGAGG (i.e., recombined *Sal*I and *Xho*I linker DNAs) at the site of the deletion.

cells was at most 10^{-3} times the amount in wild-type-transfected cells (data not shown). Furthermore, the *Dpn*I-resistant DNA was sensitive to cleavage with *Mbo*I (Fig. 2, lane 7) and, thus, had replicated in monkey cells.

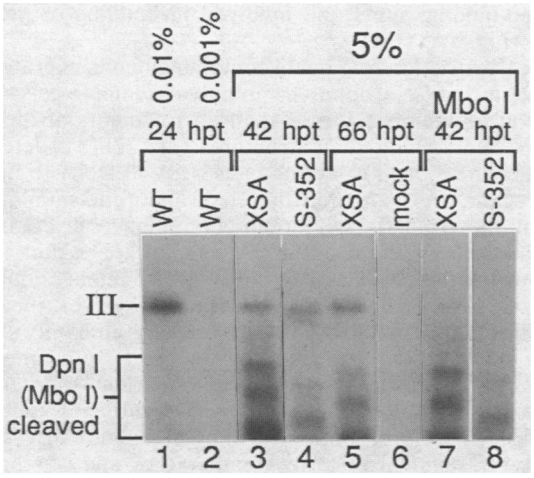

FIG. 2. Autoradiogram of Southern blot used to quantitate the relative accumulation of replicated viral DNA. Viral DNA was isolated from COS cells at 24, 42, and 66 h posttransfection. The samples in lanes 1 through 6 were treated with restriction endonucleases *Dpn*I and *Acc*I. In lanes 7 and 8, portions of the samples in lanes 3 and 4 were treated additionally with *Mbo*I. Each lane contained the DNA isolated from 5% of a 100-mm tissue culture dish, except for lanes 1 and 2, which contained the DNA isolated from the percentage of a dish indicated by the number at the top of the lane. III, Linear viral DNA; WT, wild type; hpt, hours posttransfection.

The amount of replicated viral DNA observed in the DNA samples obtained from the cells transfected with the replication-negative mutant S-352 (Fig. 2, lane 4) was similar to that observed in the cells transfected with mutant XSA (Fig. 2, lane 3). Therefore, although mutant XSA may have replicated very slightly, the amount of replicated DNA observed was comparable to the extremely small amount seen in a replication-negative mutant. Furthermore, the replicated viral DNA present in the S-352 sample was sensitive to cleavage with *Bgl*I (data not shown). Therefore, this small amount of DNA was a contaminant (probably having arisen by reversion [8], recombination with the SV40 DNA present in COS cells, or experimental contamination with replication-competent SV40 DNA) rather than S-352 DNA, which lacks half of the *Bgl*I recognition site present in wild-type SV40 DNA (Fig. 1). These findings indicate that the replicated viral DNA observed in the XSA samples was present at a level similar to our level of detection in the experiment. Therefore, we conclude that the late end (nucleotides 25 to 31) of the A-T stretch is very important and, possibly, essential for viral DNA replication.

The early end (nucleotides 14 to 20) of the 17-base-pair A-T region contains the TATA box for positioning the major initiation site for early gene transcription (1, 9, 11); mutations in this TATA box can affect the accumulation of early RNA (21). Therefore, we were interested in determining whether the XSA mutation might also affect early gene transcription. To analyze this, we recombined XSA with *dlA*4000, a mutant containing a frameshift deletion in the amino-terminal end of the T-antigen genes (Fig. 3A) (13), to form the double mutant XSA/*dlA*4000. Since the *dlA*4000 mutation inactivates large T antigen, it eliminates both the autoregulation of early gene transcription and the replication of viral DNA as variables. XSA/*dlA*4000 and *dlA*4000 were

then transfected in parallel into CV-1P monkey cells. Whole-cell RNA was isolated at 40 h posttransfection and analyzed by nuclease Sl mapping as described elsewhere (G. Z. Hertz and J. E. Mertz, submitted for publication).

The data presented in Fig. 3B show that XSA/*dlA*4000 produced the same amount of early RNA as did *dlA*4000, which contains a wild-type promoter-origin region. Therefore, the substitution mutation in XSA did not affect the level of early gene transcription—at least in the absence of functional T antigen. Taken together with the data of others (1, 9, 11), these findings suggest that the early end but not the late end of the 17-base-pair A-T region is important for early gene transcription. However, it is not yet known whether the XSA mutation affects the sites of transcriptional initiation.

There are several plausible reasons why the 17-base-pair A-T region is important for the initiation of SV40 DNA replication: (i) replication may require a region with a low melting temperature so that an initiation complex can form on single-stranded DNA; (ii) this region may be the recognition site for a specific factor involved in replication; (iii) the 8-base-pair A stretch within the 17-base-pair A-T region may enable bending of the DNA, thereby either allowing nearby sequences to configure properly or facilitating the formation of a DNA conformation needed for the assembly of an initiation complex. This last possibility arises because double-stranded DNA molecules having stretches of five to eight A's have been shown to exhibit a stable bent structure under some conditions (12, 15). In addition, a sequence including a stretch of six A's has been shown to be both necessary for high-affinity binding of the SV40 large T antigen to T-antigen-binding site I and involved in bending of the viral DNA (Fig. 1A) (19).

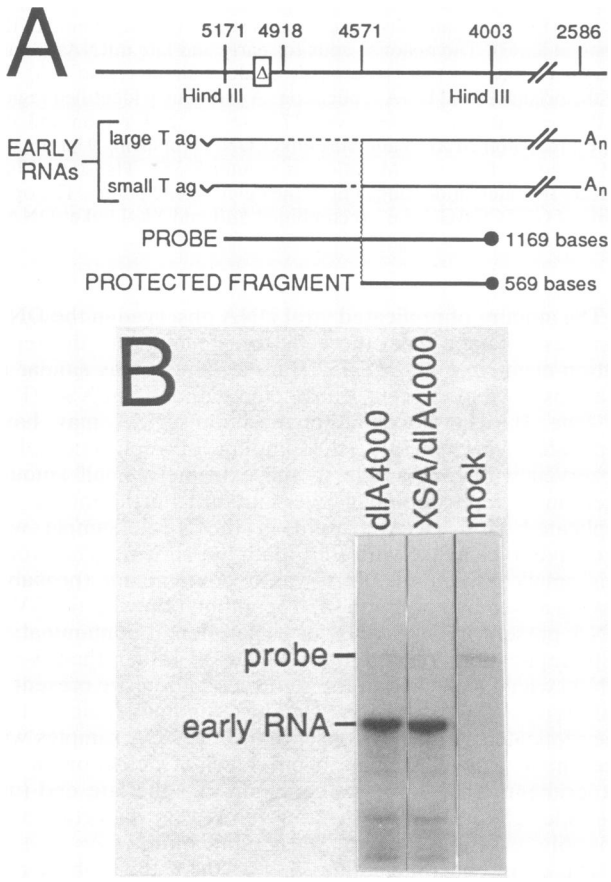

FIG. 3. Nuclease S1 mapping of early RNA produced in CV-1P cells by mutants *dlA*4000 and XSA/*dlA*4000. RNA was isolated at 40 h posttransfection and analyzed as described elsewhere (Hertz and Mertz, submitted). (A) RNA was hybridized with a probe 5′ end labeled with ^{32}P at nucleotide residue 4003 (a *Hin*dIII site) and extending through nucleotide residue 5171 (a *Hin*dIII site). The box containing the triangle indicates the location of the deletion in mutant *dlA*4000. Zigzag lines and dashed lines denote the locations of cap sites and introns, respectively. The black dots indicate the labeled end of the probe. (B) Autoradiogram from nuclease S1 mapping analysis. Each nuclease S1 reaction contained the RNA isolated from 10% of a 100-mm tissue culture dish. probe, Full-length probe.

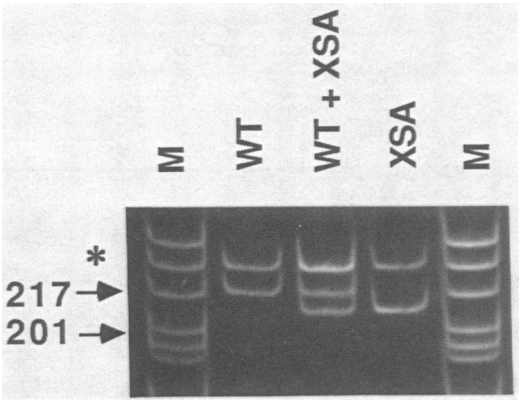

FIG. 4. Wild-type (WT) and XSA DNAs cleaved with restriction endonucleases *Sph*I and *Hin*dIII and electrophoresed at 4°C. DNA was electrophoresed in a 12% polyacrylamide gel (acrylamide/bisacrylamide ratio, 39:1) at 10 V/cm in 89 mM Tris–89 mM boric acid–2.5 mM EDTA. Afterwards, the gel was stained with ethidium bromide and photographed over short-wavelength UV light. Only the relevant portion of the gel is shown here. M, Size markers (pBR322 DNA cleaved with *Hpa*II). (The 242-base-pair size marker, indicated by the asterisk, exhibits a slower mobility than expected for its size; however, it contains a 6-base-pair adenine stretch and, therefore, may also be bent.) Numbers at left are base pairs.

DNA restriction fragments containing bends migrate more slowly during electrophoresis in polyacrylamide gels—especially at low temperatures—than do fragments of the same size not containing such structures (18, 22). To determine whether the 8-base A stretch causes SV40 DNA to bend in this region, we compared the electrophoretic mobilities at 4°C of the *Hin*dIII-*Sph*I origin-containing restriction fragments of the wild type and XSA (Fig. 1A). The data in Fig. 4 show that the 200-base-pair wild-type fragment comigrated with a 217-base-pair DNA size marker. However, the corresponding 200-base-pair XSA fragment migrated only slightly more slowly than expected for its size. Both fragments comigrated with the appropriate size marker when electrophoresed in a denaturing polyacrylamide gel (data not shown). Therefore, both the anomalous mobility of the wild-type fragment at a low temperature and our findings that the analogous DNA fragment from the XSA mutant, in which 3 of the 8 base pairs of the A stretch are altered, migrated more closely to its expected size suggest that the 17-base-pair A-T region contains a bend. The slight mobility shift seen with the XSA fragment may be due to (i) the presence of the bent region in T-antigen-binding site I (Fig. 1A) (19) or (ii) the remaining stretch of five A's in the 17-base-pair A-T region (Fig. 1B).

While this manuscript was in preparation, reports from two other laboratories which reached similar conclusions

concerning the role of the 17-base-pair A-T region appeared (4, 8). However, the work presented here is significant in that it was carried out under conditions in which the upstream promoter elements have been shown to be very important for origin function (13) and in which the complete viral genome, including those upstream promoter elements, remained intact, whereas their assays were performed under conditions in which these elements were either physically absent (4) or physiologically unimportant (8). In addition, the finding of Deb et al. (4) and Gerard and Gluzman (8) that other mutants with different base substitutions in this region are also defective in replication rules out the hypothesis that the XSA mutant studied here was defective in replication because its base substitution created a novel sequence that inhibited replication.

In conclusion, besides containing the TATA box for early gene transcription, the 17-base-pair A-T region contains sequences essential in *cis* for the replication of SV40 DNA. In addition, sequences in this region form a bend that exists in wild-type DNA. Whether this bend facilitates the initiation of viral DNA replication remains to be determined.

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