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Sequence-specific functions of the early palindrome domain within the SV40 core origin of replication

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ABSTRACT

The early palindrome domain within the SV40 core origin of replication is essential for the initiation of replication. Studies with single point mutants in this region suggested that the early palindrome domain does not function as a cruciform structure(1), but may be involved in the initiation of SV40 DNA replication in a sequence-specific manner. Two mutants, base-substituted at a primase initiation site nucleotide 5214(2), showed dramatic decreases in DNA replication in monkey cells. Despite earlier reports to the contrary (3,4), disruption of the cruciform configuration or polypyrimidine tract does not invariably lead to lack of replication function, as some mutants unable to form this structure replicate normally. Gel retention assays and DNase I footprinting with the nuclear proteins of monkey cells showed that the 5'GAGGC3' pentanucleotide repeats on either side of early palindrome domain interact with monkey nuclear protein. The early palindrome domain may affect the interaction of SV40 DNA with nuclear protein, and participate in SV40 DNA replication.

INTRODUCTION

Simian virus 40 (SV40) represents a simple model system for studying the mechanisms of DNA replication in eukaryotic cells(5). Previous studies have established the approximate boundaries of the minimal core region involved in SV40 DNA replication(6) and devided the core origin into three functional domains(3). One of these domains, the early palindrome domain(15-bp), is located on the early end of the core origin and can form a curciform structure(1).

Deb *et al.*(3) firstly suggested that the early palindrome domain was required for origin activity, but its functional role was not defined. Recently, Hurwitz and colleagues(4) suggested that the left arm of the early palindrome domain is the critical melted region for unwinding by SV40 large T antigen.

A valuable goal for studying eukaryotic DNA replication is to identify the cellular proteins that make up the replication apparatus and decipher their regulation. At present the SV40 system is the best viral system to study cellular replication preteins. It is clear from many recent reports that the numorous cellular factors are required for SV40 DNA replication (7,8,9,10,11). The origins of DNA replication in the genomes of the closely related primate papovaviruses SV40, BK, JC, and SA12 bear several sequences with high degrees of nucleotide identity(3). The left arm of the early palindrome domain shows the most highly conserved sequences. This fact raised the possibility that the early palindrome domain within the core origin functions as a cruciform in initiation of DNA replication. To address this question, we employed the oligonucleotide-directed site-specific mutagenesis, gel retension technique, and DNaseI protection technique to assay the replication efficiencies and the binding patterns of nuclear proteins to wild-type and mutant SV40 origin DNAs.

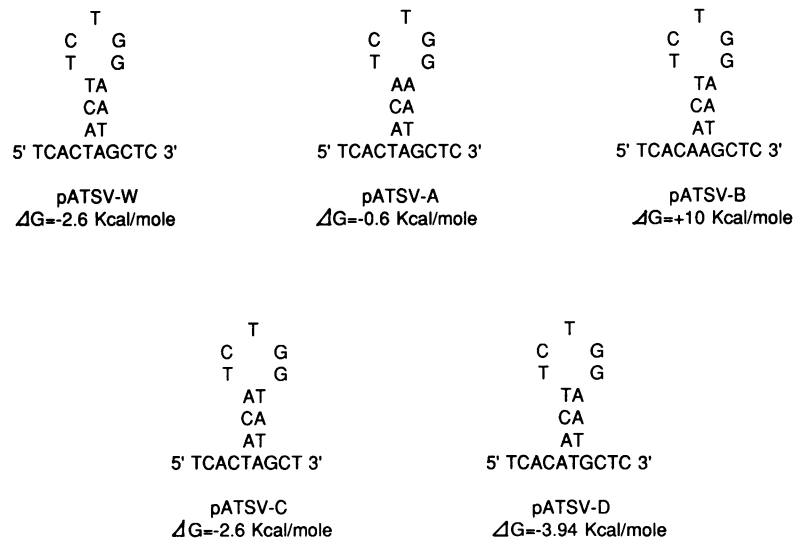

Figure 1. Altered stem structures of the early palindrome domain by oligonucleotide-directed site-specific mutagenesis. pATSV-A and pATSV-B have an additional mismatch within the stem of the early palindrome domain by one base substitution. pATSV-C and pATSV-D have the normal stems restored by additional base substitutions which allowed base-pairing with the bases substituted initially.

In this paper, we describe the sequence-specific function of the early palindrome domain of the SV40 replication origin and the binding activity of the monkey cellular factor on wild-type and mutant SV40 origin DNAs.

MATERIALS AND METHODS

Enzymes

All restriction enzymes were obtained from Bethesda Research Laboratories (BRL), New England Biolabs(NEB), or KOSCO Biotech (in KOREA). The large fragment of DNA polymerase I, S1 nuclease, and T4 polynucleotide kinase were purchased from NEB, and T4 DNA ligase from KOSCO Biotech.

Cells and DNA

The african green monkey kidney (AGMK) cell lines, CV-1 and COS-1, were cultured in DME medium supplemented with 5% fetal calf serum. Purified SV40 DNA (wt830) from infected CV-1 was digested with KpnI and TaqI, and 794 bp fragment containing the replication origin was purified from agarose gel. Bacteriophage M13mp19 RF DNA was isolated from infected *E.coli* JM103. The 794 bp *KpnI-TaqI* fragment was cloned into the *KpnI-AccI* site of M13mp19 RF DNA. This single-stranded recombinant viral DNA was used as template for oligonucleotide-directed site-specific mutagenesis (12).

For DNA replication assay, SV40 origin-containing fragments of wild type and four kinds of mutant were inserted in M13mp19 RF DNA, and then were subcloned into the plasmid pAT153 (13) after digestion with *EcoRI* and *PstI*, which were designated pATSV-W, pATSV-A, pATSV-B, pATSV-C, and pATSV-D, respectively (Fig. 1).

DNA replication

COS-1 cells were transfected with 1 μg of plasmid per 75 cm^2 flask using DEAE-dextran (14). Forty eight hours post-transfection, plasmid DNA was purified from the transfected

cells using the procedure of Hirt (15). The purified plasmid DNA was digested with *MboI*, an restriction endonuclease that cleaves only unmethylated DNA molecules replicated in the eukaryotic cells. Digested DNA samples were transferred to nitrocellulose filter (0.45 μm, Millipore) according to the method of Southern (16) and hybridized to ^{32}P-labeled probe prepared by the primer extension method. The filter was washed and exposed to X-ray film (Kodak XAR film). To ensure equivalent replication effciency, plasmid DNA was digested with *DpnI* and used to transform competent *E.coli* HB101. *DpnI* resistant DNA molecules represent the unmethylated plasmids replicated in eukaryotic cells. The ratio of the number of transformants induced by mutant DNA to that induced by the wild-type DNA (pATSV-W) was calculated, and is expressed as a percentage of wild-type efficiency.

Preparation of labeled restriction fragments

In order to prepare the labeled templates for protein binding assays, the 46-bp *StuI-BglI* fragments containing the early palindrome domain and the T antigen binding site I (5'-GAGGC-3' pentanucleotide repeats) were purified from pATSV-series of plasmids by agarose gel electrophoresis using the NA-45 DEAE-membrane (S & S). These eluted fragments were 3'-end labeled with [α-^{32}P]ddATP (Amersham; sp. act. 3,000 Ci/mmol) and 3'-labeling kit (Amersham) containing terminal deoxynucleotidyl transferase (TdT) according to the procedure recommended by the supplier.

Alternatively, the 110-bp *HindIII-NcoI* fragments were purified and 3'-end labeled for DNase I footprinting assay. [α-^{32}P]dCTP (Amersham; sp. act. 3,000 Ci/mmol) and the large fragment of *E.coli* DNA polymerase I were used to 3'-end label DNA. Labeled DNAs were separated from unincoporated nucleotides by repeated ethanol precipitation.

Nuclear extracts

Nuclear extracts were made from tissue-cultured cell lines (CV-1 and COS-1 ; monkey cell lines, HeLa ; human cell line, NIH 3T3 ; mouse cell line) essentially as described by Dignam *et al.* (17), except that the high salt nuclear extraction buffer contained 0.35 M rather than 0.43 M NaCl. All buffers contained 1 mM phenylmethylsulfonyl fluoride as protease inhibitor. Nuclear extracts generally contained 3 mg of protein per ml as measured by the Bradford assay (18) with bovine serum albumin as a standard.

DMS methylation

End-labeled DNA fragments were partially methylated at the N7 of guanine and the N3 of adenine residues with dimethylsulfate (19). This methylation reaction was quenched with 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, and 200 mg of glycogen per ml. Methylated DNA was ethanol precipitated twice, rinsed with 95% ethanol, dried, suspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and used as template in a standard DNA-protein binding reaction as below.

Protein-DNA binding assay

Binding reaction mixtures included 500 ng of poly(dA-dT)-poly(dA-dT) (Pharmacia) or 1 mg of salmon sperm DNA, and 10 fmol of labeled restriction fragments in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 10% glycerol, and 6 mg of nuclear proteins adjusted to 0.15 M NaCl. The amounts of protein and labeled DNAs were varied in some experiments, as stated in the RESULTS. The total volume of each reaction mixture was 30 ml. The reaction was allowed to proceed for 15 min, generally at 25°C. The reaction mixture was subsequently subjected to gel electrophoresis on 12% polyacrylamide gels (acrylamide /bisacrylamide = 44/0.8) in 0.375 M Tris-glycine, pH 8.8. Gels were electrophoresed at 20 mA with 40 mM Tris-glycine, pH 8.5 as a running buffer (20).

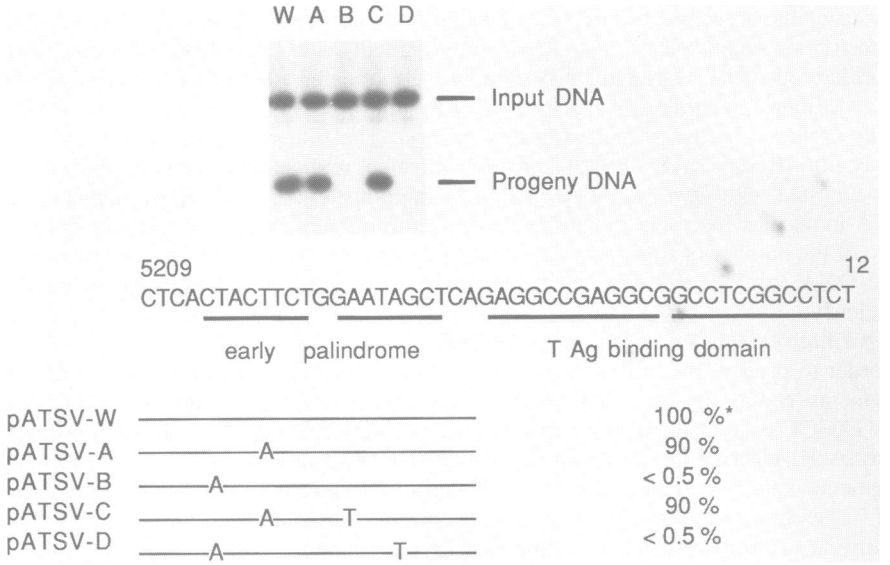

Figure 2. Identification and mapping of sequence-specific replication of pATSV-series. The autoradiogram shows the replication efficiency of base substitution mutants. For preparation of hybridization probes, SV40 sequencing primer was hybridized to single stranded M13 DNA containing SV40 origin of replication and extended by primer extension method. Input DNA (methylated) and progeny (replicated and unmethylated in monkey cells) are indicated. The replication efficiencies were expressed as percentage of the corresponding ratio for pATSV-W to derive the relative *in vivo* replication efficiency.

Samples were applied in 5% glycerol, 10 mM DTT and 0.05% bromophenol blue. Following electrophoresis, gels were soaked in 5% glycerol, dried, and autoradiographed.

DNase I protection

DNase I footprinting was performed essentially as described by Clark *et al.*(21) and Levy *et al.*(22) with some modifications. DNA-protein binding reactions (as described above) were scaled up to 50 ml in the same buffer lacking glycerol and containing 20 fmol of labeled DNAs and 30 mg of nuclear proteins. After 20 min at 20°C, DNase I (Worthington) was added to 16 mg/ml and incubated for 4 min at 30°C. DNase I digestion was terminated by the addition of 10 mM EDTA, 0.2% SDS, and 100 mg/ml proteinase K. Following 15 min at 60°C, DNA was recovered by phenol/ chloroform extraction, ethanol-precipitated, washed with 70% ethanol, dried, and redissolved in 5 ml of 98% formamide, 20 mM NaOH, 1 mM EDTA with bromophenol blue and xylene cyanol FF. DNA was denatured at 90°C for 2 min, cooled on ice, and reaction product was electrophoresed on 8% polyacrylamide gels containing 7 M urea. Gels were fixed in 10% acetic acids, dried, and exposed to X-ray film in the presence of an intensifying screen. Sequence markers were prepared by the chain termination dideoxy sequencing method (23).

RESULTS

Replication of SV40 origin-containing plasmid in vivo

The replication efficiencies of each mutant in COS-1 cells were determined in three independent experiments. A representative gel is shown in figure 2. Below the autoradiogram, the mean replication efficiency of each mutant relative to that of the wild-

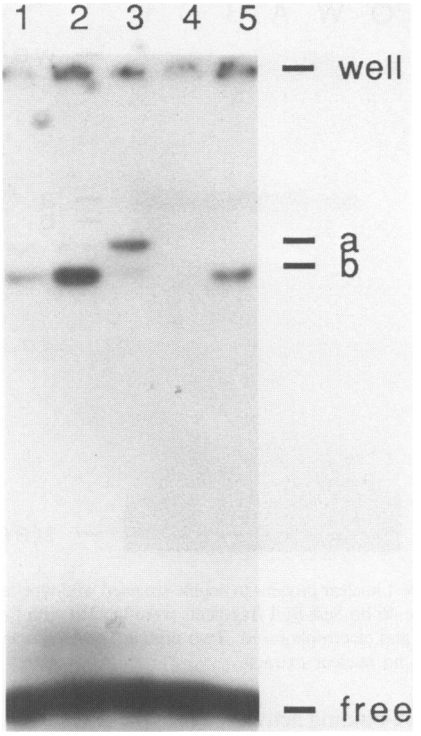

Figure 3. Occurrence of origin binding activity in nuclear extracts of primate and rodent cell lines. Binding of 6 μg of nuclear proteins to the 3′ end labeled *BglI-StuI* origin fragment of pATSV-W was tested in a gel retention assay. Two protein-DNA complex bands(a and b) were separated from the unbound DNA(free).
Lane 1, nuclear extracts from COS-1. 2, from CV-1. 3, from HeLa. 4, from NIH 3T3. 5, from CV-1.

type pATSV-W is expressed as a percentage (see MATERIALS AND METHODS), and the substituted bases of each mutant are mapped.

The plasmid DNA grown in a *dam*$^+$ *E.coli* strain was methylated and thus rendered sensitive to cleavage by *DpnI*. Since mammalian cells do not contain this methylase, DNA replication produces hemimethylated and unmethylated DNA, both of which are sensitive to cleavage by *MboI*.

Interaction of SV40 origin DNA with nuclear proteins from various cell lines

To determine whether the early palindrome domain within the origin of SV40 DNA replication carries binding sites for cellular proteins, we used the end-labeled restriction fragment, 46-bp *StuI-BglI* origin fragment, bearing the early palindrome domain, T antigen binding site I, and a part of T antigen binding site II as a template in a gel retention assay. Specific complexes formed between the labeled DNA and proteins present in nuclear extracts of primate and rodent cells were detected by their slower electrophoretic migration relative to unbound DNA in nondenaturing gels. Two prominent protein-DNA complexes were formed in each of the primate extracts (Fig. 3). These bands were not detected in extracts made with rodent cells, however.

This survey of various cell lines was conducted to determine the best sources of the

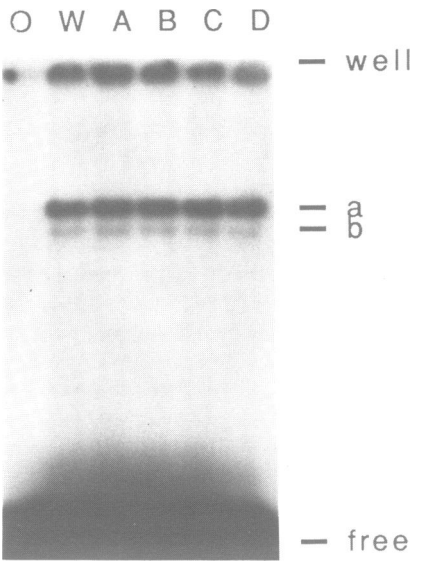

Figure 4. Specific binding of CV-1 nuclear proteins to double-stranded wild-type(lane W) and mutant(lane A,B,C,and D) SV40 origin fragments. The 46-bp *StuI-BglI* fragments were labeled with [α-^{32}P]ddATP at 3′ end, subjected to bind with nuclear proteins, and electrophoresed. Two protein-DNA complex bands(a and b) were separated from the free DNA. Lane O: no nuclear extracts.

SV40 origin-specific DNA-binding activity. From these results, we have used CV-1 nuclear extracts for all subsequent experiments.

Binding of cellular proteins to the SV40 origin of DNA replication

To facilitate the correlation of replication activity of the mutant SV40 origin DNAs with protein-binding activity, gel retention assays were performed with 46-bp *StuI-BglI* fragments of wild-type (pATSV-W) and mutant SV40 origin DNAs (pATSV-A, -B, -C, and -D).

Figure 4 shows that two DNA-protein complexes (band a and b) were detected with each of the SV40 origin templates. There was not a difference in the gel retention patterns of wild-type and mutant templates.

The specificity of the protein-origin DNA interactions was tested in competition assays. Binding of a nuclear protein to the labeled template is diminished if the unlabeled competitor DNA carries a specific binding site for the protein. The competition assay was performed with unlabeled and labeled 46-bp fragment from wild-type origin in pATSV-W. This assay confirmed the specificity of binding activities detected in the complex proteins(a) and (b) and the non-specific binding activity in the complex (c) (Fig. 5).

Methylation of templates by DMS inhibits protein-binding

The 46-bp *StuI-BglI* fragments from pATSV-series were end-labeled and partially methylated at the N7 of guanine and the N3 of adenine residues with dimethylsulfate (23).

Binding of these DNAs to nuclear proteins from CV-1 cells was studied using a gel retardation assay in which unbound DNA fragments are separated from protein-DNA complexes. In figure 6, it is clear that nuclear proteins binds with much reduced efficiency to the methylated DNAs. Also, figure 6 shows the more intensive non-specific binding complex (a and c position) and the weaker specific binding complexes (b position). From

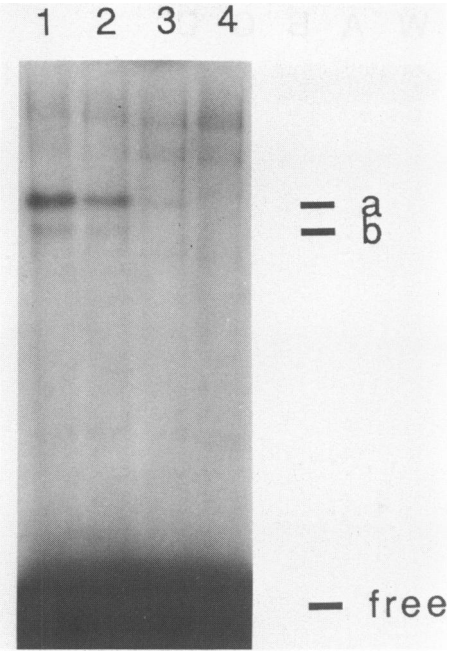

Figure 5. Competitive protein-DNA binding assay. Binding of 6 μg of CV-1 nuclear proteins to 20 fmol of labeled origin fragments (wild-type) was assayed in the presence of 10-(lane 2), 20-(lane 3), and 50-fold(lane 4) amounts of unlabeled fragments as a competitor. Lane 1: no competitor.

the result of figure 6, it can be concluded that the inhibition of the protein-binding is due to methylation by DMS.

DNase I protection analysis

To identify the binding sites of the nuclear proteins on the SV40 genome, a DNase I protection experiment was carried out. Using ^{32}P-labeled 110-bp *NcoI-HindIII* fragments digested from pATSV-series as probes, two areas of the SV40 origin DNA were protected from DNase I digestion in the presence of CV-1 nuclear proteins (Fig. 7). The area from position 5195 to 5207 contains the 5′-GAGGC-3′ pentanucleotide repeats (T antigen binding site I), and also the region from 5238 to 3 is the center of 27-bp perfect palindrome (T antigen binding site II). Both binding sites of CV-1 cell nuclear proteins are located in T antigen binding sites. Interestingly, the DNase I protection patterns of mutant origin fragments were altered at the early palindrome domains (from 5213 to 5225). The patterns of DNase I protection of the replicated (pATSV-A and pATSV-C) and non-replicated (pATSV-B and pATSV-D) mutant origins were different. A new band appeared in cases of pATSV-B and pATSV-D, the mutants which showed a dramatic decrease of replication.

DISCUSSION

It is known that the imperfect inverted repeats in SV40 core origin can form cruciform structure (1). If a cruciform structure exists in the early palindrome domain and is functional in SV40 DNA replication, the replication efficiencies of pATSV-A and pATSV-B mutants

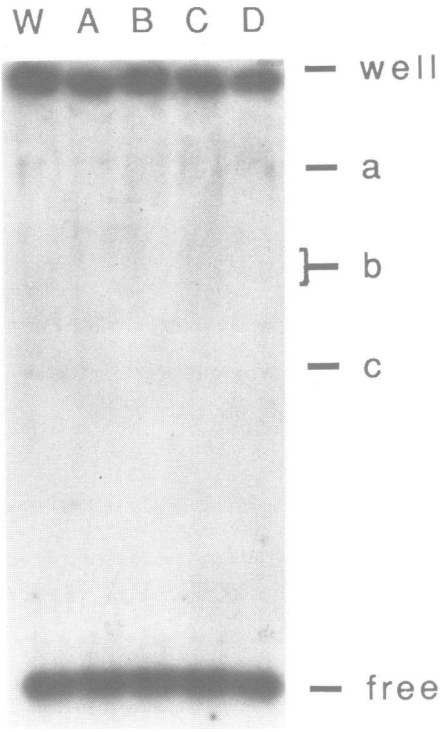

Figure 6. Inhibition of protein binding to DNA by DMS methylation. DNA fragments from wild-type(W) and mutants(A, B, C, and D) were methylated with DMS, and binding reaction was done as a method described in text. Nonspecific binding complexes(a and c) and specific binding complexes(b) with reduced intensity were indicated.

which contain an additional mismatch in stem region should be reduced. Those mutants pATSV-C and pATSV-D which restore the stem should be similar to that of wild-type. Our results showed that pATSV-A and pATSV-C could replicate to the level of wild type, and pATSV-B and pATSV-D showed a dramatic decrease in DNA replication. There was a report that a mutant with T-to-C substitution at nucleotide 5217 failed to replicate (3). In contrast, the mutant DNA pATSV-A with T-to-A substitution at the same position was replicated to the same level as wild-type. For such reasons, it is likely that the early palindrome domain is not important solely in the formation of a functional cruciform structure, but rather is a sequence-specific functional domain in SV40 DNA replication.

Why was the replication of pATSV-B and the pATSV-D suppressed in monkey cells? One possibility is that modification of T antigen binding patterns is induced by base substitutions at these positions. In T antigen binding assay, we observed that all mutant origins among pATSV-series bound with T antigen (data not shown), but we don't know whether the modification of T antigen binding pattern occurred or not. Also, the origin regions in pATSV-series showed DNA bending structures normally (data not shown).

How was the early palindrome region implicated in initiation of SV40 DNA replication? Borowiec and Hurwitz(4) suggested that the left arm of the early palindrome domain is the critical melted region for unwinding by T antigen binding. The critical melting sequences

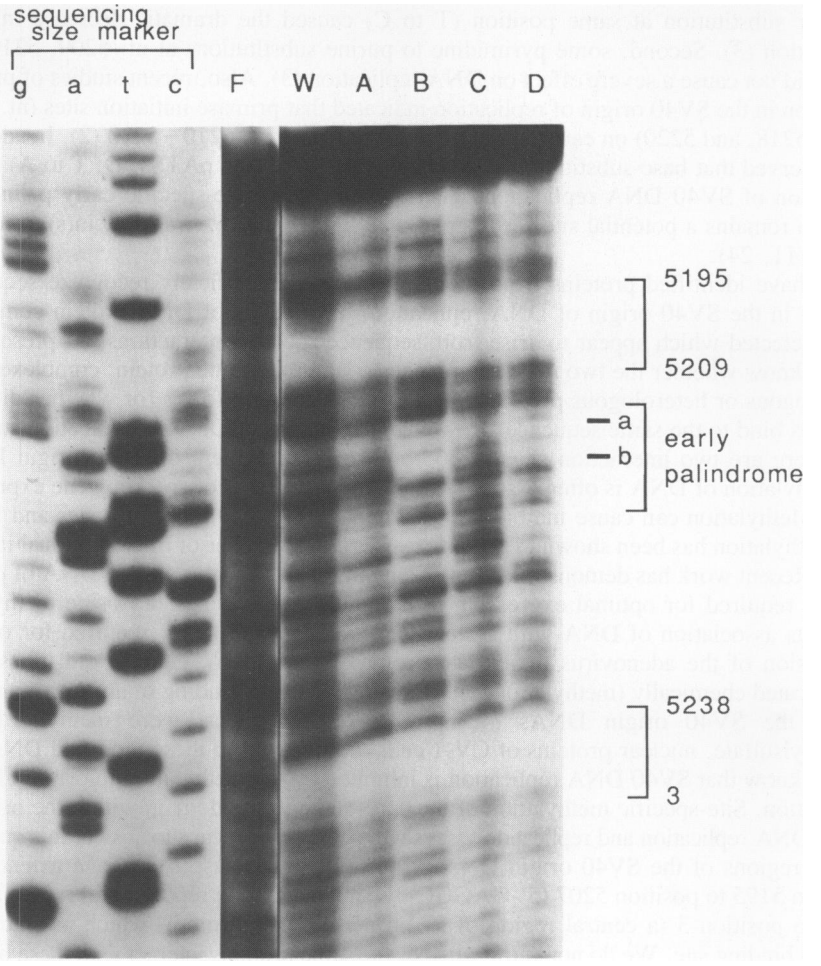

Figure 7. DNase I footprint of CV-1 nuclear protein(s) bound to origin regions in wild-type(W) and mutant DNAs(A, B, C, and D).End-labeled 110-bp *Hind*III-*Nco*I origin fragments were cut with DNase I in the presence of CV-1 nuclear proteins in 150 mM NaCl. The areas with bracket are the protected regions by protein binding. In the early palindrome domain, the band (a) of wild-type(W), pATSV-A, and pATSV-C disappeared, and the band (b) disappeared only in pATSV-B and pATSV-D. The left side of panel (g,a,t,c) is size marker(sequenced by Sanger's method) and lane F is DNase I footprint of wild-type origin fragment in the absence of nuclear proteins.

were predicted to be in a region in which nine of eleven nucleotides on the late strand are pyrimidines (i.e., nt 5210−5220; 5′-TCACTACTTCT-3′). They suggested that this polypurine/ polypyrimidine asymmetry (pPu/pPy) is predisposed to form unstable duplex structures which are more easily melted than non-pPu/pPy tracts, and T antigen binding to the pPu/pPy tract can further destabilize this tract and shift the equilibrium of the duplex DNA toward melting, resulting in the formation of single stranded DNA. Our results do not substantiate their hypothesis. First, a pyrimidine to purine substitution of pATSV-A and pATSV-C (nt 5217; T to A) did not cause a decrease in DNA replication. However,

another substitution at same position (T to C) caused the dramatic decrease in DNA replication (3). Second, some pyrimidine to purine substitutions at nt. 5209, 5210, and 5222 did not cause a severe effect on DNA replication (3). Also, recent studies of primase initiation in the SV40 origin of replication indicated that primase initiation sites (nt. 5210, 5214, 5218, and 5220) on early strand are located within nt. 5210−5220 (2). In addition, we observed that base-substitution at nt. 5214 (pATSV-B and pATSV-D; T to A) causes inhibition of SV40 DNA replication. Therefore the sequence-specific early palindrome domain remains a potential site for interaction with cellular or viral protein(s) except for T Ag (11, 24).

We have identified proteins from monkey cells that specifically recognize sequences located in the SV40 origin of DNA replication. Two bands of DNA-protein complexes were detected which appear to arise from sequence-specific interactions. At present, we do not know whether the two major complexes contain the same protein, complexed with homologous or heterologous proteins in different oligomeric forms, or whether different proteins bind to the same sequence. However, the pattern of DNase I protection suggests that there are two interaction sites in 46-bp *StuI-BglI* fragment of SV40 origin DNA.

Methylation of DNA is often associated with the repression of specific gene expression (25). Methylation can cause inhibition of transcription of a number of genes and loss of the methylation has been shown to activate expression a number of transcriptional inactive gene. Recent work has demonstrated that methylation prevents binding to DNA of protein factors required for optimal expression or induction of the genes. Cytosine methylation prevents association of DNA with a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter (26). Also, guanine and phosphates modificated chemically (methylation) interferes with protein binding of ara *C* protein (27). When the SV40 origin DNAs (46-bp *StuI-BglI* fragment) were methylated with dimethylsulfate, nuclear proteins of CV-1 cells did not bind to these modified DNA. We do not know that SV40 DNA replication is inhibited by the methylation in origin of DNA replication. Site-specific methylation of the early palindrome domain within the origin of SV40 DNA replication and replication assays will provide the means to answer this question.

The regions of the SV40 origin DNA protected from DNase I cleavage extend from position 5195 to position 5207 (5′-GAGGC-3′ pentanucleotide repeats) and from position 5238 to position 3 (a central region of 27-bp perfect palindrome), which are part of T antigen binding site. We do not know whether the same proteins interact with these binding sites. From our result, it is possible that the protein(s) may interact with T antigen. Stillman and his colleagues (10) fractionated a cellular factor required for formation of the complex with T antigen and ATP. In the case of phage lambda replication system, protein-DNA and protein-protein interactions also result in the formation of a specific initiation complex. Again, a site-specific DNA binding protein (O), together with the phage encoded *P* protein first interact with a defined origin sequence. Subsequently, the two *E.coli* proteins *dnaJ* and *dnaK* promote the correct placement of the dnaB helicase, which then results in an unwound structure. This complex can then act as a substrate for priming and elongation (28).

The functional role of the early palindrome domain in SV40 DNA replication was not defined. Hurwitz and his colleagues (4) insisted that the early palindrome domain is melted easily for unwinding of DNA. One another suggestion is that the early palindrome domain is a binding site to cellular protein(s) (11). The patterns of DNase I protection of wild-type and mutants in Fig. 7 suggest the early palindrome domain may affect the interaction of SV40 DNA with nuclear protein. For example, base-substitution at position 5,214 (mutations

pATSV-B and -D) may induce inadequate conformation of nuclear protein bound to SV40 DNA. Clearly, purification of the cellular factor(s) and SV40 replication assays will provide the means with which to answer this question.

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