Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1605–1609, April 1975

T Antigen Binds to Simian Virus 40 DNA at the Origin of Replication

(ferritin-conjugated antibodies/electron microscopy/DNA mapping/simian virus 40 antigen/ *Escherichia coli* DNA unwinding protein)

STEVEN I. REED, JILL FERGUSON, RONALD W. DAVIS, AND GEORGE R. STARK

Department of Biochemistry, Stanford University School of Medicine, Stanford, Calif. 94305

Communicated by Paul Berg, February 10, 1975

ABSTRACT **A technique employing ferritin-conjugated antibody has been developed to visualize specific protein–DNA complexes in the electron microscope and has been used to demonstrate the preferential binding of simian virus 40 (SV40) T antigen at or near the origin of replication of SV40 DNA, 0.67 fractional length clockwise from the *Eco*RI restriction endonuclease cleavage site. Purified covalently closed supercoiled circles of SV40 DNA were treated with partially purified T antigen and the complex was stabilized by crosslinking with glutaraldehyde. Hamster anti-T antigen γ-globulin, ferritin-labeled goat anti-hamster γ-globulin, and glutaraldehyde were then added sequentially. The location of the bound ferritin cores was measured with respect to the *Eco*RI cleavage site and the orientation of the cores relative to the ends of the DNA was determined with respect to the locations of *Escherichia coli* DNA unwinding protein, which binds to covalently closed supercoiled SV40 DNA at either of two preferred sites, 0.46 or 0.90 fractional length clockwise from the *Eco*RI cleavage site.**

T antigen, a protein found in the nucleus of all simian virus 40 (SV40)-infected and SV40-transformed cells, has eluded definitive characterization since its discovery. However, in recent years several important advances have been made. Apparent values of molecular weight determined by electrophoresis of highly purified preparations in sodium dodecyl sulfate/polyacrylamide gels range from about 70,000 to 100,000 (1, 2), and sedimentation values under nondenaturing conditions indicate that the molecular weight of the native antigen is much larger (3, 4). Although there is little direct information about the role(s) of T antigen in the life cycle of SV40, conjecture has been stimulated by two recent observations. First, SV40 mutants in the A complementation group are defective in the initiation of viral DNA synthesis (5) and in the induction of T antigen (6). Second, T antigen binds strongly to DNA-cellulose at low pH and low ionic strength (4). Taken together, these findings suggest that T antigen may be the product of the SV40 A gene and that it may be involved in initiating viral DNA synthesis. We now report further evidence consistent with such a role: T antigen binds preferentially to SV40 DNA at or near the origin of replication.

MATERIALS AND METHODS

Cell Lines. CV-1 and MA-134, established lines of African green monkey kidney cells, were obtained from Dr. Paul Berg's laboratory and C13/SV28, a cloned SV40-transformed baby hamster kidney line (7), was a gift of Dr. Chris Wiblin. CV-1 and MA-134 cells were grown on Lux plastic plates in Eagle's Medium as modified by Dulbecco (Gibco) with 10% fetal bovine serum (Microbiological Assoc.) in a CO_2 incubator. C13/SV28 cells were grown in suspension in Dulbecco's modified Eagle's medium with 10% calf serum (Microbiological Assoc.).

SV40 Virus and DNA. Strain WT-830*, a derivative of SV-S (8), was obtained from Dr. Paul Berg's laboratory and a non-defective virus stock was prepared as described by Pagano *et al.* (9). For preparing DNA, MA-134 cells were infected at 0.01 plaque-forming unit per cell. After 11 days, the cells were lysed by the method of Hirt (10) and DNA in the supernatant was extracted with chloroform: isoamyl alcohol (24:1). After centrifugation to equilibrium in CsCl/ethidium bromide gradients (11), the SV40(I) DNA (covalently closed supercoiled circles) was removed with a syringe. The ethidium bromide was removed by extraction with butanol, and the DNA was analyzed by electron microscopy.

Enzymes and Proteins. *Eco*RI restriction endonuclease was purified as described by Yoshimori (12). *Hemophilus parainfluenzae* restriction endonuclease II (*Hpa*II) was a gift of Drs. John Morrow and Janet Mertz, and was prepared by a modification of the second method described by Sharp *et al.* (13). *Escherichia coli* DNA unwinding protein and horse spleen ferritin were generous gifts of Dr. Joel Weiner (14) and Dr. Norman Davidson, respectively.

SV40 T Antigen. T antigen was partially purified from SV40-infected CV-1 cells or from C13/SV28 cells as described in Fig. 1. "Mock" T antigen used in control experiments was prepared from confluent uninfected CV-1 cells by the same procedure. Equivalent fractions were chosen from comparable separations on DEAE-cellulose (see Fig. 1).

γ-Globulins. Anti-T sera have usually been obtained from tumor-bearing hamsters. To obtain antisera with higher than usual activity, we injected partially purified T antigen from SV40-transformed hamster cells directly into hamsters. A large dose of antigen can be injected in this way, and normal hamster components present in the antigen preparation would not be expected to be highly immunogenic. Male golden hamsters were injected with 0.7 ml of T antigen from C13/SV28 cells (Fig. 1C) without adjuvant every other day for 2 weeks. After a 4-week interval, the hamsters were boosted

Abbreviations: SV40, simian virus 40; SV40(I) DNA, covalently closed supercoiled circles of SV40 DNA.

* M. Herzberg, J. E. Mertz, P. Berg, J. R. Cameron, and R. W. Davis, in preparation.

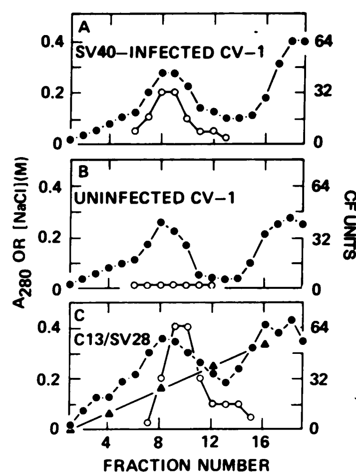

FIG. 1. DEAE-cellulose chromatography of partially purified extracts, (A) from SV40-infected CV-1 cells, (B) from uninfected CV-1 cells, (C) from C13/SV28 (SV40-transformed) hamster cells. Confluent layers of uninfected CV-1 cells, or of CV-1 cells infected with SV40 virus at a multiplicity of 25 plaque-forming units per cell and harvested 36 hr after infection, were scraped free with a rubber policeman. C13/SV28 cells grown in spinner culture were harvested by sedimentation at low speed. In a typical preparation, cells giving a packed volume of 3 ml were washed once by sedimentation through Tris–saline (20 mM Tris·HCl, pH 7.6, 1 mM Na_2HPO_4, 8 mM KCl, 0.137 M NaCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$). Nuclei were prepared by diluting the packed cells 10-fold in a solution containing 10 mM triethanolamine·HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.5% NP40 (Shell), a mild, nonionic detergent. The cells at 4° were agitated intermittently with a vortex mixer. This lysis procedure with $CaCl_2$ maintains most of the T antigen in the nucleus (ref. 1; Reed and Stark, unpublished observations). When lysis was complete, as judged by microscopic examination, the nuclei were washed by sedimentation through 2 M sucrose at 50,000 rpm for 30 min in a Beckman Type 50 rotor. The pelleted nuclei were resuspended in 15 ml of the lysis buffer, sonicated in 4 half-minute pulses at 0° and sedimented at 10,000 rpm for 10 min in an International 856 rotor. The pellet was resuspended in 15 ml of a solution containing 10 mM triethanolamine·HCl, pH 7.4, 0.3 M NaCl, and 5 mM EDTA, homogenized in a Dounce apparatus, and sonicated. After centrifugation at 10,000 rpm for 10 min in an International 856 rotor, the supernatant solution was brought to 33% saturation with ammonium sulfate and, after incubation at 0° for 1 hr, the precipitate was collected by sedimentation and resuspended at 0° in 2 ml of 50 mM Tris·HCl buffer, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol. The suspension was dialyzed extensively against the same solution, and chromatographed on a 1 × 10 cm column of DEAE-cellulose (Whatman DE52) with a 20 ml linear gradient from 0 to 0.4 M NaCl. T antigen was assayed by micro complement fixation (7) with minor modifications. Instead of incubating for 12 hr at 0°, we substituted a 1 hr incubation at 37° to save time and have found that this gives reproducible results. The titer is defined as the dilution of a 25 μl aliquot of material that protects one-half of the added erythrocytes from complement-mediated lysis. For T antigen–SV40 DNA binding experiments, one of the peak fractions from the infected CV-1 profile [fraction 8, (A)] and the equivalent fraction from the uninfected CV-1 profile [fraction 8, (B)] were brought to 25% in glycerol and 50 mM in potassium phosphate buffer, pH 7.0. Peak fractions in the C13/SV profile [fractions 8–11, (C)] were pooled and brought to 25% in glycerol. All samples were stored in liquid nitrogen until needed. (●) A_{280} readings for the eluted fractions; (○) complement fixing (CF) units; (▲) salt concentration determined by conductivity.

with similar injections for another 2 weeks. Ten days after the last injection the animals were bled and sera were prepared. Antibodies were titered by micro complement fixation (15) with extracts of SV40-infected CV-1 cells and uninfected CV-1 cells. Sera with antibody titers of greater than 500 against T antigen and no detectable activity against the uninfected cell extracts were pooled and adsorbed with an insolubilized extract of uninfected CV-1 cells (16) to rule out the possibility of contamination by low levels of nonspecific antibodies which might react with some component other than T antigen. The γ-globulin fraction was then purified (17), yielding material with a complement fixation titer of 16. Most of the reduction in titer is due to dilution, although about 50% of the total amount of antibody is lost during this procedure. Goat antiserum to hamster globulin was purchased from Antibodies, Inc., Davis, Calif., and the γ-globulin fraction was purified (17). Horse-spleen ferritin was activated with glutaraldehyde and linked to this γ-globulin (18).

Binding of T Antigen and Antibodies to SV40(I) DNA. One hundred twenty microliters of SV40(I) DNA (15 μg/ml) in 10 mM potassium phosphate buffer, pH 7.0, 0.1 M KCl, 0.1 mM EDTA, 10 mM $MgCl_2$ were incubated with 30 μl of T antigen from SV40-infected CV-1 cells (Fig. 1A) or 30 μl of mock T antigen from uninfected CV-1 cells (Fig. 1B) for 5 min at 37°; in some experiments neither was added. The mixture was chilled to 0° and 36 μl of 0.4% glutaraldehyde in 10 mM potassium phosphate buffer, pH 7.0, were added (19). After incubation at 37° for 10 min, 60 μl of anti-T γ-globulin were added and, after 10 min, 60 μl of ferritin-conjugated goat anti-hamster γ-globulin were added. After an additional 10 min at 37°, the mixture was chilled to 0°, 42 μl of 0.4% glutaraldehyde were added, and the mixture was brought to 37° for a final 20 min. Five hundred fifty microliters of 0.1% glutaraldehyde in 10 mM potassium phosphate buffer, pH 7.0, were added and the mixture was heated to 60° for 5 min to destroy an exonuclease activity that partially digested the linear molecules produced by the subsequent *Eco*RI cleavage. Finally, the sample was dialyzed into 10 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA.

Reactions with DNA Unwinding Protein. Twenty microliters of SV40(I) DNA (2 μg/ml), with or without bound ferritin cores, in 10 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, were mixed with 4 μl of purified *E. coli* DNA unwinding protein in 50 mM imidazolium chloride buffer, pH 6.8, 0.3 M KCl at 37° for 5 min. The mixture was chilled to 0° and 2.5 μl of 1% glutaraldehyde in 10 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, were added, and fixation was carried out for 10 min at 37°.

Cleavage Reactions with Restriction Endonucleases. *Eco*RI endonuclease reactions were carried out in 0.1 M Tris·HCl buffer, pH 7.5, 7 mM $MgCl_2$ at 37° for 10 min. *Hpa*II endonuclease reactions were carried out in 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA at 37° for 5 hr.

Electron Microscopy. Samples were mounted for electron microscopy using 40% formamide (20). Grids were rotary shadowed lightly with platinum, then examined and photographed with a Philips EM 300 electron microscope. Measurements were made with a Hewlett-Packard 9864A Digitizer and 9810A Calculator with a fully smoothed length calculation

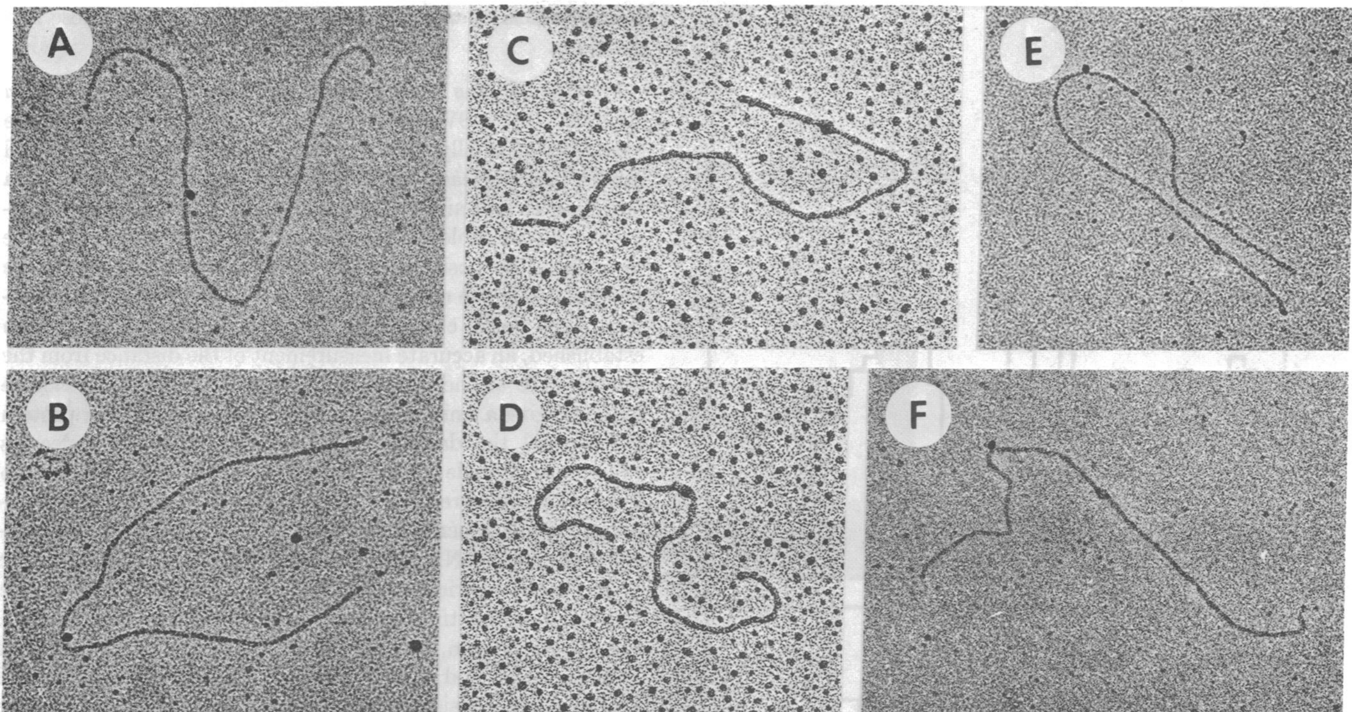

FIG. 2. Electron micrographs of SV40 DNA prepared in various ways. (A, B) SV40(I) DNA incubated with T antigen, anti-T γ-globulin, and ferritin-conjugated anti-hamster γ-globulin and cleaved with *Eco*RI endonuclease. (C, D) SV40(I) DNA treated with *E. coli* DNA unwinding protein and cleaved with *Eco*RI endonuclease. (E, F) Same as (A, B) except that the sample was treated with *E. coli* DNA unwinding protein before cleavage with *Eco*RI endonuclease. The DNA was mounted for electron microscopy as in *Materials and Methods*. The photographs in A, B, E, and F were chosen randomly and are not intended to show molecules with a ferritin core at the preferential site. (×50,000).

program giving an accuracy of 0.5% and a greater degree of reproducibility on sample figures of known length.

RESULTS

Ferritin Cores Can Be Bound to SV40 DNA. After serial incubation of SV40(I) DNA with T antigen, anti-T γ-globulin, and ferritin-conjugated anti-hamster λ-globulin, electron dense ferritin cores can be seen bound to DNA as well as in the background field. These structures are not seen when ferritin-conjugated γ-globulin is omitted. SV40 linear DNA molecules produced by cleavage with the *Eco*RI endonuclease are shown with ferritin cores bound in Fig. 2A and B. In order to map the positions of these bound cores, a marker for orientation must be added to the DNA.

E. coli DNA Unwinding Protein Provides an Orientation Marker Relative to the EcoRI Endonuclease Cleavage Site. When purified *E. coli* DNA unwinding protein is incubated with SV40(I) DNA and fixed with glutaraldehyde, regions of the DNA duplex containing 50 to 100 base pairs appear to be melted irreversibly in the electron microscope. These regions are smaller but similar in appearance to the regions melted by the gene 32 protein of phage T4 (21). Electron micrographs of such molecules after cleavage with the *Eco*RI endonuclease are shown in Fig. 2C and D. The melted regions (melts) are distributed along the DNA relative to the *Eco*RI endonuclease cleavage site as shown in Fig. 3A. The majority are present at either of two positions relative to the cleavage site. With the *Hpa*II restriction endonuclease, which cleaves SV40 DNA once at 0.73 SV40 fractional length clockwise from the *Eco*RI cleavage site (13), the melts are distributed as shown in Fig. 3B. Together these two distributions give assignment for the two preferred sites for the unwinding protein at 0.46 and 0.90 fractional length relative to the *Eco*RI endonuclease cleavage site, with an error of about ±0.02. Thus, any point on a linear SV40 DNA molecule cleaved by *Eco*RI endonuclease can be oriented unambigu-

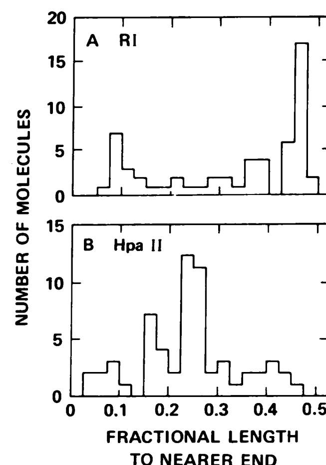

FIG. 3. Restriction endonuclease cleavage of SV40(I) DNA–*E. coli* DNA unwinding protein complexes. SV40(I) DNA was incubated with *E. coli* DNA unwinding protein and cleaved with either *Eco*RI endonuclease or *Hpa*II endonuclease. The distance from the unwinding protein melt to the nearer of the two ends is presented as a fraction of the total length of the complex. (A) Data from 57 complexes cleaved with *Eco*RI; (B) data from 60 complexes cleaved with *Hpa*II.

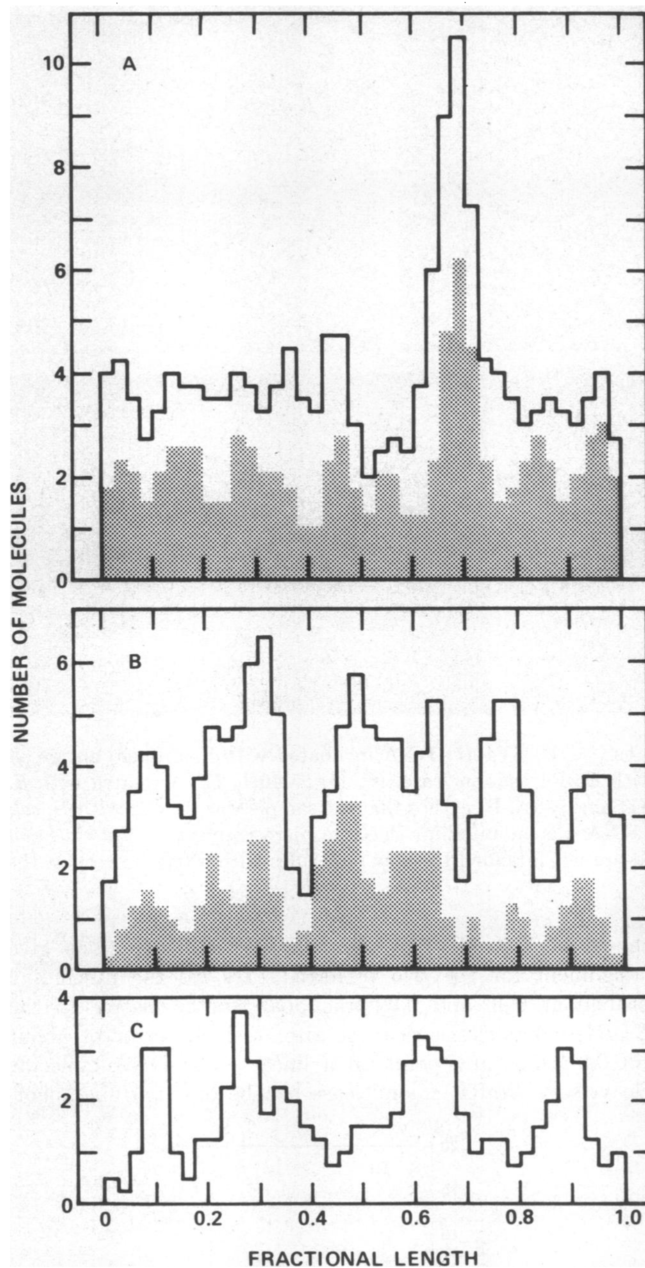

FIG. 4. Positions of ferritin cores found in association with SV40 DNA. Samples were prepared after incubating SV40(I) DNA with (A) SV40 T antigen; (B) mock T antigen; (C) no addition. Molecules with both a ferritin core and an unwinding protein melt were subjected to a preliminary screening. If the distance from the unwinding protein melt to the nearer end was between 0.075 and 0.15 SV40 fractional length, the nearer end was scored at the right *Eco*RI end (melt at 0.9 fractional length); if the distance from the melt to the nearer end was between 0.425 and 0.495 SV40 fractional length, the nearer end was scored as the left *Eco*RI end (melt at 0.46 fractional length). In this manner, an exact location could be assigned on the SV40 map for most of the ferritin cores. It should be noted that a small fraction of the melts occur at either 0.10 or 0.54 SV40 fractional length (Fig. 3). If these were incorrectly assigned to the positions 0.90 or 0.46, a maximum misassignment of about 10% of the ferritin cores could result. In presenting the data, we have used a histogram interval of 2.5%, which is slightly greater than the standard deviation of the measurements for the length of the whole DNA molecule and is consistent with the accuracy of the measurements. The histogram format imposes an arbitrary distortion of data through discontinuity. In order to compromise between correcting for the discontinuity imposed by the 2.5% interval and preserving the information, we have used a simple smoothing function so that each point more accurately simulates a Gaussian distribution. Each complex to be scored is given a weight of one. One-half is added to the histogram interval in which the measurement falls and one-fourth is added to each of the adjacent intervals. In A and B the white and shaded areas represent data from two independent experiments, presented additively. Histogram A represents 163 molecules, B represents 153 molecules, and C represents 70 molecules.

ously if it has an unwinding protein melt at one of these two positions.

Ferritin Binding Mediated by T Antigen Can Be Mapped on the SV40 DNA. SV40 DNA with ferritin cores bound can be reacted sequentially with *E. coli* DNA unwinding protein and *Eco*RI endonuclease to yield linear molecules with both a ferritin core and an unwinding protein melt. Electron micrographs of such molecules are shown in Fig. 2E and F. In these molecules, the position of the melt allows one to determine unequivocally the orientation of the bound ferritin cores relative to the *Eco*RI cleavage site. Once this orientation has been established, an accurate measurement of the distance from the ferritin core to the nearer end of the DNA molecule can be made to give a unique location. With the unwinding protein marker, all the data can be obtained from the molecules present in a single preparation. It is much more difficult to resolve the preferred site of T antigen binding from the background if the experiment is done with two separate preparations of linear DNA, one cleaved with *Eco*RI and the other with *Hpa*II restriction endonuclease. A distribution of the positions of ferritin cores, determined, using *E. coli* DNA unwinding protein, from 163 molecules (Fig. 4A) has a peak between 0.65 and 0.70 fractional length from the *Eco*RI cleavage site, consistent with preferential binding at a unique location. The same peak was found in two independent experiments. A background of ferritin cores along the entire length of the DNA is also observed. The origin of replication of SV40 DNA has been mapped within the *Hemophilus influenzae* restriction endonuclease C fragment near the A–C junction (22), corresponding to the same interval of 0.65 to 0.70 fractional length. If mock T antigen is used or if antigen is omitted, the ferritin cores are distributed randomly along the entire length of the DNA (Fig. 4B and C, respectively).

The fraction of DNA molecules with ferritin bound varied between 1 and 15% in different preparations of the same sample, and there was similar variation from one part of an electron microscope grid to another. Apparently the fraction of DNA molecules with ferritin bound that sticks to the grid is variable. Since we do not know the fraction of DNA molecules in solution that have ferritin bound, we cannot conclude that the controls without T antigen (Fig, 4B and C) account entirely for the background observed. That is, there may be some nonspecific binding of T antigen to SV40 DNA in addition to the specific binding between 0.65 and 0.70 fractional length.

DISCUSSION

Partially purified T antigen was used so that we could be confident that the material had not become altered to a

functionally inactive form. Although T antigen from SV40-transformed cells has been purified nearly to homogeneity (23, 24), if purification is taken beyond the DEAE-cellulose chromatography step, the antigen is much more labile (S. I. Reed and G. R. Stark, unpublished observations). Changes in the sedimentation properties of the antigen have also been associated with purification (4). Infected permissive cells were used rather than transformed cells because most transformed lines are derived from nonpermissive hosts of SV40 and host range restriction might involve, among other things, inability to form T antigen that can function in the replication of viral DNA.

Because T antigen binds preferentially near the origin of DNA replication, it could be involved directly in the initiation of SV40 DNA synthesis in lytic infection. The way now lies open for testing the nature of such an involvement in more functional terms. It would also be useful to show that T antigen is related to the product of gene A of SV40. To this end, one can explore the possibility that T antigen from cells infected by temperature-sensitive mutants in gene A might have temperature-sensitive properties.

A major limitation at the present stage of development is the high background. A peak of the magnitude reported here would be lost if the genome being probed were much larger than the size of SV40. Undoubtedly, with refinements of the technique, the signal to noise ratio will be improved and more general applications will be possible. The method requires that an antibody be available against the molecule in question, but the use of two kinds of antibodies allows one the latitude of using a single ferritin-labeled reagent with a variety of antigens, provided that the primary antibodies are prepared in the same species. More importantly, one needs physical markers on the DNA for orientation. We have used a special property of SV40(I) DNA, its affinity for DNA unwinding protein at specific sites, probably due to a high content of A+T at these sites (25–27). A more general technique might employ heteroduplexes of wild-type DNAs with mutant DNAs containing small deletions or insertions to generate marker loops (28). We believe that this technique should find other applications in mapping specific interactions between proteins and DNA.

This work was supported by Research Grants GB-36807 from the National Science Foundation, Public Health Service Grant GM 19177 from the National Institutes of General Medical Sciences, and a Cottrell Research Grant from the Research Corporation. S.I.R. is a U.S. Public Health Service Trainee. J.F. is supported by a predoctoral fellowship from the National Science Foundation.

1. DelVillano, B. C. & Defendi, V. (1973) *Virology* **51**, 34–46.
2. Tegtmeyer, P. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 9–15.
3. Osborn, M. & Weber, K. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 267–276.
4. Carroll, R. B., Hager, L. & Dulbecco, R. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3754–3757.
5. Tegtmeyer, P. (1972) *J. Virol.* **10**, 591–598.
6. Robb, J. A., Tegtmeyer, P., Ishikawa, A. & Ozer, H. L. (1974) *J. Virol.* **13**, 662–665.
7. Wiblin, C. N. & MacPherson, I. A. (1972) *Int. J. Cancer* **10**, 296–309.
8. Takemoto, K. K., Kirschstein, R. L. & Habel K. (1966) *J. Bacteriol.* **92**, 990–994.
9. Estes, M. K., Huang, E.-S. & Pagano, J. S. (1971) *J. Virol.* **7**, 635–641.
10. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
11. Radloff, R., Bauer, W. & Vinograd, J. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 1514–1521.
12. Yoshimori, R. N. (1971) Ph.D. Dissertation, University of California, San Francisco Medical Center.
13. Sharp, P. A., Sugden, B. & Sambrook, J. (1973) *Biochemistry* **12**, 3055–3063.
14. Weiner, J. H., Bertsch, L. L. & Kornberg, A. (1975) *J. Biol. Chem.* **250**, 1972–1980.
15. Sever, J. L. (1962) *J. Immunol.* **88**, 320–329.
16. Avrameas, S. & Ternyck, T. (1967) *J. Biol. Chem.* **242**, 1651–1659.
17. Palacios, R., Palmiter, R. D. & Schimke, R. T. (1972) *J. Biol. Chem.* **247**, 2316–2321.
18. Olsen, B. R. & Prockop, D. J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2033–2037.
19. Koller, Th., Sogo, J. M. & Bujard, H. (1974) *Biopolymers* **13**, 995–1009.
20. Davis, R., Simon, M. & Davidson, N. (1971) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 21, pp. 413–428.
21. Deluis, H., Mantell, N. J. & Alberts, B. (1972) *J. Mol. Biol.* **67**, 341–350.
22. Danna, K. J. & Nathans, D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3097–3100.
23. Spira, G., Popescu, M., Cymbalista, S., Biezunski, N. & Goldblum, N. (1972) *Archiv für die gesamte Virusforschung* **37**, 236–242.
24. Livingston, D. M. & Henderson, I. C. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 283–289.
25. Mulder, C. & Delius, H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3215–3219.
26. Morrow, J. F. & Berg, P. (1973) *J. Virol.* **12**, 1631–1632.
27. Risser, R., Hopkins, N., Davis, R. W., Delius, H. & Mulder, C. (1974) *J. Mol. Biol.* **89**, 517–544.
28. Mertz, J. E. & Berg, P. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 4879–4883.