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Differential affinities of simian virus 40 large tumor antigen for DNA

(calf thymus DNA/simian virus 40 multiorigin variant DNA/immunoprecipitation/polyacrylamide gel electrophoresis/new and aged large tumor antigens)

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ABSTRACT **The binding of simian virus 40 (SV40) large tumor antigen (T antigen) to DNA was analyzed by using the salt-sensitive affinities of the protein for various DNAs immobilized on cellulose. At least two types of interactions could be distinguished that differed in their stability. Higher salt concentrations were required to elute T antigen from SV40 DNA than from calf thymus DNA; and even greater salt concentrations were required for the elution of T antigen from multiorigin SV40 DNA compared to wild-type SV40 DNA. This would indicate that T antigen can bind weakly or strongly to DNA, depending on the DNA sequence. It was also found that a greater proportion of rapidly labeled or newly synthesized T antigen binds more efficiently and tightly to multiorigin SV40 DNA than to long-labeled or older forms of T antigen. This approach can be utilized not only to distinguish between different forms of T antigens which vary in their affinities for DNA but also for rapidly obtaining highly enriched T antigen preparations.**

Simian virus 40 (SV40) large tumor antigen (T antigen), or the viral A gene product, has been implicated by genetic studies in the initiation of viral DNA replication (1) and the control of viral RNA transcription (2, 3) as well as in virus-induced cell carcinogenesis (4–8). Because viral DNA replication (9, 10) and RNA transcription (11) both initiate at approximately the same region of the genome, known as the origin (0.67 map unit on the genome), a specific interaction of the T antigen with these sequences is implied. Biochemical studies with partially purified T antigen (12, 13) or a highly purified closely related protein (14) have demonstrated a unique binding of this protein to DNA sequences at the origin. Because T antigen has also been localized in numerous studies in the nucleus of infected and transformed cells, as well as in isolated chromatin (15), its interaction with cellular and integrated viral DNA is also likely. Analysis of the variable affinities of T antigen for cellular and viral DNA is a way to begin to assess the biochemical role of T antigen in cell transformation. Carroll *et al.* (16) and Rundell *et al.* (17) have shown that T antigen binds to, and can be eluted from, double-stranded calf thymus DNA bound to cellulose. We have extended this approach to study the differential affinities of T antigen for various DNAs linked to cellulose.

MATERIALS AND METHODS

Cells and Viruses. BSC-1 and Vero monkey lines were grown in Dulbecco's modified Eagle's medium (GIBCO) with 10% calf-serum. The standard wild-type SV40 used was a plaque-purified stock of strain 777 grown as described (18). The multiorigin F161 variant of SV40 was prepared and propagated as described by Oren *et al.* (19).

Extractions of T Antigen from Infected and Transformed Cells. Monolayer cultures labeled with [^{35}S]methionine (40 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) were extracted as described (20) except that the extraction buffer contained 20 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and 50 μg of phenylmethylsulfonyl fluoride per ml, and 2-mercaptoethanol was omitted.

DNA-Cellulose Chromatography. Viral DNA was extracted from cells infected with either wild-type SV40 or the F161 variant according to published procedures (19). The viral DNA used for binding to cellulose was purified by equilibrium density gradient centrifugation. Wild-type SV40 DNA and F161 DNA were bound to cellulose by the methods of Alberts (21). Calf thymus DNA cellulose was obtained from P-L Biochemicals. Samples to be chromatographed were brought to pH 6.0 or 7.0, as specified in the text by addition of 5-μl portions of 1.0 M acetic acid, and loaded onto a 1-ml DNA-cellulose column (approximately 0.2–0.5 mg of DNA per ml of cellulose), equilibrated with 5 mM potassium phosphate/0.1 M NaCl/10% (vol/vol) glycerol/0.5% Nonidet P-40, pH 6.0, or 5 mM sodium phosphate/0.1 M NaCl/10% glycerol/0.5% Nonidet P-40, pH 7.0. All buffers used in this study contained 40 μg of phenylmethylsulfonyl fluoride per ml. After extensive washing of the column with equilibration buffer, further elution of proteins was performed by applying a series of buffers containing increasing concentrations of NaCl as specified in each experiment. The effluents of the load and wash fractions, as well as of the following steps, were collected in 0.25-ml fractions, and the three peak fractions of each step were pooled and further analyzed.

Immunoprecipitation. Aliquots of each elution step (250 μl, unless otherwise specified) were brought to pH 7.0 and 0.15 M NaCl, mixed with 5–20 μl of hamster anti-T antigen antiserum (kindly provided by D. Gidoni), and incubated at 23°C for 1 hr. This antiserum, although strongly reactive with the M_r 90,000 T antigen, had only a weak reaction with the M_r 17,000 polypeptide. Formaldehyde-inactivated *Staphylococcus aureus*, prepared according to Kessler (22), was next added (5 μl of a 10% suspension per 1 μl of antiserum), and incubation at 23°C was continued for an additional 20 min, followed by four 1.0-ml washes with phosphate-buffered saline. The final pellet was heated at 95°C for 5 min in electrophoresis sample buffer (20). After recentrifugation at 8000 × *g*, the supernatant was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis and Autoradiography. Samples were analyzed by electrophoresis through 10% polyacrylamide slab gels at 150

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

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V. Gels were fixed and stained with Coomassie brilliant blue, impregnated with 2,5-diphenyloxazole (23), dried, and exposed to preflashed AGFA Curix x-ray film at −70°C.

RESULTS

Salt-Sensitive Affinities of T Antigen to Different DNAs. Binding of the SV40 T antigen (M_r 90,000) to various DNAs was studied with the aid of DNA-cellulose columns. The DNA was derived from calf thymus, wild-type plaque-purified SV40, or F161 [a population of SV40 consisting predominantly of a host-substituted variant of SV40 (19) in which the origin-containing segment is repeated four times in the DNA molecule]. Most preparations of F161 DNA contained approximately 70% variant and 30% wild-type SV40. [^{35}S]Methionine-labeled extracts of BSC-1 cells infected with wild-type SV40 were brought to pH 6.0 and passed through each of the three columns. The bound proteins were eluted with increasing salt concentrations (0.1 to 1.0 M NaCl, all in pH 6.0 buffer). SV40 T antigen eluted at different concentrations was immunoprecipitated with anti-T antigen antiserum. The immunoprecipitates were then subjected to polyacrylamide gel electrophoresis and autoradiography.

The T antigen was identified in this and subsequent experiments by its specific immunoreactivity with anti-SV40 T antigen antiserum and its comigration on gels with authentic M_r 90,000 T antigen synthesized in cell-free systems in response to SV40 E strand mRNA. Many of the smaller polypeptides in these autoradiograms probably were cleavage products of T antigen generated during the extraction or DNA binding procedures (24–26). Although the antiserum used for this study had low activity against the small (M_r 17,000) tumor antigen, it was established in subsequent experiments (unpublished data), that the small tumor antigen does not bind to any DNA by this procedure, as previously shown (27).

Although the T antigen was present in all fractions of the eluates, the amount that was eluted with 1.0 M NaCl varied according to the type of DNA immobilized to the column: it was very small when calf thymus DNA was used but increased with wild-type SV40 and was highest with multiorigin F161 DNA (Fig. 1). These findings suggested a correlation between the affinity of binding and the relative abundance of SV40 origins of replication in the DNA used. This difference in affinities could be made more pronounced if more stringent criteria were imposed on the initial binding stage. Thus, when both column and extract were adjusted to pH 6.0 and 0.75 M NaCl, no binding to calf thymus DNA-cellulose was observed at all whereas there was still considerable binding to SV40 DNA-cellulose (Fig. 2).

To emphasize the difference between the loose and tight bindings, the pH of the initial binding conditions was raised to 7.0 in order to decrease the initial amount of nonspecific interaction with DNA (Fig. 3). T antigen appeared to have mainly weak affinity for calf thymus DNA and multiple affinities for SV40 DNA that ranged from weak (0.35 M) to strong (1.0 M). Generally, when the binding was performed at pH 7.0, a higher proportion of the T antigen did not bind at all to DNA, presumably because of molecules with little or no affinity for DNA. When calf thymus, SV40, and F161 DNAs now were compared, there was a dramatic correlation between the type of DNA bound to the column and the proportion of T antigen eluted with high-salt buffer (Fig. 4, *Upper*). Immunoprecipitation of the eluted fractions from each column with anti-T antigen antiserum showed again that almost all the T antigen was eluted from calf thymus DNA at 0.35 M NaCl but, in contrast, with F161 DNA, the great majority of T antigen that bound to the column was eluted only by 1.0 M NaCl.

When aliquots of all fractions bound to and eluted from the different DNAs were analyzed directly without immunoprecipitation (Fig. 4 *Lower*), the majority of [^{35}S]methionine-labeled DNA binding proteins in the cell were eluted by 0.35 M NaCl. A few had even greater affinity (0.5 M) especially for calf thymus DNA. However, with the multiorigin F161 DNA, the 1.0 M NaCl fraction contained only one prominent band of labeled protein. Although direct confirmation of this polypeptide as T antigen awaits tryptic peptide mapping, it is highly likely that this is the viral early gene product because: (*i*) it has electrophoretic mobility identical to that of the M_r 90,000 T antigen; (*ii*) it is specifically immunoreactive with SV40 anti-T antigen antiserum; (*iii*) it is not present when the binding and elution steps are performed with extracts of uninfected cells. This system, therefore, can be used both as a sensitive assay for testing the efficiency of binding of any given T antigen to the SV40 origin of replication and a rapid method to obtain active T antigen in a significantly enriched form.

Differential Affinities of New and Old T Antigens for DNA. It is possible that newly synthesized T antigen differs from the older accumulated T antigen in its DNA affinities. This possibility is suggested from experiments in which inter-

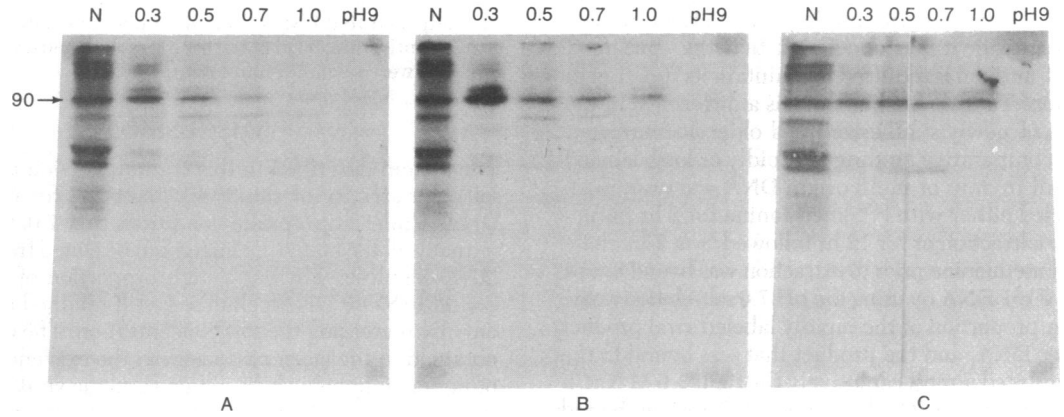

FIG. 1. Elution of SV40 T antigen from DNA-cellulose columns at pH 6.0. BSC-1 cells (7×10^6) infected with SV40 were labeled for 46–48 hr with [^{35}S]methionine (40 μCi/ml) prior to extraction with 0.6 ml of Nonidet P-40 buffer (20). A 0.5-ml portion of extract containing 0.15 M NaCl was adjusted to pH 6.0 and passed through calf thymus (*A*), SV40 (*B*), or F161 (*C*) DNA-cellulose columns that were preequilibrated with pH 6.0 buffer. Proteins were eluted with successive increases in NaCl concentration (0.3–1.0) as shown. Volumes taken for immunoprecipitation with anti-T antigen antiserum were 50 μl of fraction N (nonbound) and 250 μl of all the others. All fractions were adjusted to 0.15 M NaCl before immunoprecipitation by dilution with the appropriate amount of pH 6.0 buffer from which NaCl was omitted.

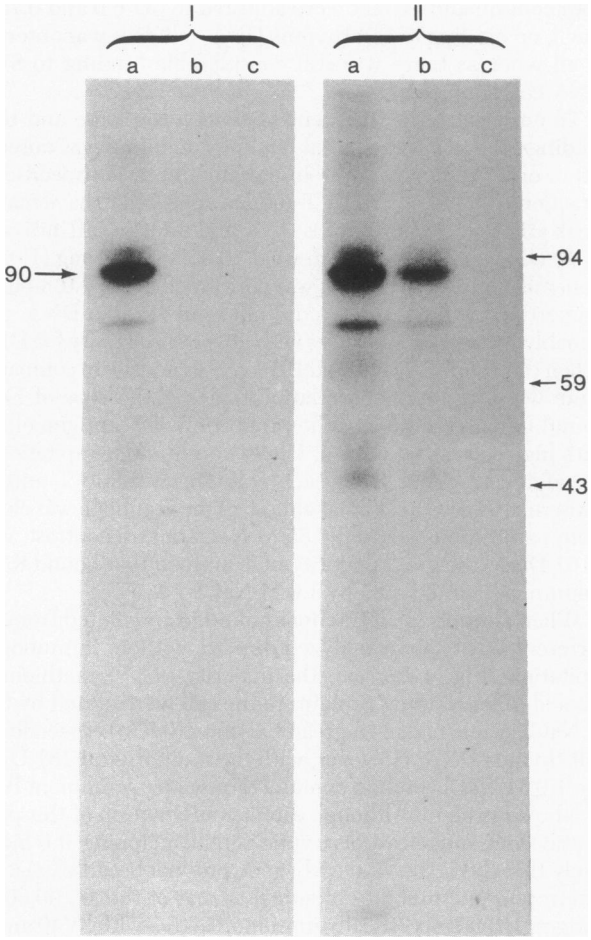

FIG. 2. Binding of T antigen at pH 6.0, in high salt to calf thymus DNA (I) and SV40 DNA (II). Extracts were prepared as in Fig. 1 and adjusted to pH 6.0 in buffer containing 0.75 M NaCl prior to passage through the columns which were equilibrated with the same buffer. For further details see legend to Fig. 1. Autoradiograms are anti-T antigen immunoprecipitates of material eluted at pH 6.0 in 0.75 M NaCl (lane a), at pH 6.0 in 1.0 M NaCl (lane b), or at pH 9.0 in 1.0 M NaCl (lane c).

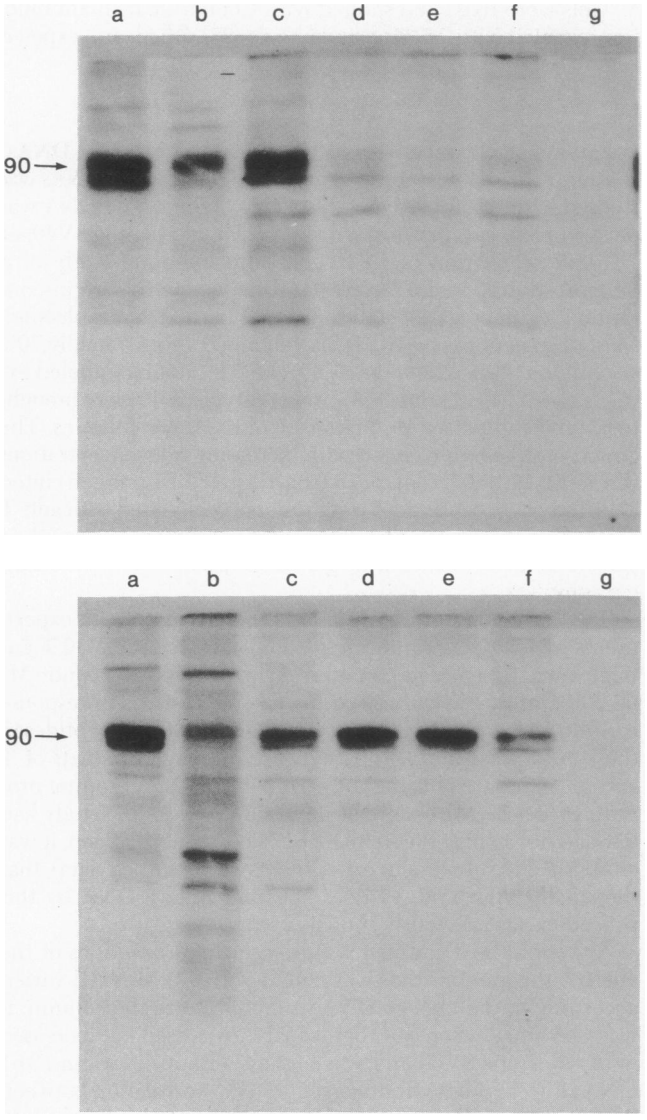

FIG. 3. T antigen binding to and elution from calf thymus and SV40 DNAs at pH 7.0. Extracts prepared as in Fig. 1 were adjusted to pH 7.0 and bound to calf thymus (*Upper*) and SV40 (*Lower*) DNA-cellulose columns. Bound material was eluted with pH 7.0 buffer containing various concentrations of NaCl. Lanes: a, 0.1 M NaCl; b, 0.2 M; c, 0.35 M; d, 0.5 M; e, 0.7 M; f, 1.0 M; g, 1.0 M at pH 8.5. Eluates were adjusted to 0.1 M NaCl and immunoprecipitated with anti-T antigen antiserum; 50 μl of the 0.1 M fraction and 250 μl of the other fractions were taken for immunoprecipitation.

feron, applied after infection, produced a marked inhibition of viral DNA replication (28). Because there was a concomitant reduction in the newly synthesized, but not the previously accumulated, viral T antigen and no detectable effect on host protein synthesis (29), it was concluded that the continued production of T antigen is required to maintain its function in DNA replication. To test whether there is a difference in the DNA affinities of newly synthesized and older accumulated T antigen, the comparative binding of rapidly or long-labeled T antigen to calf thymus or multi origin DNA was examined. T antigen labeled either with [^{35}S]methionine for 2 hr immediately prior to extraction or for 12 hr followed by a 2-hr chase with unlabeled methionine prior to extraction was bound to calf thymus and to F161 DNA by using the pH 7.0 salt-elution assay. A much higher proportion of the rapidly labeled viral product bound to either DNA, and the product that was bound to the F161 DNA exhibited almost exclusively the tight 1.0 M NaCl-sensitive binding (Fig. 5). The long-labeled T antigen bound less efficiently to either DNA, and a much higher proportion of it appeared to have a weaker (0.4 M NaCl-sensitive) affinity for the multiorigin DNA. Thus, the notion that newly synthesized T antigen is required for DNA replication function is born out by this biochemical assay.

DISCUSSION

The system described in this communication is based on the selective affinity of the SV40 T antigen for its homologous DNA. Under appropriate conditions (pH 7.0, 0.35 M NaCl), virtually all the bound T antigen can be eluted from calf thymus DNA-cellulose, whereas a high proportion of the T antigen bound to SV40 DNA-cellulose is still retained and is released only by increasing the salt concentration. The elution pattern obtained in the latter case suggests the existence of two types of interactions between T antigen and the viral DNA. The first type is similar in its stability to that exhibited with heterologous DNA, whereas the other is much more stable and appears to be unique to viral origin-containing DNA sequences. When DNA of F161, a viral variant possessing four replication origins per molecule, was substituted for wild-type SV40 DNA on the column, the interaction pattern was almost exclusively of the

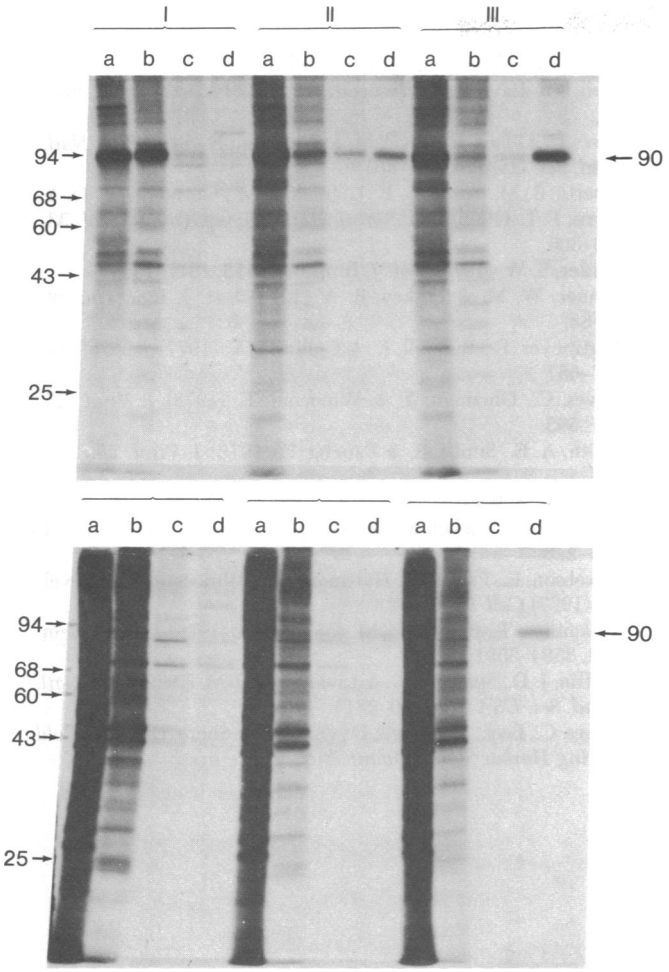

FIG. 4. T antigen binding to and elution from calf thymus (I), SV40 (II), and F161 (III) DNA-cellulose at pH 7.0. Extracts prepared as in Fig. 1 were adjusted to pH 7.0 and bound to the columns. Nonbound material was eluted from each at 0.1 M NaCl (lane a), followed by stepwise elution of bound proteins at 0.35, 0.5, and 1.0 M NaCl (lanes b, c, and d). (*Upper*) Anti-T antigen immunoprecipitates of 50 μl of 0.1 M NaCl eluate and 250 μl of the other eluates after adjustment to 0.1 M NaCl. (*Lower*) Autoradiogram of the corresponding material from the eluted pools, prior to immunoprecipitation, directly applied (4 μl in a, 40 μl in b, c, and d) to the polyacrylamide gel.

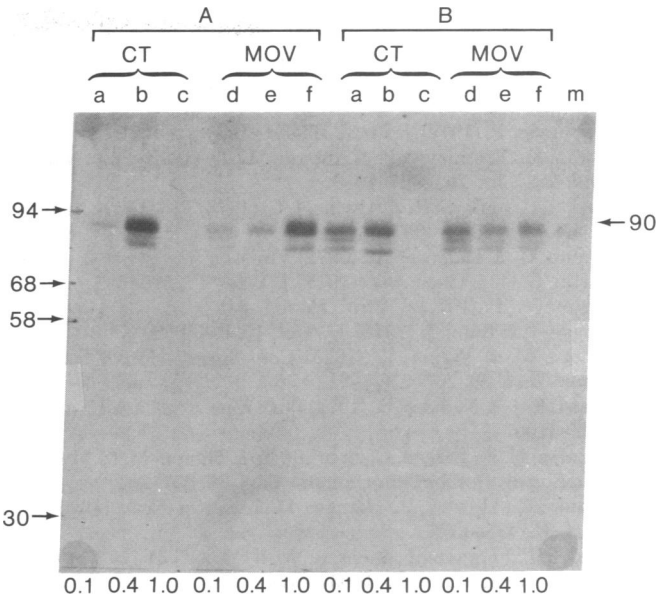

FIG. 5. Comparative affinities of newly synthesized and older T antigen for calf thymus and F161 DNA-cellulose. To obtain newly synthesized T antigen, cells were labeled with [^{35}S]methionine at 46–48 hr after infection and then extracted in Nonidet P-40 buffer as for all other experiments. To obtain older T antigen, cells were labeled with [^{35}S]methionine at 36–46 hr after infection, followed by replacement of label-containing medium with unlabeled methionine-containing medium at 46–48 hr and then extracted as above. Extracts were bound to calf thymus (CT) or F161 multiorigin variant (MOV) DNA-cellulose columns in 0.1 M NaCl at pH 7.0 and eluted with 0.4 and 1.0 M NaCl. (*A*) Affinities of rapidly labeled T antigen. (*B*) Affinities of long-labeled T antigen. Lanes: a and d, 0.1 M NaCl; b and e, 0.4 M NaCl; c and f, 1.0 M NaCl; m, M_r 90,000 marker T antigen.

latter, stable type. This was true only when the conditions used for binding were close to physiological (pH 7.0, 0.15 M NaCl). When the experiment was performed at pH 6.0, much more of the T antigen bound to the F161 DNA-cellulose was elutable under the same conditions that removed this protein from calf thymus DNA-cellulose. These observations suggest that the tight interaction involves the viral origin of replication (or sequences associated with the signal that specifies the start of viral replication), which is in agreement with the data obtained by using DNA binding in solution (12, 14). However, it should be kept in mind that we do not have direct proof for the role of the variant's origins in the tight binding because the F161 molecules also contain considerable additional stretches of DNA, of both viral and cellular origin (19, 30).

The presence of a subpopulation of T antigen molecules that do not bind to DNA, noted herein and by others (31), may reflect the fact that T antigen can exist in a nonbinding form, due either to modifications or the formation of more complex structures. Such forms recently have been found to sediment more rapidly than the actively binding T antigen in sucrose gradient ultracentrifugation (32). Chromatography on multiorigin DNA-cellulose may thus serve as a rapid and simple procedure for the isolation of the DNA binding, and therefore presumably active, forms of T antigen.

The existence of conditions that differentiate between specific and nonspecific binding of the SV40 T antigen to DNA may serve important analytical purposes. In particular, it will be valuable in determining the factors that control the specific interaction between this protein and the viral origin of replication. The method uses crude cell extracts, thus abolishing the need for extensive purification steps and minimizing the possible effects of artifacts created during this purification. (It is realized, however, that many artifacts can also arise in crude extracts.) In addition, the system can use small amounts of proteins, provided that they are sufficiently labeled. By the choice of appropriate binding and elution conditions, subtle differences in the binding of various T antigen populations to the viral DNA may be examined. We have observed a marked decrease in the strength of this binding as the T antigen molecule ages. However, it is noteworthy that the effect is also at the nonspecific binding stage—i.e., the binding to calf thymus DNA is also affected. The mechanism responsible for this "aging" of the T antigen is as yet unknown but may involve the formation of rapidly sedimenting T antigen complexes.

The temporal decrease in affinity of T antigen for the viral DNA may play an important biological role. This protein has a regulatory function at the levels of DNA replication and viral transcription, both most probably being effected through its binding to the viral DNA, at or around the origin of replication. For such a mechanism to be efficient, the functional half-life of the regulatory protein should be relatively short. It is suggested that this half-life is controlled by modulating the ability of the T antigen to bind to DNA.

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