The EMBO Journal vol.4 no.11 pp.2933–2939, 1985

T antigen and template requirements for SV40 DNA replication *in vitro*

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Communicated by P.W.J.Rigby

A cell-free system for replication of SV40 DNA was used to assess the effect of mutations altering either the SV40 origin of DNA replication or the virus-encoded large tumor (T) antigen. Plasmid DNAs containing various portions of the SV40 genome that surround the origin of DNA replication support efficient DNA synthesis *in vitro* and *in vivo*. Deletion of DNA sequences adjacent to the binding sites for T antigen either reduce or prevent DNA synthesis. This analysis shows that sequences that had been previously defined by studies *in vivo* to constitute the minimal core origin sequences are also necessary for DNA synthesis *in vitro*. Five mutant T antigens containing amino acid substitutions that affect SV40 replication have been purified and their *in vitro* properties compared with the purified wild-type protein. One protein is completely defective in the ATPase activity of T antigen, but still binds to the origin sequences. Three altered proteins are defective in their ability to bind to origin DNA, but retain ATPase activity. Finally, one of the altered T antigens binds to origin sequences and contains ATPase activity and thus appears like wild-type for these functions. All five proteins fail to support SV40 DNA replication *in vitro*. Interestingly, in mixing experiments, all five proteins efficiently compete with the wild-type protein and reduce the amount of DNA replication. These data suggest that an additional function of T antigen other than origin binding or ATPase activity, is required for initiation of DNA replication.

Key words: ATPase/mutant tumor antigens/origin binding/origin sequences

Introduction

Replication of the small circular chromosome of simian virus 40 (SV40) is an excellent model for studying the process of replication of cellular chromosomes (see DePamphilis and Wassarman, 1982, for review). The genome of 5243 bp contains a single origin of DNA replication and encodes the large tumor (T) antigen, the only viral protein required for SV40 DNA replication. Furthermore, SV40 DNA exists in a chromatin structure that is similar to cellular chromatin. Recently, it has become possible to examine the mechanism of SV40 DNA replication in cell-free extracts prepared from monkey or human cells (Ariga and Sugano, 1983; Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985) and future characterization of these reactions should lead to a detailed understanding of the process of eukaryotic DNA replication.

The SV40 DNA replication origin was first defined by studies *in vivo* using two methods. First, DNA synthesis was shown to initiate at a unique region in the viral chromosome, proceed bidirectionally around the DNA and terminate when the replication forks met (Danna and Nathans, 1972; Hay and DePamphilis, 1982). Second, mutations in recombinant SV40 DNA sequences have been constructed and the minimal sequences required to support efficient, extrachromosomal replication of the DNAs *in vivo* were determined (Subramanian and Shenk, 1978; Gluzman *et al.*, 1979; DiMaio and Nathans, 1980, 1982; Shortle and Nathans, 1979; Myers and Tjian, 1980; Fromm and Berg, 1982; Bergsma *et al.*, 1982; Jones *et al.*, 1984; reviewed by DePamphilis and Wassarman, 1982). These studies have demonstrated that a 65-bp sequence is sufficient for origin function *in vivo*. This sequence contains the SV40 T antigen binding site II, part of T antigen binding site I and an AT-rich region (nucleotides 5208–30 in Figure 1). However, one report suggests that DNA sequences that lie adjacent to this minimal core sequence affect the levels of DNA synthesis (Bergsma *et al.*, 1982). Using a cell-free system for replication of SV40 DNA, we have examined the template DNA sequence requirements for efficient DNA replication and compared the results with those obtained *in vivo*.

The SV40 T antigen is a multifunctional protein that is required for regulation of transcription of both early and late virus genes, for initiation of DNA replication and for cell transformation (reviewed by Rigby and Lane, 1983). A number of biochemical properties have been shown to reside with the protein. T antigen binds to three distinct sites in the origin-promoter region of SV40 DNA (see Figure 1) and DNA binding is required for both transcription regulation and DNA synthesis. In addition, T antigen contains a DNA-independent ATPase activity (Giacherio and Hager, 1979; Tjian and Robbins, 1979), a nucleotide-binding activity (Clertant *et al.*, 1984) and can be modified by adenylation (Bradley *et al.*, 1984) and phosphorylation (Tegtmeyer *et al.*, 1977). A number of mutants in the gene encoding T antigen have been isolated that affect the DNA replication function of T antigen (Gluzman and Ahrens, 1982; Manos and Gluzman, 1984, 1985; Wilson *et al.*, 1982; Stringer, 1982; Clark *et al.*, 1983; Margolskee and Nathans, 1984; Kalderon and Smith, 1984) and some of their biochemical properties have been determined. We have examined the ability of five altered T antigen proteins to support DNA synthesis *in vitro*. These studies suggest that a T antigen function, in addition to the ATPase and DNA binding activities, is required for DNA replication.

Results

Origin sequences required for DNA replication

A functional origin sequence is required in the template DNA for efficient DNA replication in cell-free extracts from monkey and human cells (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985). To delineate the minimum DNA sequences that are required for DNA replication, a series of deletion mutants were constructed that removed DNA sequences from either the late side (pS1 series) or the early side (pSV0 series) of the central palindrome that contains the T antigen binding site II (Figure 1C). These plasmid DNAs were purified and increasing amounts of each were utilized as template DNA in reactions that contained

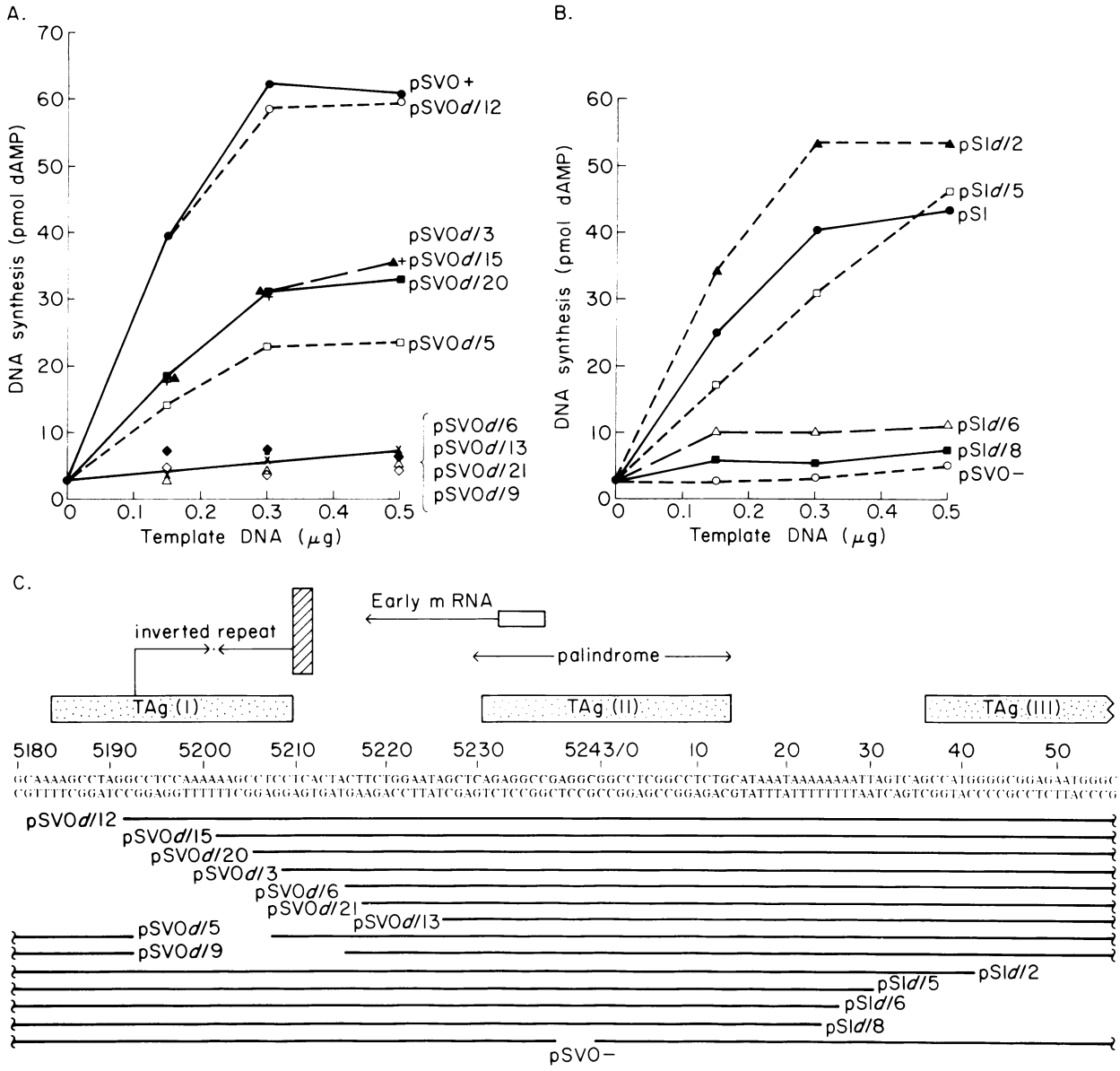

Fig. 1. **(A)** DNA synthesis with deletion mutant DNAs of the pSV0 series as template in 50 μl reactions containing 293 cell cytosol extract and 0.5 μg of T antigen as previously described (Stillman and Gluzman, 1985). After 90 min at 37°C, the amount of incorporated [α-^{32}P]dAMP was determined by acid precipitation. **(B)** Same as **A** except that deletion plasmids of the pS1 series and pSV0− were used as template DNA. **(C)** Nucleotide sequence and features of the SV40 origin region. Above the sequence are indicated the T antigen binding sites, the 27-bp palindrome, the small inverted repeat and the start sites and direction of transcription for early mRNA (reviewed by DePamphilis and Wassarman, 1982). The vertical hatched region at nucleotides 5210−5211 shows the transition point between lagging and leading strand replication as determined by Hay and DePamphilis (1982). Below the sequence: the DNA sequences contained in various deletion plasmids (sequences present are indicated by the solid lines). The nucleotide position of each deletion end-point (last base-pair remaining in the plasmid) is as follows: pSV0*dl*12, 5192; pSV0*dl*15, 5202; pSV0*dl*20, 5206; pSV0*dl*5, 5208; pSV0*dl*3, 5209; pSV0*dl*6 and pSV0*dl*9, 5216; pSV0*dl*21, 5218; pSV0*dl*13, 5227; pS1*dl*8, 24; pS1*dl*6, 26; pS1*dl*5, 30; pS1*dl*2, 41 and pSV0−, deleted 5239−5242 inclusive.

purified SV40 T antigen (from the Ad5SVR115 vector) and 293 cell cytosol extracts. Plasmids containing deletions of SV40 sequences from the early transcription side of the origin (Figure 1A) demonstrated a complex relationship between the level of DNA synthesis and DNA sequences present. Only one deletion plasmid, pSV0*dl*12 replicated as well as the wild-type pSV0+ plasmid. Three plasmids, pSV0*dl*3, pSV0*dl*15 and pSV0*dl*20, which remove most of the T antigen binding site I, replicated approximately one half as efficiently as wild-type, indicating that the sequences between the end points for *dl*12 and *dl*15 are required for efficient DNA replication. Deletions beyond nucleotide 5211 (*dl*6, *dl*9, *dl*13 and *dl*21) reduced DNA synthesis by >90% of wild-type levels. However, this pattern was complicated by the results obtained with pSV0*dl*5, which has a deletion end point that leaves one base more than pSV0*dl*3, but replicated less efficiently than pSV0*dl*3. The difference between the plasmids, apart from the extra base pair at position 5208, is the presence of different flanking sequences on the origin distal side of the deletion. This result also suggests that DNA sequences on the early side (left in Figure 1) of nucleotide 5211 affect the level of DNA replication but are not absolutely required.

These plasmids were also transfected into CMT3 cells that express the SV40 T antigen from the mouse metallothionein promoter and the amount of DNA synthesized at 50 h

Table I. Replication of plasmid DNA sequences *in vivo*

Plasmid	*Dpn*I-resistant DNA (c.p.m.)
Early side pSV0+	4421
pSV0*dl*12	4838
*dl*15	4774
*dl*20	5980
*dl*5	3362
*dl*3	4540
*dl*6	3090
*dl*9	184
*dl*21	158
*dl*13	75
Late side pS1	2904
pS1*dl*2	4514
*dl*5	2866
*dl*6	457
*dl*8	82
None	75

Each plasmid DNA (100 ng) was transfected into 2×10^6 CMT3 cells in the presence of DEAE-dextran (250 μg/ml) for 1 h and then the excess DNA was removed and the cells were treated with 100 μM chloroquine in medium for 5 h. The chloroquine was removed and cells were incubated for a further 47 h at 37°C in medium containing 1 μM $CdSO_4$ and 100 μM $ZnCl_2$, which induce the metallothionein promoter. The cells were lysed and DNA extracted by the procedure of Hirt (1967), then the DNA digested with *Dpn*I and either *Bam*HI (pS1 plasmids) or *Cla*I (pSV0 plasmids) prior to agarose gel electrophoresis. Only replicated DNA is resistant to digestion by the *Dpn*I enzyme (Peden *et al.*, 1980). The DNA was transferred to nitrocellulose from the gel following nicking by acid treatment using standard procedures (Maniatis *et al.*, 1982) and the blot hybridized with ^{32}P-labeled, nick-translated pXf3 plasmid DNA containing SV40 nucleotides 5211–2533. The bands were detected by autoradiography and then cut out and counted using a liquid scintillant. Standard amounts of DNA on the blot showed that the response was linear up to 2000 c.p.m. per band (25 ng DNA).

post-transfection determined by blot hybridization (Table I). Except for pSV0*dl*6 which replicated *in vivo* but failed to replicate *in vitro*, the replication results obtained were qualitatively similar to those described above. The difference between the results obtained with pSV0*dl*6 may also be related to differential effects of flanking sequences *in vivo* and *in vitro*. The plasmid pSV0*dl*6 had the same restriction pattern before and after DNA replication *in vivo* (data not shown), which indicated that rearrangement of origin sequences was not occurring.

In contrast to results obtained with deletion mutants on the early side of the origin, analysis of deletions on the late side of the origin yielded a well-defined boundary (Figure 1B). Plasmids pS1, pS1*dl*2 and pS1*dl*5 all replicated *in vitro* with similar efficiency, but pS1*dl*6 and pS1*dl*8 replicated poorly or not at all. Plasmid pSV0–, which contains a four-base deletion in the middle of the palindrome, did not support DNA replication (Stillman and Gluzman, 1985 and Figure 1B). Similar results were observed *in vivo* with these plasmids (Table I).

Purification of replication-defective T antigens

A number of SV40-transformed permissive monkey cells had been isolated and shown to contain integrated SV40 DNA that contained mutated T antigen genes (Gluzman and Ahrens, 1982; Manos and Gluzman, 1984, 1985). The mutations in the T antigen prevented DNA replication from SV40 origin sequences in these permissive cells, but the cell transformation function of T antigen remained intact. The genes encoding these altered T antigens have been cloned (Gluzman and Ahrens, 1982; Manos and Gluzman, 1984, 1985) and were subsequently incorporated into an adenovirus vector that is capable of producing large amounts of T antigen when infected into human 293 cells. The defective T antigens produced from these vectors contain amino acid substitutions as follows; C2, lysine to asparagine at amino acid number 516; C6.2, asparagine to threonine (153); C8A, lysine to glutamic acid (224); C11, a double mutant, proline to serine (522) and proline to histidine (549), and T22, histidine to glutamine (203) (see Manos and Gluzman, 1985). These proteins were purified to apparent homogeneity by immunoaffinity chromotography (Simanis and Lane, 1985) and 1 μg of each sample, as well as the wild-type T antigen (R112) was subjected to SDS-polyacrylamide gel electrophoresis (Figure 2). The proteins migrate as full-length polypeptides with slightly different apparent mol. wts., due to the amino acid substitutions and differences between SV40 strains 776 and 777 (Manos and Gluzman, 1985).

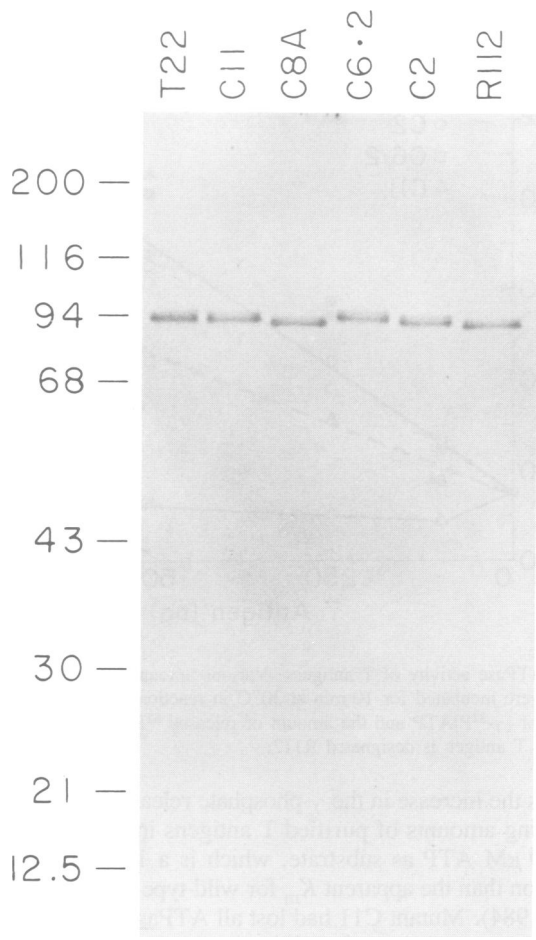

Fig. 2. SDS-polyacrylamide gel electrophoresis of immunoaffinity-purified wild-type and defective SV40 T antigens. Proteins were purified from 293 cells that had been infected with recombinant adenoviruses that overproduce the T antigens T22, C11, C8A, C6.2, C2 and wild-type (R112). 1 μg of each purified protein was subjected to SDS-polyacrylamide gel electrophoresis and the gel stained with Coomassie brilliant blue. The numbers at left are mol. wt. standards ($\times 10^{-3}$).

ATPase activity of purified T antigens

Although a preliminary characterization of the mutant T antigens using immunoprecipitated protein from crude cellular extracts had been published (Gluzman and Ahrens, 1982; Manos and Gluzman, 1984, 1985), it was necessary to examine both ATPase activity and origin binding with the purified T antigens. Figure

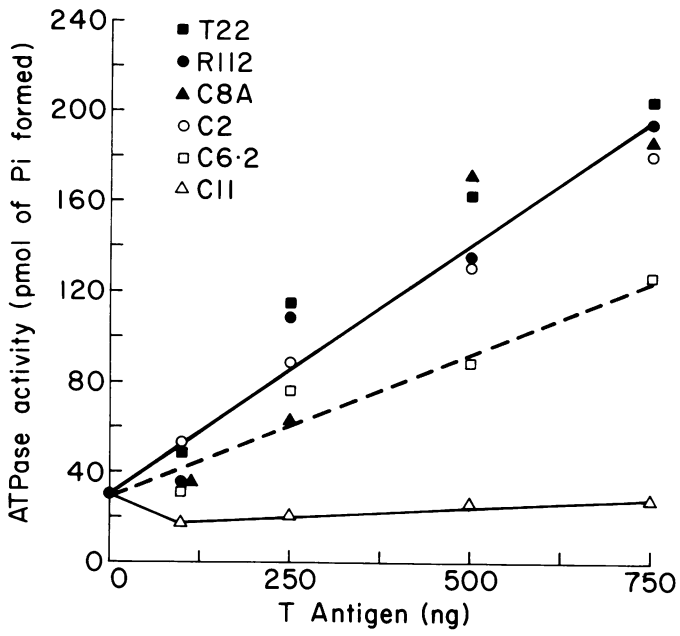

Fig. 3. ATPase activity of T antigens. Various amounts of each purified T antigen were incubated for 10 min at 30°C in reaction mixtures containing 100 μM of [γ-^{32}P]ATP and the amount of released ^{32}P was determined. The wild-type T antigen is designated R112.

3 shows the increase in the γ-phosphate released from ATP with increasing amounts of purified T antigens in reactions containing 100 μM ATP as substrate, which is a 10-fold higher concentration than the apparent K_m for wild-type T antigen (Clertant *et al.*, 1984). Mutant C11 had lost all ATPase activity, whereas all other T antigen mutants retained wild-type or near wild-type levels of ATPase activity.

Binding of T antigens to the origin-promoter DNA

Purified mutant and wild-type T antigens were mixed with ^{32}P-labeled, SV40 DNA *Bst*NI restriction fragments in buffer containing 150 mM NaCl, and the DNA-protein complexes were subsequently isolated by binding to nitrocellulose. The bound fragments were identified by eluting them from the nitrocellulose followed by polyacrylamide gel electrophoresis (Figure 4). The origin-promoter region is entirely contained within the *Bst*NI G fragment, which was selectively retained when mixed with wild-type (R115), C8A and C11 T antigens. The altered C2, C6.2 and T22 proteins did not preferentially bind the origin fragment even at high concentrations. Identical results were obtained when the binding was performed in a buffer containing 40 mM NaCl, which is similar to conditions used for DNA replication experiments described below (data not shown). These results are similar to those reported earlier, except for mutant C2 which was previously reported to be functional for origin binding using an immunoprecipitation assay (Prives *et al.*, 1983). We do not know the reason for this variation, but the results were identical when both high (150 mM) and low (40 mM) concentrations of NaCl were used in the buffer, indicating that the different binding was not NaCl dependent.

DNA replication with purified T antigens

Figure 5A demonstrates that wild-type T antigen stimulated SV40 DNA replication when added to 293 cytosol extracts, whereas none of the altered T antigens could support DNA replication. The mutant T antigen C8A was completely defective for supporting DNA replication even though it retained wild-type ATPase and origin-binding activities. It was of interest to determine whether the various replication-defective T antigens, particularly those that did not bind to the origin sequences, could compete with wild-type T antigen for replication functions. Figure 5B shows that all five of the altered T antigens reduced the amount of DNA replication when added in equal amounts or in 2- or 3-fold excess to reactions containing 1 μg of wild-type T antigen. Fifty percent inhibition of replication was obtained with an equal molar amount of each defective and wild-type T antigen, indicating that the inhibition was efficient.

Discussion

The replication of SV40 DNA requires a specific interaction between the T antigen and DNA sequences located at a unique site on the virus chromosome. These T antigen binding sequences in site II must be correctly spaced with respect to each other, suggesting that critical protein-protein interactions play an important role in initiation of DNA synthesis at the origin (Jones *et al.*, 1984; DeLucia *et al.*, 1983; Tegtmeyer *et al.*, 1983; Cohen *et al.*, 1984). Results presented herein demonstrate that the cell-free system for the replication of SV40 DNA is a faithful representation of the *in vivo* mechanism. The DNA sequences required for DNA replication *in vitro* are similar to those previously identified as being required for origin function *in vivo* (Subramanian and Shenk, 1978; Gluzman *et al.*, 1979; DiMaio and Nathans, 1980, 1982; Shortle and Nathans, 1979; Myers and Tjian, 1980; Fromm and Berg, 1982; Bergsma *et al.*, 1982; Jones *et al.*, 1984; this report). Deletion of the origin distal third of T antigen binding site I did not affect the level of DNA synthesis, but deletions further into T antigen binding site I reduced replication by one half. Mutations in this region are known to affect the level of replication and also to confer cold sensitivity on replication *in vivo* (see DePamphilis and Wassarman, 1982). This region contains an inverted repeated sequence that is conserved in papovavirus origins (DePamphilis and Wassarman, 1982 and Figure 1). Deletions to the right of nucleotide 5208 (Figure 1) delete essential replication sequences *in vivo* and *in vitro*, although replication *in vivo* is affected by flanking sequences (compare pSV0*dl*6 and pSV0*dl*9, Table I). This region (nucleotide 5210–5211) is the region of transition between leading (continuous) and lagging (discontinuous) strand replication observed *in vivo* (Hay and DePamphilis, 1982) and may play an essential role in the initiation of replication on the DNA strand encoding late proteins (bottom sequence, Figure 1).

In contrast to the complex boundary for the origin on the early side, the boundary on the late side is well defined. Deletions into the 17-bp AT-rich sequence (nucleotides 15–31, Figure 1) reduce and eventually inactivate origin function, suggesting that these AT base pairs play a crucial role in initiation of DNA synthesis *in vitro*. The AT-rich sequence contains the 'TATA' homologous region that is required for early gene transcription. Similar results have been obtained from studies *in vivo* (Fromm and Berg, 1982; Bergsma *et al.*, 1982; Wasylyk *et al.*, 1983). The mutants pS1*dl*5 and pS1*dl*2 replicate as efficiently as wild-type pS1 DNA *in vitro* and *in vivo*, demonstrating that early promoter elements such as the SP1 binding sites (Dynan and Tjian, 1983) are not required for DNA replication. This conclusion, while different from results *in vivo* presented earlier (Bergsma *et al.*, 1982), agrees with the *in vivo* results of Myers and Tjian (1980). These results suggest that factor SP1-dependent early transcription is not required for initiation of DNA replication, but does not exclude the possibility that transcription may be re-

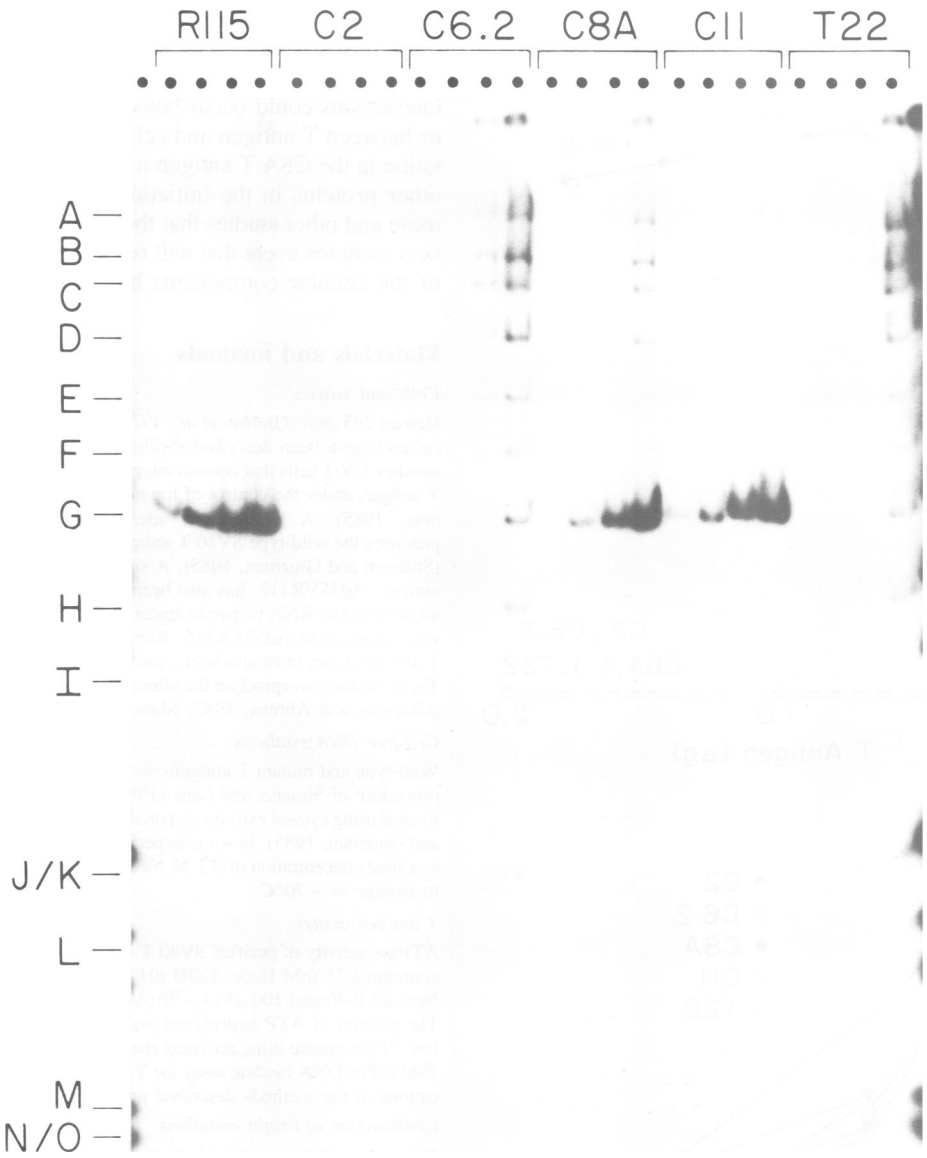

Fig. 4. DNA binding of T antigens. Four different amounts of wild-type (R115) and defective T antigens (40 ng, 150 ng, 400 ng and 1 μg, left to right) were mixed in 10 μl with 20 ng of ^{32}P-end-labeled, *Bst*NI-digested SV40 DNA in reaction mixtures containing 25 mM Pipes, pH 6.8, 0.2 mM dithiothreitol, 10 μg/ml bovine serum albumin and 150 mM NaCl for 10 min at room temperature. The samples were then applied to nitrocellulose and washed with buffer containing 20 mM sodium phosphate pH 7.0, 1 mM EDTA and 0.03% β-mercaptoethanol. The bound DNA was eluted from each filter with SDS and protease, precipitated with ethanol and identified by polyacrylamide gel electrophoresis and autoradiography. The left lane shows a control reaction without T antigen. The letters show the SV40 *Bst*NI fragments.

quired for origin function. The related papovavirus origins of replication present in the human viruses BK and JC will also support DNA replication in the presence of the SV40 T antigen (Li and Kelly, 1985), suggesting that conserved features of these DNAs are sufficient for the initiation of DNA replication.

One important role for origin sequences is to interact with the virus-encoded T antigen. Three altered T antigens analyzed in this report that did not bind to the origin region failed to support DNA replication *in vitro*. However, two other altered T antigens that bound to origin DNA, also failed to support cell-free DNA synthesis. One of these proteins (C11) lacked any detectable ATPase activity, which provides more circumstantial evidence for a role of the T antigen ATPase activity in the initiation of DNA replication, a conclusion previously drawn (Clark *et al.*, 1983). The other protein, C8A, retained both origin-binding activity and ATPase activity but still failed to support efficient DNA replication. This suggests that T antigen has another function in the initiation of replication, perhaps mediated by interaction with cellular replication factors that are required for the initiation reactions. Consistent with this hypothesis, although not the only possibility, is the ability of T antigens defective in origin binding or ATPase activity to compete with the wild-type protein and reduce the level of DNA synthesis. It is particularly interesting that origin binding was not required for the ability of the altered T antigens to inhibit replication, suggesting that some protein-protein interactions occur prior to origin binding.

The available data suggest that the SV40 T antigen binds specifically to the origin sequences in the template DNA and most probably has to retain ATPase activity for correct initiation of DNA synthesis to occur. However, an additional function(s) of the T antigen must now be considered. One possibility is another as yet undiscovered enzymatic activity for T antigen. Alternative-

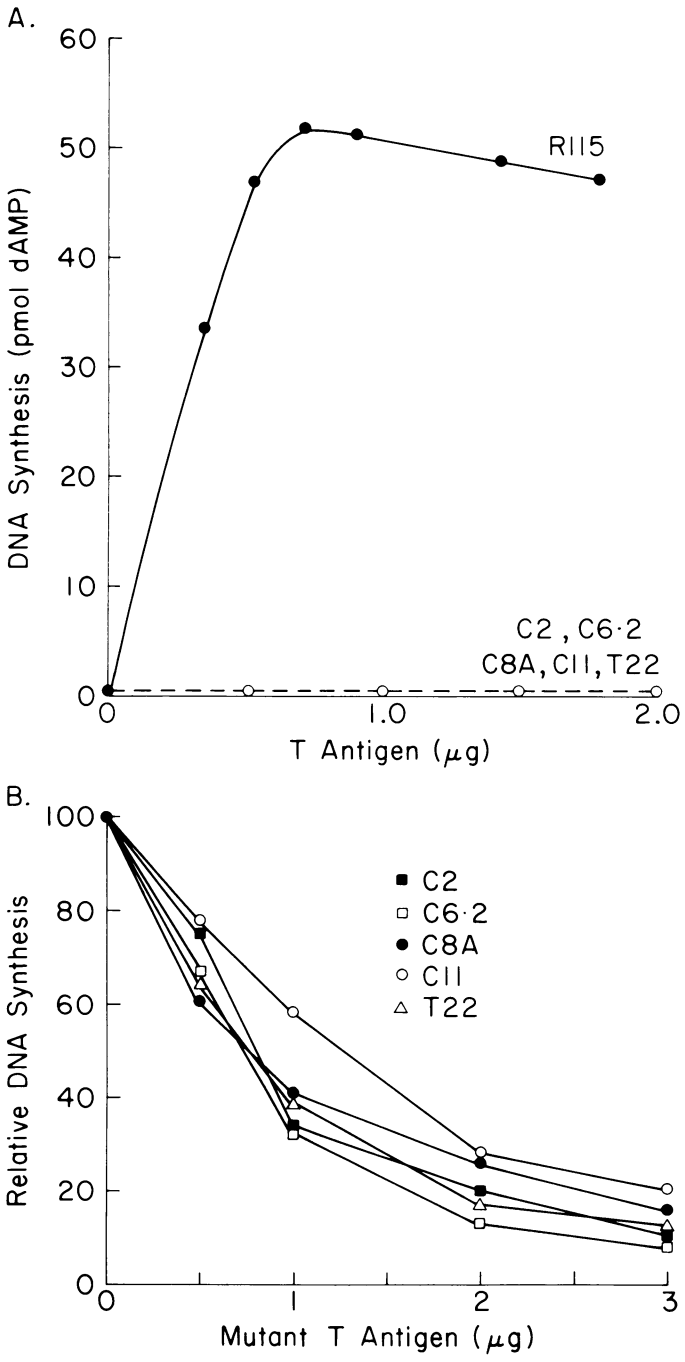

Fig. 5. (A) Stimulation of DNA synthesis with wild-type (R115) but not defective (C2, C6.2, C8A, C11 or T22) T antigens. Reaction mixtures contained various amounts of purified T antigens, 200 μg of 293 cell cytosol (S100 fraction) and 0.3 μg of pSV40 DNA as previously described (Stillman and Gluzman, 1985). DNA synthesis was for 1 h at 37°C and measured by incorporation of labeled [α-^{32}P]dAMP into acid-precipitable materials. **(B)** Inhibition of DNA replication by defective T antigens. Increasing amounts of defective T antigens (C2, C6.2, C8A, C11 and T22 as shown) were added to reaction mixtures containing 1 μg of wild-type (R115) T antigen, 200 μg of 293 cell cytosol (S100 fraction) and 0.3 μg of pSV40 DNA (Stillman and Gluzman, 1985) and then incubated for 1 h at 37°C. DNA synthesis was measured by the incorporation of [α-^{32}P]dAMP into acid-insoluble material. The amount of DNA synthesis in the reactions containing only wild-type T antigen (52–67 pmol dAMP in different experiments) was set at 100.

ly, it is likely that complex protein-protein interactions occur prior to the initiation of DNA replication and that some of these interactions do not require prior origin binding. The protein-protein interactions could occur between different T antigen molecules or between T antigen and cellular replication proteins. The mutation in the C8A T antigen may affect its ability to interact with other proteins in the initiation complex. Thus it is clear from these and other studies that the initiation of DNA replication will be a complex event that will require purification and identification of the cellular components before elucidation.

Materials and methods

Cells and viruses

Human 293 cells (Graham *et al.*, 1977) grown in both monolayer and suspension cultures have been described (Stillman and Gluzman, 1985). CMT3 cells are monkey CV-1 cells that contain integrated copies of the wild-type gene for SV40 T antigen under the control of the metallothionein promoter (Gerard and Gluzman, 1985). A recombinant adenovirus vector, Ad5SVR115, that overproduces the wild-type SV40 T antigen in infected 293 cells has been described (Stillman and Gluzman, 1985). A similar vector that overproduces wild-type T antigen, Ad5SVR112, has also been used in these studies. This virus lacks the adenovirus late RNA tri-partite leader sequences in T antigen mRNA, but is otherwise identical to Ad5SVR115. Recombinant adenoviruses that contain mutant T antigen genes have also been constructed in a similar manner to Ad5SVR115. These vectors overproduce the altered T antigens C2, C6.2, C8A, C11 and T22 (Gluzman and Ahrens, 1982; Manos and Gluzman, 1984, 1985).

Cell-free DNA synthesis

Wild-type and mutant T antigens were purified to apparent homogeneity by the procedure of Simanis and Lane (1985). DNA synthesis experiments were performed using cytosol extracts prepared from human 293 cells as described (Stillman and Gluzman, 1985). In some experiments, the 293 cytosol extract was adjusted to a final concentration of 0.1 M NaCl and centrifuged at 100 000 *g* for 1 h prior to storage at −70°C.

T antigen assays

ATPase activity of purified SV40 T antigens was measured in reaction mixtures containing 25 mM Hepes/KOH pH 7.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Nonidet P-40 and 100 μM [γ-^{32}P]ATP (200 c.p.m./pmol) for 10 min at 30°C. The amount of ATP hydrolyzed was measured by determining the increase of free ^{32}P phosphate using activated charcoal (norit) as described (Simanis and Lane, 1985). The DNA binding assay for T antigens was performed using minor modifications of the methods described previously (Myers and Tjian, 1980).

Construction of origin mutations

Deletion mutations were made from the early transcription side (left side in Figure 1) of the origin in plasmid pSV0+ which contains the *Hind*III-*Sph*I SV40 origin fragment cloned into pAT153 DNA (Li and Kelly, 1985). Deletions from the late transcription side (right side in Figure 1) of the origin were made in plasmid pS1, which contains the large *Hpa*II-*Bam*HI fragment of SV40 cloned into plasmid pXf3 (Maniatis *et al.*, 1982). Deletions were constructed as follows: (i) late side; plasmid pS1 was digested with *Nco*I which removes nucleotides 42 to 333 (SV40 numbering system), followed by *Bal*31 exonuclease digestion and the ends filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I. A *Bgl*II linker was inserted in place of the deleted sequences. (ii) Early side; plasmid pSV0+ was first digested with *Stu*I (nucleotide 5190), followed by *Bal*31 digestion and then the DNA was cleaved with *Hind*III. The ends of the DNA were filled in with the Klenow fragment of DNA polymerase I and the DNA self ligated. The plasmids were grown in *E. coli* DH 1 (Hanahan, 1983) and the deletion end points determined by DNA sequencing using the Maxam-Gilbert technique (Maniatis *et al.*, 1982).

Acknowledgements

We thank R.Roberts for comments on the manuscript and B.Faha, J.Brody and J.Cossetti for valuable technical assistance. This work was supported by a Cancer Center Grant to Cold Spring Harbor Laboratory (CA-13106) and grants to B.W.S. from the National Institutes of Health (AI 20460) and the Rita Allen Foundation. R.D.G. was supported by National Research Service Award (CA-07493) from the National Cancer Institute.

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Received on 15 July 1985; revised on 6 August 1985

Note added in proof

We have recently constructed recombinant plasmids containing combinations of deletion end-points shown in Figure 1. One such plasmid, pRG53, contains SV40 sequences from nucleotide 5209-30 inclusive (i.e., 65 bp) and this plasmid DNA replicates with similar efficiency to pSV0*dl*3 and pS1*dl*5 in the cell-free system.