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DNA Binding Properties of Simian Virus 40 Temperature-Sensitive A Proteins

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Wild-type simian virus 40 A protein (large T antigen) bound to three tandem regions of simian virus 40 DNA. The binding regions were defined by the ability of A protein to protect simian virus 40 DNA from digestion with limited (footprint assay) or excess (fragment assay) amounts of DNase I. At low concentrations, protein first bound to region I, which maps 30 to 45 base pairs to the early side of the origin of replication. At higher concentrations, A protein also protected region II and then region III. Region II spanned approximately 65 base pairs and corresponded in location to the functional origin of replication that contains a unique *BglI* site along with an adjacent adenine-thymine-rich region. Region III was adjacent to the late boundary of region II, but its distal limit was not well defined. Twelve distinct temperature-sensitive (*ts*) A proteins were purified and examined for their ability to bind in regions I to III. Three classes of *ts*A protein were defined on the basis of thermal stability. Class I *ts*A protein displayed wild-type binding either with or without a heat shock. Unheated class II *ts*A protein exhibited wild-type binding, but after a heat shock bound very poorly to the origin of replication. Class III *ts*A protein was defective in its binding even without a heat shock and only protected region I. Classes II and III were coded by mutants mapping in two distinct regions of the genome. For all of the *ts*A proteins examined, there was a positive correlation between the thermolability of origin binding in vitro and the temperature sensitivity of these mutants for DNA replication and transcriptional autoregulation in vivo. This correlation adds support to the essential role of origin binding by A protein in viral DNA replication and early transcription repression.

Simian virus 40 (SV40) A protein (SVA), or large T antigen, plays a role in both productive and transforming infection (33). During lytic growth the SVA protein is required for the initiation of each round of viral DNA replication (24). Replication originates from a 65-base pair noncoding region centered at the unique *BglI* site (6). Mutations within this region often cause defective viral DNA replication (9, 18, 22), however, some of these mutations can be compensated by second-site revertants that map in the *A* gene (22). The 65-base pair origin region also encompasses one of the three adjacent DNA binding regions of SVA protein (17, 26) and the related adenovirus-SV40 D2 hybrid protein (31, 32). This conjunction of binding and mutational data has implicated origin binding as a critical feature in SVA protein's role in viral DNA replication.

The 5′ ends of SV40 early mRNA transcripts also map within the origin region (2, 7, 8, 12). Repression of early transcription in vivo is mediated by SVA protein (1, 13, 28), and recent in vitro studies have confirmed that this inhibition of early transcription is dependent upon binding of SVA protein to sequences in the origin region (10, 17, 21). Thus, SVA protein modulates two essential viral activities, replication and transcription, by interacting with the same region of SV40 DNA.

We recently characterized the origin binding of wild-type (WT) SVA protein purified from lytically infected CV-1 cells (26). This protein protects three adjacent regions of SV40 DNA from DNase digestion. We address the protected areas as regions rather than sites because alternative interactions occur between SVA protein and region II, suggesting that region II has more than one binding site within it. In location, the three protected regions are similar to Tjian's sites I, II, and III (32); however, region II extends beyond site II in the late direction (Fig. 1). Region II contains the unique *BglI* site and can be isolated as a discrete, DNase-protected, 65-base pair fragment that had not been described previously. In addition, the boundaries

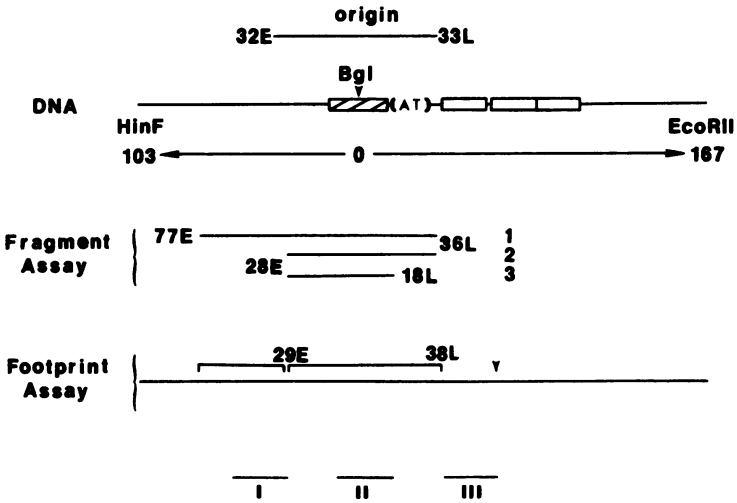

FIG. 1. Summary of the regions of SV40 DNA protected by SVA protein. The SV40 DNA between a *Hinf* and an *Eco*RII cleavage site is shown. This DNA contains the origin of replication as defined by viable deletion mutants (6). Within the origin region is a palindrome (cross-hatched box) containing the *BglI* site and an adenine-thymine (AT)-rich region. Adjacent to the origin on the late side are three 21-base pair repeats (open boxes). The numbers indicate distances to the early and late side of *BglI* in base pairs. The fragment assay indicates the location of mapped fragments 1, 2, and 3. The footprint assay indicates the location of predominant hyposensitive sites (brackets) and the hypersensitive site (arrow). At the bottom are the three locations of D2 protein-DNA contact defined by Tjian (32).

of region II correspond extremely well with the limits of the functional origin of replication defined by viable deletion mutants (6). Thus, the binding that generates region II may be the same binding that is necessary and sufficient for the initiation of replication.

Temperature-sensitive (*ts*) SVA mutants, with the exception of *ts*A1642, are very defective at elevated temperatures for the initiation of DNA replication and for the repression of early transcription (3, 5, 19, 24). We wished to determine whether or not the functional defects of the *ts*A proteins could be associated with defective binding in regions I to III. Previous in vitro studies have demonstrated a general thermolability for DNA binding by a few *ts*A proteins (11, 29), but the origin specific binding of a large number of mutants has not been examined in detail. We partially purified 12 distinct *ts*A proteins from lytically infected CV-1 cells and focused on their origin binding properties. The *ts*A proteins were grouped into three classes based on their in vitro DNA binding phenotypes.

MATERIALS AND METHODS

Cells and viruses. All viruses were grown in cultures of the CV-1 line of simian cells obtained from the American Type Culture Collection. The cells were grown in Eagle basal medium containing 2% fetal bovine serum. Stocks of WT SV40 strain VA 45-54 and the various *ts*A mutants were prepared and titrated as previously described (28). Mutants A1609, A1637, and A1642 were isolated by Tevethia and Ripper (30); mutants A207 and A241, by Chou and Martin (4); and mutants A7, A28, A30, A47, A57, and A58, by Tegtmeyer et al. (27, 28). Productive infection with each virus was carried out as previously described (25), except that the cells were incubated at 32°C for 96 h.

Protein purification. SVA protein from WT and *ts*A infections was purified by the method of Tegtmeyer and Andersen (25). The partially purified proteins were in 20 mM PIPES [piperazine-*N*,*N*'-bis (2-ethanesulfonic acid)]–1 mM NaCl–0.1 mM EDTA–1 mM dithioerythritol–10% glycerol at pH 7.0 (PNE buffer) with 100 μg of bovine serum albumin per ml.

Preparation of DNA. SV40 DNA was extracted from WT-infected CV-1 cells and purified according to Spillman et al. (23). Labeling by nick-translation with [^{32}P]dCTP (500 to 800 Ci/mmol; New England Nuclear Corp.) yielded specific activities of 3×10^7 to 8×10^7 cpm/μg. Labeled DNA was extracted with 2 volumes of phenol-chloroform (1:1) and separated from unincorporated radiolabel by gel filtration, using Al.5m agarose (Bio-Rad) in PNE. In later experiments the phenol extraction and gel filtration were omitted with no effect on the subsequent DNA binding and protection by SVA protein. The pBR322 DNA was obtained from Bethesda Research Laboratories.

DNase footprinting. The conditions for template preparation and labeling have been reported previously (26). The origin-containing portion of the *Eco*RII-G fragment cut with *Hinf* was used as the template for all experiments. Template strands were labeled at the 3' ends with avian myeloblastosis virus reverse transcriptase. To label the late strand only, [^{32}P]dATP (2,000 to 3,000 Ci/mmol) was used in the reverse transcription reaction with unlabeled dCTP, dGTP, and dTTP. Alternatively, early strands alone could be labeled with [^{32}P]dGTP (2,000 to 3,000 Ci/mmol) and unlabeled dATP, dCTP, and dTTP. Approximately 0.1

μg of SVA protein was incubated with 0.002 μg of end-labeled template in the presence of 0.02 μg of pBR322 DNA for 60 min at 4°C in 100 μl of PNE. To evaluate the effect of heating on *ts*A proteins, prebinding heat shocks were performed. For the preshock, SVA protein samples were removed from a 4°C bath and submerged in a 39.5°C water bath for 5 min. After heating, the protein samples were returned to the 4°C bath for 5 min before the addition of DNA. After binding, the samples were DNase treated and prepared for analysis on 8% polyacrylamide-urea gels as previously described (26).

Fragment assay. Binding reactions consisted of 0.01 μg of SVA protein incubated with 0.003 μg of nick-translated SV40 DNA for 60 min at 4°C. Binding was performed in 1.5-ml Eppendorf tubes in a final volume of 100 μl PNE. Preshocking of protein samples was performed as described for the footprint assay. Postshocking was performed by first incubating the protein and DNA for 60 min at 4°C and then submerging the protein-DNA mixture in a 39.5°C water bath for 5 min. The heated mixture was returned to the 4°C bath for 5 min before addition of DNase I. After completion of the binding reaction, samples were digested for 5 min at 4°C with 5 U of DNase I (Worthington Diagnostics) in 2 μl of PNE with 0.5 M $MgCl_2$ and 0.05 M $CaCl_2$. A 1-ml amount of 20 mM PIPES–1 mM NaCl–10 mM EDTA, pH 7.0, was added to terminate the digestion. Protein-DNA complexes were collected on nitrocellulose filters (Schleicher & Schuell) and washed five times with 1 ml of 20 mM PIPES–100 mM NaCl–1 mM EDTA, pH 7.0, and five times with 1 ml of PNE. DNA fragments were extracted from the filters by incubation for 1 h at room temperature in 50 μl of 10 mM Tris-borate (pH 8.3)–1 mM EDTA–10% glycerol–0.05% xylene cyanol–0.05% bromophenol blue–0.2% sodium dodecyl sulfate. The extracted material was analyzed on 12-cm 12% nondenaturing gels as previously described (26). Increased resolution was achieved by electrophoresis on 30-cm gels at a constant temperature of 20°C (Hoefer Scientific Instruments).

RESULTS

Footprint analysis of WT and *ts*A proteins. We previously reported the footprint pattern for WT SVA protein on the early strand of SV40 DNA (26). We have now extended the analysis by examining the interaction of WT SVA protein with both strands of SV40 DNA and by footprinting with *ts*A proteins. Figure 2 shows the footprint pattern generated by increasing amounts of WT SVA protein bound to the early and late strands. On either strand, the protection was first observed in region I. This is in agreement with previous studies that have determined region I to be the highest-affinity binding site for A protein and the related D2 protein. As the protein concentration increased, protection was observed in region II as well. On the early strand footprint, region II extended from 29 nucleotides to the early side of the *Bgl*I site to 36 to 38 nucleotides to the late side of *Bgl*I. However, since region II extended into a DNase-insensitive region of the early strand, the late boundary could not be determined with certainty. Confirmation of the late boundary of region II was obtained on the late-strand footprint which does not exhibit a highly DNase-insensitive region. Thus, as in our earlier study, region II was approximately 65 nucleotides in length and spanned the *Bgl*I site (26). Furthermore, this expanded region II correlated extremely well with our ability to isolate this same region as a discrete fragment of SV40 DNA protected from excess DNase by SVA protein (26). The extent of region II was significantly larger than the site II described by Tjian (32).

At high protein concentrations protection was observed in a third region to the late side of region II. The distal boundary of hyposensitive region III was not determined precisely, but a characteristic feature of this late-region protection was a hypersensitive site some 60 nucleotides to the late side of *Bgl*I. This hypersensitive site can be seen in the early-strand footprint (Fig. 2f), but is less obvious on the late strand.

Figure 2 also shows several artifactual bands. Origin-containing DNA fragments from SV40 have a number of regions of potential intrastrand base pairing. Unless stringent denaturing conditions are maintained before and during the gel electrophoresis, intrastrand pairing occurs and gives rise to discrete bands migrating faster than fully denatured template. Such artifactual bands are superimposed on the fragment ladder generated by DNase treatment. These snapback bands can be observed in Fig. 2a, g, and h, which show template DNA alone. Later experiments used a constant-temperature gel electrophoresis apparatus at 60°C and eliminated this problem.

The footprint patterns of WT SVA protein and 12 *ts*A proteins were determined. Parallel protein samples were either unheated or heat shocked at 39.5°C before addition of the template DNA. Comparison of the protection patterns of unheated and preheated samples was used to categorize the *ts*A proteins, and three classes were defined by this procedure (Fig. 3). The amounts of WT and *ts*A proteins used for comparison were similar as judged by immunoprecipitation of uniformly radiolabeled protein. Furthermore, in the assays of DNA binding by WT, class I, and class II proteins, the amounts of protein were functionally similar without heat treatment.

Figure 3 shows the characteristic protection patterns of WT SVA protein and a representative of each *ts*A class. Unheated WT protein exhibited the usual protection in regions I and II. Preshocking WT protein had little effect on its ability to protect regions I and II. Class I *ts*A protein displayed a resistance to a preshock

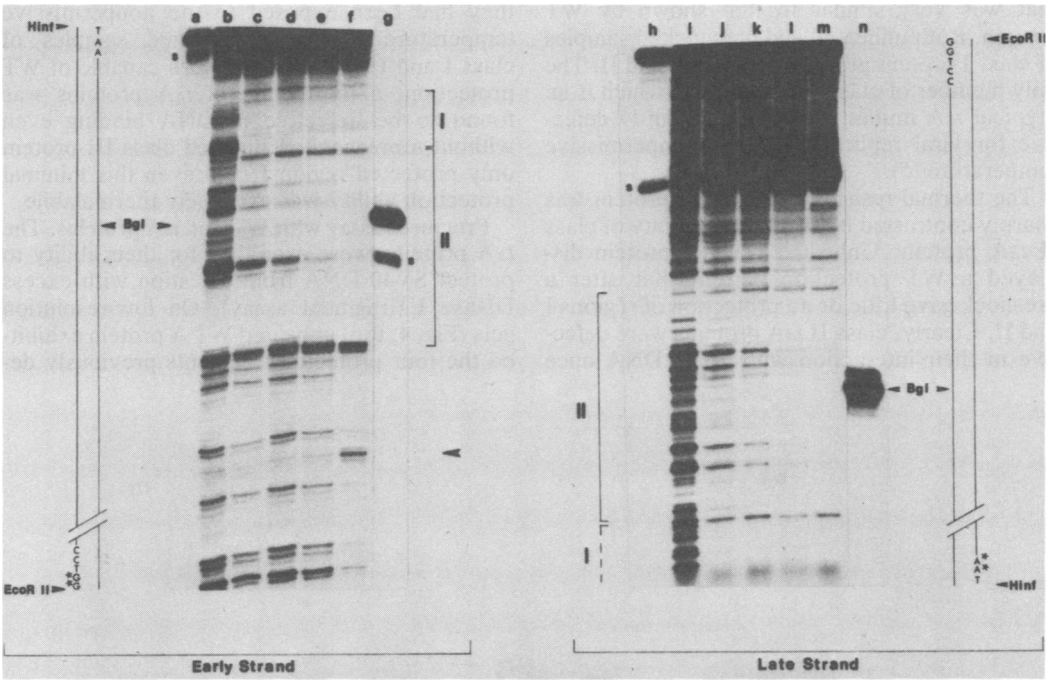

FIG. 2. DNase footprint of the early and late strands of SV40 origin DNA protected by SVA protein. The respective templates utilized are depicted. The procedures for template labeling, SVA binding, and gel analysis are described in the text. Protected regions I and II are indicated by brackets, and the hypersensitive site is shown by an arrow. Samples of the end-labeled templates without DNase I digestion are shown in lanes a, g, h, and n. The samples in lanes g and n were digested with *BglI*. The s designates snapback artifacts discussed in the text. The molar ratio of SVA monomers per SV40 template was: 0 (b, i); 10 (c, j); 20 (d, k); 40 (e, l); and 200 (f, m).

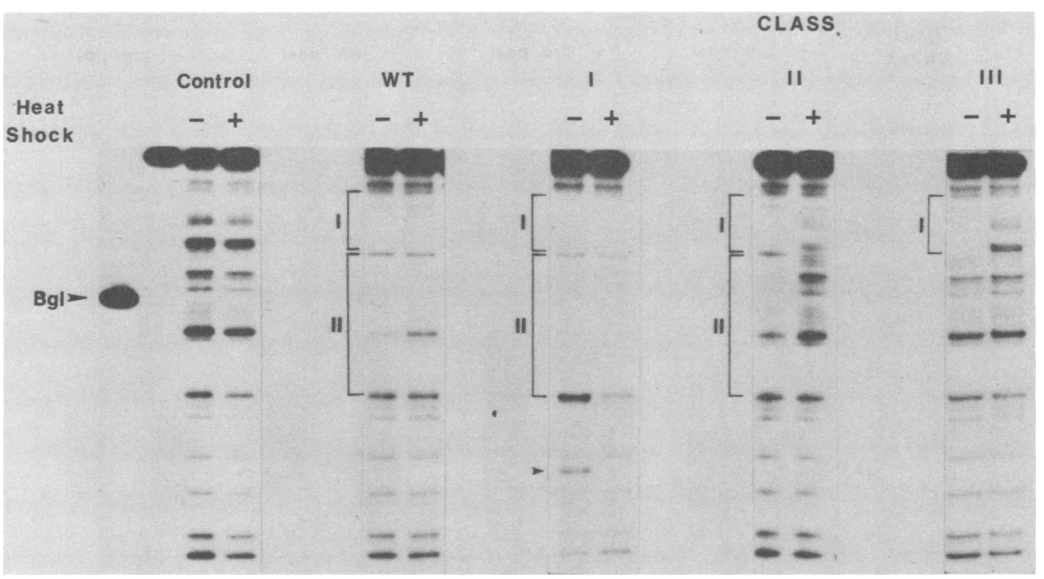

FIG. 3. Summary of the *ts*A classes by DNase footprinting. The template for these reactions was the *Eco*RII-G–*Hinf* fragment labeled on the early strand as in Fig. 2. Protein samples were either untreated or heat shocked for 5 min at 39.5°C before incubation with the template. The binding, DNase digestion, and gel electrophoresis are described in the text. The first and second lanes show *BglI*-cut template DNA and uncut template DNA, respectively, neither of which was DNase digested. The control lanes show template incubated with bovine serum albumin at 100 μg/ml in PNE with 10% glycerol. The *ts*A proteins used as representatives of classes I, II, and III were *ts*A1642, *ts*A58, and *ts*A30, respectively.

that was very similar to that shown by WT protein. Both unheated and preshocked samples of class I protein protected regions I and II. The only member of class I was *ts*A1642 which is an atypical *ts*A mutant that is only slightly defective for viral replication at the nonpermissive temperature (5).

The thermal resistance of class I protein was sharply contrasted by the thermolability of class II *ts*A protein. Unheated class II protein displayed a WT protection pattern, but after a preshock gave little or no protection of regions I and II. Clearly, class II *ts*A proteins were defective in their interaction with SV40 DNA once they had been exposed to the nonpermissive temperature. Whereas unheated samples of class I and II *ts*A proteins were capable of WT protection, a third class of *ts*A proteins was found to be defective in DNA binding even without a preshock. Unheated class III protein only protected region I, and even this minimal protection ability was extremely thermolabile.

Fragment assay with WT and *ts*A proteins. The *ts*A proteins were examined for their ability to protect SV40 DNA from digestion with excess DNase I (fragment assay). On low-resolution gels (Fig. 4, top) unheated WT A protein exhibited the four protected fragments previously de-

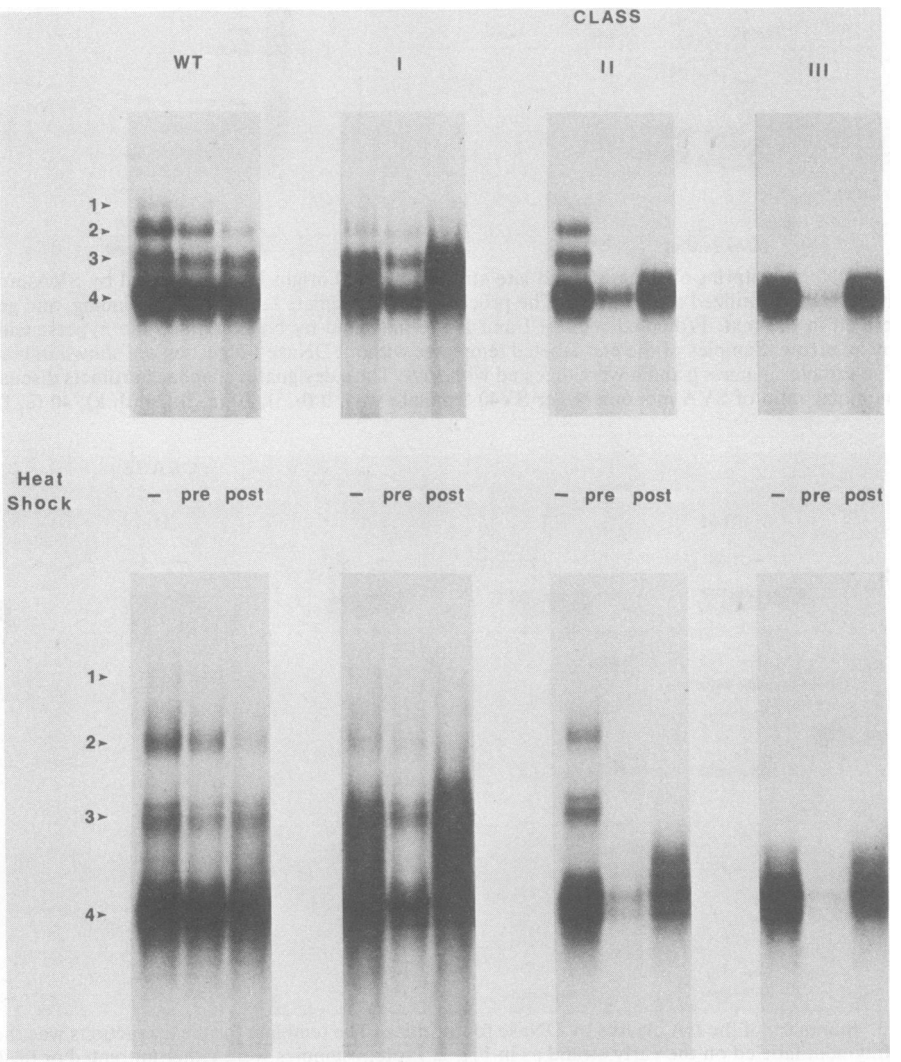

FIG. 4. Fragment assay of WT and *ts*A proteins. Details of the pre- and post-heat shock conditions, the fragment assay procedures, and the two gel procedures utilized are presented in the text. (Top) Fragment patterns on a low-resolution 12% nondenaturing polyacrylamide gel; (bottom) same samples on a high-resolution gel. Fragment classes 1 to 4 are indicated on each gel. The *ts*A proteins used as representatives of classes I, II, and III were *ts*A1642, *ts*A58, and *ts*A30, respectively.

scribed (26). However, at higher resolution (Fig. 4, bottom) the heterogeneity of fragments 1 to 4 became more apparent. Thus, what we have designated as fragments 1 to 4 actually represents classes of similar sized fragments. Preshocking WT protein for 5 min caused very little diminishment in its protection ability as evidenced by the maintenance of fragments 1 to 4 in amounts similar to the unheated sample. Both unheated and preshocked samples of class I protein also exhibited fragments 1 to 4 in similar amounts. However, the unheated class I protein occasionally showed a blurring of the protection pattern between fragments 3 and 4. As no such perturbation was detected in the footprint of this same sample of *ts*A1642 (Fig. 3), the nature of this phenomenon was unclear. Unheated class II protein gave a WT fragment pattern, but after heating gave only weak fragment 4 protection. Unheated class III protein yielded only an altered fragment 4 and preheating greatly reduced the amount of fragment 4 protected. These fragment assay results were consistent with the footprint results. In both assays unheated class I and II proteins displayed a WT protection pattern, whereas unheated class III protein gave defective protection. By either assay WT and class I proteins were not significantly affected by preheating, whereas class II and III proteins were very thermolabile.

Effect of template binding on thermolability of *ts*A proteins. The classification of the *ts*A proteins was based on the effect of heating free protein before incubation with template DNA. In vivo, SVA protein is both free and associated with viral DNA (15, 20). Temperature shift-up in vivo causes the loss of *ts*A58 protein but not WT SVA protein from chromatin (15). This suggests that *ts*A protein bound to SV40 DNA rapidly loses its binding ability at elevated temperatures. To examine whether or not template binding affected in vitro thermolability, a postbinding heat shock was performed (Fig. 4). SVA protein and SV40 DNA were incubated for 1 h on ice and then heat shocked for 5 min before DNase treatment. Although a protective effect of template binding on the thermal stability of *ts*A protein was not evident by footprinting (data not shown), the fragment assay clearly demonstrated a partial protection (Fig. 4). WT protein exhibited a slight decrease in the protection of fragments 1 to 4 after the postshock, but this may have been caused by an increased rate of dissociation at the higher temperature rather than by protein inactivation. The class I *ts*A protein also maintained the WT fragment pattern after a postshock, further confirming its similarity to WT protein. Postshocking of class II proteins was similar to preshocking in that the binding that produced fragments 1, 2, and 3 was still extremely thermolabile. However, for both the class II and III *ts*A proteins, the protection of fragment 4 was stabilized by template binding before the heat shock. In addition, the fragment 4 appearing after a postshock of these two classes of *ts*A proteins was clearly more heterogeneous than the fragment 4 produced by WT SVA protein.

DISCUSSION

WT SVA protein protects adjacent regions of SV40 origin DNA. These regions were detected as hyposensitive areas on a DNase footprint or as a set of four protected fragment classes that remained after complete DNase digestion. Protein titration indicated that SVA protein bound first to region I, as previously demonstrated (17). Briefly heating WT A protein before or after incubation with template DNA caused only a slight quantitative diminishment in the extent of protection by either assay, but no qualitative difference. Extended heating resulted in the gradual loss of protection ability by WT A protein (data not shown).

The assignment of the 12 *ts*A mutants to

TABLE 1. Classification of 12 *ts*A proteins

Class	Virus	DNA binding[a]		Location[b]	DNA replication[c]	
		−Shock	+Shock		33°C	40°C
	WT	1–4	1–4		+	+
I	A1642	1–4	1–4	B	+	+/−
II	A7	1–4	4	B	+	−
	A28			I	+	−
	A58			I	+	−
	A207			I	+	−
	A241			I	+	−
III	A30	4	0	H	+	−
	A40			H	+	−
	A47			H	+	−
	A57			H	+	−
	A1609			H	+	−
	A1637			H	+	−

[a] Examined with the fragment assay. The protected fragments observed with unshocked and preshocked samples are listed.

[b] The *Hin*dII+III fragment within which each *ts*A lesion maps. Mapping data is from Lai and Nathans (14) and Cosman and Tevethia (5).

[c] The in vivo replication ability of WT SV40 and the 12 *ts*A mutants. Symbols: +, WT level of replication; +/−, slightly defective replicative ability; −, <10% of WT replication. Replication data are from Tegtmeyer (24; unpublished data), Chou et al. (3), and Cosman and Tevethia (5).

classes I to III is presented in Table 1. The DNA binding phenotypes of the *ts*A proteins are described in terms of the fragments protected by unheated and preshocked samples, because the fragment assay was more sensitive than the footprint assay for detecting low levels of binding. The genomic locations of the *ts*A mutants and the mutant phenotypes for DNA replication in vivo are indicated also. In all cases, we observed that the genomic locations of the *ts*A mutations of the class II proteins differed from those of the class III proteins. The mutations of class II mapped to *Hin*dII+III fragments B and I, whereas the mutations of class III mapped to fragment H. This consistent difference in location emphasizes that the phenotypic differences in DNA binding by the two classes are a result of genetic differences and not an artifactual consequence of the DNA binding assay in vitro. Since nothing is known of the relationship between the primary and tertiary structure of SVA protein, it is not clear whether the class II and III mutations affect the DNA binding site of A protein directly or indirectly through an overall conformational change. Nonetheless, the class III alterations map closer to the putative DNA contact region of the protein identified by Shortle et al., using protein pseudorevertants of an origin *cis* mutation (22). This fact may explain the greater defectiveness of the class III proteins in DNA binding.

The class II *ts*A proteins were classically thermolabile for DNA binding in vitro; unshocked samples exhibited WT protection, whereas heat-shocked samples were defective for origin binding. Our prototype for class II was *ts*A58. Temperature shift-up in vivo of *ts*A58-infected cells inhibits viral DNA replication within 10 min even though levels of the accumulated protein are not significantly altered by a shift of this duration (24; unpublished data). This rapid inhibition suggests that *ts*A58 loses its DNA binding ability very rapidly in vivo. However, it previously has been reported that *ts*A58 protein is not thermolabile for DNA binding in vitro (17). We do not know the explanation for this discrepancy.

The *ts*A proteins of class III are unusual because they failed to protect origin DNA even before they had been exposed to an elevated temperature. Since the class III *ts*A mutants replicate normally at the permissive temperature, their A proteins must have WT function in vivo. Failure to observe WT protection in vitro with unheated class III proteins suggested that they were easily damaged during extraction and purification. The defect is not due simply to underproduction or more rapid turnover of the class III *ts*A protein, since we have observed that *ts*A30 accumulates to WT levels in vivo at the permissive temperature (unpublished data). As noted above, the mutations in all of the class III *ts*A proteins map to the *Hin*dII+III-H fragment (Table 1). This common location suggested that the portion of the SVA protein coded by this fragment may be particularly important in determining the functional lability of SVA protein. The lability could affect either protein-DNA interactions or protein-protein interactions.

Class III *ts*A protein was particularly useful in examining fragment 4 protection. Previously, the location of the binding that produced fragment 4 was defined only partially as this fragment was not amenable to mapping by cleavage with *Bgl*I (26). We had observed that rebinding of A protein to isolated fragment 2, which encompasses region II only, generates a fragment 4. These rebinding data suggest that at least a portion of fragment 4 could derive from region II. However, due to its extreme heterogeneity, it was quite possible that fragment 4 also reflected binding to and protection of sequences not in region II. This possibility was confirmed by the results of the fragment and footprint assays with class III *ts*A protein. Unheated class III *ts*A protein protected only fragment 4 after excess DNase digestion (Fig. 4) and under the same conditions protected region I of the footprint most completely. Thus, it is likely that a significant portion of fragment 4 reflects binding in region I. The comparison between the fragment and footprint assays is valid because similar molar ratios of protein relative to total DNA and origin DNA were used in both assays.

The fragment assay showed an apparent qualitative difference between WT and class III protein. The distribution of bands within class III fragment 4 was more heterogeneous than within fragment 4 produced by WT protein. This same heterogeneity was evident in fragment 4 generated by postshocking class II protein. In both cases fragment 4 exhibited new protected bands whose size was larger than seen with fragment 4 from WT protein. However, this heterogeneity in fragment 4 can be observed with WT protein at low protein concentrations (unpublished data). Therefore, a heterogeneous fragment 4 probably results from a low concentration of functionally active A protein. The functional inactivation of the *ts*A proteins presumably would have been caused by heating in the case of the class II proteins and by damage incurred during purification for the unheated class III proteins.

An additional feature of the class II and III *ts*A proteins was that template binding before a heat shock only stabilized the interaction that generated fragment 4 (Fig. 4). The explanation for this phenomenon is not known, but it may simply reflect a high affinity between SVA pro-

tein and SV40 DNA in region I. SVA protein bound in region I may be protected from thermally induced conformational changes due to its tight interaction with the DNA in region I, whereas binding in lower-affinity regions II and III may not confer such protection. Alternatively, different aggregation states of SVA protein may be responsible for the generation of different sized protected fragments. These different binding forms may have slightly different thermostabilities, with the form that produces fragment 4 being the most stable. For example, a monomer or dimer could be more stable than a tetramer.

Regardless of the structural form(s) of SVA protein involved in binding, the in vitro binding phenotypes of all *ts*A proteins examined were consistent with origin binding being necessary for replication and repression of early transcription in vivo. All of the mutants of classes II and III are defective for both replication and autoregulation of SVA production (3, 19, 24). Correspondingly, all class II and III *ts*A proteins were extremely defective for origin binding after a brief exposure to an elevated temperature. In contrast, the class I *ts*A protein was not significantly thermolabile for DNA binding in vitro, and this mutant is only mildly defective in vivo at the nonpermissive temperature for replication and autoregulation (5). Thus, there was a strong positive correlation between the temperature sensitivity of in vitro origin binding and in vivo defectiveness for replication and autoregulation.

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