Free and Viral Chromosome-Bound Simian Virus 40 T Antigen: Changes in Reactivity of Specific Antigenic Determinants during Lytic Infection

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Simian virus 40 (SV40) large T antigen (TAg), both free and bound to mature 70S and replicating 90S SV40 chromosomes, was prepared from lytically infected cells. The relative reactivity of the different TAg-containing fractions toward 10 monoclonal antibodies directed against three different regions in SV40 TAg and toward an antibody against the p53 protein was measured. The results for free TAg indicated that all of the determinants in both the amino-terminal (0.65 to 0.62 map units) and carboxy-terminal (0.28 to 0.17 map units) regions were highly reactive, whereas all five determinants located between 0.43 and 0.28 map units in the midregion of TAg were poorly reactive. For TAg bound to replicating chromosomes, all but one of the antibodies specific for TAg were highly reactive. Thus, antigenic sites in the middle of TAg, the region important for nucleotide binding and ATP hydrolysis (an activity required for viral DNA replication), were more accessible in TAg-replicating DNA complexes. As replicating molecules matured into 70S chromosomes, three or more determinants at different locations in TAg bound to chromatin became two- to fivefold less reactive, indicating other changes in TAg structure. Overall, at least nine different antigenic determinants in the TAg molecule were identified. Anti-p53 was reactive with about 10% of the free TAg and the same amount of SV40 chromosomes of all ages, suggesting that p53-TAg complexes are not preferentially associated with either replicating or mature viral chromosomes. When the reactivity of both mature and replicating labeled SV40 chromosomes with polyclonal tumor anti-T was measured as a function of time after purification, TAg bound to mature chromosomes appeared to dissociate about fourfold faster than that bound to replicating chromosomes. The relative amount of TAg in various subcellular fractions was measured by an enzyme-linked immunosorbent assay. Approximately 1.3% of the total TAg was estimated to be associated with SV40 chromosomes in infected cells. Based on the relative amounts of TAg and viral DNA in the 70S and 90S fractions, replicating chromosome-TAg complexes were estimated to bind 4.8 times more TAg per DNA molecule, on the average, than mature chromosome-TAg complexes. Together, these results are consistent with major differences in TAg structure when free and associated with replicating and nonreplicating SV40 chromosomes.

Simian virus 40 (SV40) large T antigen (TAg) is a protein with an apparent M_w of about 94,000 that is required for viral DNA replication and transformation. TAg binds to three nucleotide sequences that include the SV40 origin of replication (*ori*) and the start sites for early gene transcription. The interaction of TAg at these sites results in not only initiation of viral DNA synthesis but also down-regulation of early viral RNA synthesis (for review, see reference 38). The initiation and, in some cases, maintenance of murine cellular transformation has been demonstrated to require a functional TAg (4, 37). A cellular tumor antigen, 53 kilodaltons in size, is expressed in high levels in SV40-transformed murine cells (18, 21) and is physically associated with SV40 TAg (15, 18, 21).

The preparation of SV40 chromosomes of varied age that include theta-form replicating DNA (RI), termination intermediates, and recently replicated mature and older mature viral DNA molecules has been described previously (35, 36). The fraction of each SV40 chromosomal DNA species associated with TAg has also been measured after immunoprecipitation with anti-T serum (35). TAg was preferentially associated with early-replicating SV40 chromosomes, whereas most of the late-replicating molecules (more than 70% replicated and catenated dimers) had lost TAg.

Monoclonal antibodies, because of their specificity for single antigenic determinants or epitopes, are useful reagents for defining the solution conformation or accessibility (or both) of functional and structural domains in macromolecules (for review, see reference 22). Several monoclonal antibodies specifically directed against SV40 tumor antigens have been isolated (15-17, 19, 23). These have proven useful for characterizing the binding of SV40 TAg to viral DNA (25-27, 31), identifying distinct subclasses of TAg (15), and defining the association of TAg with the p53 protein (6, 15, 21, 28). Viral DNA species associated with TAg during lytic infection have been analyzed by immunoprecipitation with hamster tumor serum (24, 29, 30, 32, 35) and a monoclonal anti-T (35). Recently, the isolation and characterization of several new hybridoma lines synthesizing antibodies specific for three different antigenic regions of TAg have been described (16). A linear map of the hybridoma anti-T binding sites in TAg has been compiled (10, 16). Many of the monoclonal antibodies were unique based on their specificity for different regions of TAg and their cross-reactivity with the human papovavirus BKV TAg.

In this paper, the relative reactivity of different SV40 chromosome-TAg complexes, as well as that of free TAg, toward 10 different monoclonal antibodies directed against different regions in SV40 TAg (15, 16) was measured. A monoclonal antibody specific for the p53 protein, PAb 122

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FIG. 1. Preparation of TAg-containing subcellular fractions from SV40-infected CV1 cells. Infected cells were fractionated with hypotonic buffer into cytosol, nuclear extract (1-h extraction time), and nuclear pellet components (see Materials and Methods). The nuclear extract (NE) was further fractionated by gradient centrifugation into mature 70S and replicating 90S chromosomes and free TAg (top five fractions of the gradient).

(15), was also analyzed. We wished to measure differences in the reactivity of determinants in both free TAg and that bound to SV40 chromosomes which could be related to the functional state and age of the SV40 chromosome complex. We were also interested in whether p53 was associated with TAg bound to either replicating or mature chromosomes in lytically infected cells. To confirm that any observed differences in reactivity were due to changes in the structure of TAg, rather than a result of the conditions of the immunoassay used, two different immunoassays were used to analyze the relative reactivity of TAg bound to different chromosome fractions. These were (i) the standard immunoprecipitation assay with fixed *Staphylococcus aureus* with and without a second antibody and (ii) adsorption of anti-T-TAg complexes to a second antibody immobilized onto microtiter wells. Both assays measured the amount of labeled chromosomal DNA associated with TAg; one was protein A dependent, whereas the other was not. Based on the relative reactivities of these monoclonal antibodies, several were chosen to compare the relative stability of TAg binding to replicating and mature chromosomes. In addition, the relative amounts of free and chromatin-bound TAg in various subcellular fractions was analyzed by an enzyme-linked immunoadsorbent assay (ELISA). This assay allows sensitive and accurate estimates of antigen concentration and thus can directly measure the amount of TAg in a sample whether it is free or chromatin bound.

MATERIALS AND METHODS

Cells and virus. CV1 cells and SV40 strain wt800 viral stocks were grown as previously described (35, 36).

Cytosol, nuclear extract, and SV40 chromosome preparation. Viral chromosomal DNA was labeled by incubating

SV40-infected cells with [^3H]thymidine (100 to 250 μCi per 150-mm dish, 60 Ci/mmol; New England Nuclear Corp.) for various times from 3 min to 18 h as previously described (35, 36). Labeled infected cells at 36 h postinfection (time of maximum DNA synthesis) were floated on an ice bath and rinsed 3 times with 10 ml of ice-cold hypotonic solution using either the Hyp A buffer of Su and DePamphilis (33) or the HBE buffer of Beard and Nyfeler (1). Hyp A buffer consisted of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.8), 5 mM KCl, 0.5 mM MgCl_2 , and 0.5 mM dithiothreitol. HBE buffer was identical, except 1 mM disodium EDTA was substituted for the MgCl_2 . Each rinse was removed by suction. The cells were scraped and then treated with six to eight strokes in a Dounce homogenizer until more than 90% of the cells were broken. About 1 ml of cell suspension was obtained per 150-mm plate. This was centrifuged at $1,400 \times g$ for 3 min at 0°C in a Beckman JS-13 rotor. The supernatant was carefully removed from the nuclear pellet, and the pellet was suspended in 2 volumes of hypotonic buffer and incubated in ice for 1 to 2 h to extract the viral chromosomes. The supernatant, or cytosol fraction, was centrifuged again at $20,000 \times g$ for 15 min to remove cell debris. The nuclear suspension was then centrifuged similarly to give a nuclear extract, containing SV40 chromosomes, and a nuclear pellet. The nuclear extract was fractionated on a 5 to 20% sucrose gradient (containing the corresponding hypotonic buffer) at 40,000 rpm for 105 min at 0°C with an SW41 rotor. For each labeling time, parallel gradients were run, each yielding mature 70S and replicating 90S chromosome peaks. Fractions were collected from the bottom of each tube, and 20- μl samples were counted in Aquasol (New England Nuclear). The relative positions of the 70S and 90S fractions were identified by long and short labeling times, respectively, and usually consisted of four fractions each. A flow diagram is shown in Fig. 1.

Buffers containing MgCl_2 gave a better separation of replicating and mature SV40 chromosomal peaks after sucrose gradient centrifugation, but EDTA-containing nuclear extracts and gradient fractions were more stable for long times with respect to endonuclease cleavage. For this CV1 cell line, extraction with either buffer for 1 to 2 h was sufficient to yield 80 to 90% of the SV40 chromosomes in the cytosol and nuclear extract fractions. The yields of viral DNA were essentially identical for the two methods, i.e., 20 μg per 150-mm plate. More than 95% of the label in the 70S and 90S was acid precipitable. The integrity and relative amounts of the viral DNA species in each chromosome fraction were assessed by agarose gel electrophoresis as previously described (35).

Radioimmunoassays (RIAs). Hybridoma supernatants PABs 100 through 109 were prepared as described previously (15, 16). All were reactive with large TAg. Hybridoma supernatant PAb 122, specific for the p53 protein, was also prepared as described previously (15). NS1 medium (parent nonsecretor myeloma line) and G11 medium (non-TAg-specific murine immunoglobulin G [IgG] secretor line; anti-cytochrome p450 provided by K. Griffin, Scripps Clinic) were used as controls. Polyclonal tumor serum from hamsters (obtained from the National Cancer Institute) was used for comparison; preimmune hamster serum was used as a control.

(i) Immunoprecipitation with anti-T and *S. aureus*: protein A-dependent RIA. The reactivity of labeled SV40 chromosomal DNA complexed to TAg was measured by immunoprecipitation with monoclonal and tumor anti-T using fixed *S. aureus* as the immunosorbent as previously described

(35). The maximum amount of labeled SV40 chromosomal DNA associated with TAG for each fraction was determined as follows. Increasing amounts of each hybridoma supernatant or tumor serum were added to a fixed amount of [³H]thymidine-labeled SV40 chromosome-TAG complex (usually 50 μ l of mature 70S or replicating 90S chromosome fraction). To each reaction mixture was added 0.1 volume of 10 $\times$ NET-NP-40 buffer (1 M NaCl, 50 mM disodium EDTA, 0.5 M Tris [pH 7.8], 0.5% Nonidet P-40 [NP-40]) so that the final reaction volume (usually 500 μ l) was isotonic with respect to salt concentration and 0.05% NP-40. This was incubated in ice (0°C) for 1 h, and then up to 200 μ l of washed fixed *S. aureus* cell suspension (10%, wt/vol; IgG-binding capacity, 2 mg/ml; The Enzyme Center) in 1 $\times$ NET-NP-40 buffer was added for an additional hour in ice. The immunoprecipitate was pelleted at 3,000 rpm for 3 min in a swinging bucket rotor at 0°C. The anti-T supernatant was saved, and a sample was counted. The pellets were gently washed twice in 1 $\times$ NET-NP-40 buffer, extracted with 100 μ l of 2% sodium dodecyl sulfate (SDS)-2% β -mercaptoethanol in boiling water for 5 min, centrifuged to remove the insoluble complexes, and counted in Aquasol. The percentage of the input labeled chromosomal DNA in the precipitate (with TAG) was calculated, as was the optimum amount of each monoclonal anti-T necessary for maximum precipitation. Controls consisted of NS1 and G11 medium and hamster preimmune serum (see above). Protein A-Sepharose (Sigma Chemical Co.) was also used instead of fixed *S. aureus*; no difference was found in the results.

For essentially all of the antibodies, 100 μ l of hybridoma medium (1 to 5 μ g of murine IgG) was sufficient to maximally precipitate up to 100 μ l of labeled chromosome fraction (up to 1 μ g of viral DNA). The effect of adding a second antibody directed against the monoclonal murine IgG and highly reactive with the protein A in fixed *S. aureus* was also measured. Some of the hybridoma antibodies are of the IgG1 subclass (16) and might not efficiently bind protein A (11). Accordingly, 5 μ g of affinity-purified rabbit anti-mouse IgG (RAM; provided by K. Griffin) was added to each standard reaction mixture containing 100 μ l of hybridoma supernatant and 100 μ l of 10% *S. aureus* suspension. These reactions were incubated for 4 h or overnight in ice before washing and quantitation because the addition of a second antibody requires longer reaction times for efficient binding to protein A complexes. Added RAM significantly improved the precipitation efficiency of PABs 106, 103, and 104. It had little effect on precipitation with PABs 100, 105, and 107. PABs 101 and 108 were less reactive (up to 20%) when RAM was added. Accordingly, RAM was routinely added to all protein A-dependent reactions with IgG1 but not IgG2a or IgG2b monoclonal antibodies.

(ii) **Immunoabsorption of anti-T-TAG complexes onto microtiter wells coated with RAM: PVC-dependent RIA.** The reactivity of thymidine-labeled replicating 90S and mature 70S SV40 chromosomes with monoclonal anti-T was also measured by using a second antibody immobilized onto polyvinyl chloride (PVC) plates as an immunosorbent. This assay also quantifies the amount of labeled chromosomal DNA associated with TAG but is independent of protein A. Microtiter PVC plates (96-well, flexible PVC U plates; Dynatech Laboratories, Inc.) were precoated with affinity-purified RAM (150 μ l per well of a 50- μ g/ml solution in 0.1 M sodium carbonate, pH 9.6) overnight at 4°C. Each well was capable of adsorbing up to 1 μ g of protein. The plates were washed once with 3% bovine serum albumin (BSA; Leptalb 7, 30% solution; Armour) in phosphate-buffered saline (PBS)

and three times with PBS. A fixed amount (50 μ l) of SV40 chromatin fraction (either gradient-purified 70S or 90S chromosomes labeled for various times) prepared in HBE buffer and a fixed amount of monoclonal anti-T (usually 50 μ l) were added together. Controls consisted of NS1 or G11 supernatants added to the labeled chromatin. HBE buffer was added to give a constant reaction volume of 150 μ l. The reactions were incubated for 2 to 4 h at 4°C. After one wash with BSA-PBS and three washes with PBS, the bottom half of each well was cut off and counted in Aquasol; no effect of the position of the well as it was counted was observed. Counting efficiencies of labeled samples adsorbed to plastic wells were 85 to 90% of samples counted directly in scintillation solution.

Quantification of TAG by ELISA. The ELISA has been used to determine the stoichiometry of SV40 TAG complexed with p53 protein in SV40-transformed cells (13). A similar method was used here to directly measure the relative amount of TAG in different subcellular fractions. Various amounts of TAG-containing fractions (Fig. 1), including cytosols, nuclear extracts, nuclei after extraction of viral chromosomes and free TAG, and 70S mature and 90S replicating chromatin fractions, were adsorbed to polystyrene microtiter plates (96 well; Nunc) and incubated overnight in HBE buffer (pH 8.0) at 4°C. The final volume was always 150 μ l. After one wash with BSA-PBS and three washes with PBS, a fixed amount of monoclonal anti-T (50 μ l was usually optimum) made to 150 μ l with HBE buffer was added to the TAG-coated plates, and the reactions were incubated for 4 h at 4°C. Four different antibodies, PABs 101, 102, 106, and 108, reactive in the two RIAs above and in this assay, were analyzed. After one wash with BSA-PBS and three washes with PBS, 150 μ l of alkaline phosphatase-conjugated RAM (Sigma), diluted 1:1,000 in BSA-PBS, was added per well for 30 min at 37°C. After five washes with PBS, 150 μ l of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) at 1 mg/ml in 1 M diethanolamine (pH 9.6)-0.5 mM MgCl₂ was added, and the reaction was developed for 30 min or longer in the dark at room temperature. The reaction was stopped by adding 50 μ l of 2 M NaOH. The absorbance at 410 nm was measured with a Titertek ELISA reader. Control values (NS1 and G11 media) were subtracted. The maximum amount of each TAG fraction that could be adsorbed, as measured by no further increase in the rate of substrate consumption, was determined for each of the antibodies, and the results were averaged. Saturation per well for the cytosol, nuclear extract, and 70S and 90S gradient-purified chromatin fractions was 5, 10, 50, and 100 μ l, respectively. The optimum amount of hybridoma anti-T necessary to maximally react with 50 μ l of 70S chromatin was essentially identical on comparing the results of this assay with that of the two RIAs. Labeled chromosomes adsorbed to uncoated wells in proportion to the amount of label added until saturation was reached.

Quantification of specific antibody by ELISA. The relative amounts of specific mouse IgG in each of the hybridoma supernatants were measured by ELISA. Fixed amounts (25 μ l) of monoclonal anti-T and control media were adsorbed to microtiter plates and assayed after the addition of enzyme-conjugated second antibody and substrate as described above. Based on the absorbance at 410 nm and the known amounts of specific murine IgG in the NS1 (none) and G11 (50 μ g/ml) media, the amount of specific anti-T IgG in each supernatant was calculated. All contained between 5 and 25 μ g of mouse IgG per ml. In particular, PABs 100 and 122 had 15 μ g of mouse IgG per ml. Thus, sufficient amounts of

TABLE 1. Relative reactivity of monoclonal anti-T with free TAG and labeled SV40 chromosome-TAG complexes by protein A-dependent immunoprecipitation

Specificity	Antibody				TAG fraction precipitated ^a			Change in reactivity	
	PAb	RAM ^b	IgG subclass	SDS ^c	Free ^d	R _{TOT} ^e	M _E ^e	Free/R _{TOT}	M _E /R _{TOT}
TAG (I) ^f	105	+	1	+	89	79	71	1.1	0.9
	108 ^g	—	2a	+	99	91	80	1.1	0.9
	109	—	2a	+	115	98	57	1.2	0.6
TAG (III) ^f	100	+	1	—	9	8	9	1.1	1.1
	102	—	2a	—	43	78	22	0.5	0.3
	103	+	1	—	18	110	60	0.1	0.5
	104	+	1	—	13	120	125	0.1	1.0
	106 ^g	+	1	—	32	110	94	0.3	0.9
TAG (IV) ^f	101	—	2a	+	83	79	40	1.1	0.5
	107	+	1	+	71	69	62	1.0	0.9
p53	122	—	2b	ND ^h	12	3	11	3.9	3.7
T Ag + p53	Tumor	—	All	+	100	100	100	1.0	1.0

^a Relative reactivity with anti-T of a fixed volume of TAG-containing fraction plus saturating amounts of antibody plus fixed *S. aureus* or protein A-Sepharose. Control values were subtracted. At least three separate experiments were averaged and normalized to the amount of TAG precipitated with tumor serum.

^b RAM added (+) or not added (—) to the immunoprecipitation reaction (see Materials and Methods).

^c Resistant (+) or sensitive (—) to SDS denaturation (16).

^d Relative amount of free TAG in cytosols after immunoprecipitation and SDS-gel analysis (Fig. 2).

^e Labeled SV40 chromosome fraction (R_{TOT} is the 5-min-labeled 90S peak; M_E is the 60-min-labeled 70S peak) analyzed in Fig. 5.

^f Regions of TAG recognized by the monoclonal anti-T: I, 0.62 to 0.65 map units; III, 0.28 to 0.44 map units; IV, 0.17 to 0.28 map units.

^g Cross-reactive with BK virus TAG (16).

^h ND, Not determined.

specific IgG were present in each of the antibody preparations used in these studies.

RESULTS

Relative reactivity of 11 monoclonal antibodies with free TAG in cytosols. We have used 10 monoclonal antibodies specific for SV40 TAG, designated PAb 100 through 109, and the anti-p53 monoclonal antibody PAb 122 to study TAG bound to viral chromosomes during lytic infection. The location of the recognition sites in TAG for these antibodies has been previously determined by using adenovirus-SV40 hybrid viruses, which synthesize shortened forms of the TAG molecule, and both TAG and small t-antigen species (10, 16). PAb 105, 108, and 109 bind near the N terminus (region I; 0.65 to 0.62 map units), PAb 100, 102, 103, 104, and 106 bind near the middle (region III; 0.43 to 0.28 map units), and PAb 101 and 107 bind near the C terminus (region IV; 0.28 to 0.17 map units) of TAG (Table 1). PAb 106 and 108 cross-react with BK virus TAG by immunofluorescence (16). PAb 122 is specific for the cellular protein p53, coprecipitating TAG associated with p53 (15).

All 11 monoclonal antibodies precipitate TAG from extracts of SV40-infected monkey cells (15, 16). We confirmed this by using cytosols prepared in HBE or Hyp A buffer (see Materials and Methods) and immunoprecipitation with saturating amounts of antibody and washed protein A-Sepharose as the immunosorbent (35). RAM was added to all reactions containing IgG1 subclass antibodies. Hamster tumor serum was analyzed for comparison. NS1 medium was used as a control. The amount of TAG in each precipitate was analyzed after SDS-gel electrophoresis and quantified by scanning Coomassie blue-stained gels. Representative gels are shown in Fig. 2, and the relative amount of TAG maximally precipitated by each antibody compared with tumor serum is shown in Table 1. Similar results were obtained after immu-

noprecipitation of cytosols from infected cells labeled in vivo with [³⁵S]methionine for 12 h.

The results indicated that determinants located in regions I (N terminus) and IV (C terminus) on free TAG were highly reactive; these antibodies precipitated as much TAG as tumor serum. However, all of the region III (middle) determinants were significantly less reactive. PAb 122 (specific for p53) reacted with 12% of the free TAG and is thus associated with p53 in SV40-infected cell extracts. Less TAG was precipitated by PAb 122 when measured with cytosols labeled for 2 to 4 h with [³⁵S]methionine. No p53 was precipitated by PAb 122 after PAb 108 was used to remove all of the TAG and its associated p53. Similar results were observed when free TAG in nuclear extracts was quantified.

Purification of replicating and mature SV40 chromosomes of various ages. SV40-infected cells at the peak of viral replication were incubated in vivo with [³H]thymidine for 5 min, 30 to 120 min, and 12 h to radiolabel viral chromosomes of different ages as previously described (35, 36). Nuclear extracts from infected cells were then fractionated into mature 70S and replicating 90S viral chromosomes (Fig. 1). Figure 3 shows a typical sucrose gradient sedimentation profile. For the 5-min-labeled sample (Fig. 3A), most of the radioactivity sedimented with the 90S peak rather than with the slower-sedimenting 70S pool. In contrast, with longer labeling times, most (30 min, Fig. 3B) or all (12 h, Fig. 3C) of the radioactivity is associated with the 70S mature chromosomal DNA peak.

In subsequent experiments, we used three labeled fractions from such sucrose gradients. The first was the 5-min-labeled 90S peak or R_{TOT}. The TAG-containing labeled chromosomal DNA species in this fraction (accounting for 40 to 60% of the label) are predominantly replicating DNA molecules elongated from 5 to 90% (35). The second fraction, M_E, was the 70S peak from cells labeled for 30 to 120 min. The TAG-containing species in this peak (accounting for

FIG. 2. Relative reactivity of free TAG in cellular extracts with 11 specific monoclonal antibodies and tumor serum. (A) Results for each antibody immunoprecipitated with protein A-Sepharose beads alone. Each reaction consisted of 750 μ l of cytosol from SV40-infected cells in Hyp A buffer and 400 μ l of hybridoma supernatant or 200 μ l of hamster tumor serum incubated for 30 min in ice. Then, 100 μ l of washed protein A-Sepharose beads (1:1 [wt/vol] suspension) in NET-NP-40-1% sucrose buffer was added to each reaction with shaking for 1 h in ice. The pellets were washed three times with NET-NP-40-sucrose buffer (centrifuging for 30 s in a Brinkman microfuge), suspended in SDS sample buffer, and boiled, and the solubilized proteins were electrophoresed on SDS gels as previously described (34). Gels were stained with Coomassie blue, destained, dried, and photographed. Lanes 12 through 15 were from a separate experiment. (B) Reactions were performed similarly, except 20 μ g of RAM (Cappel Laboratories) was added to those reactions containing IgG1 antibodies. Protein A-Sepharose was added for 4 h in ice. Molecular weight standards (S) included the following: myosin, 200,000; β -galactosidase, 116,000; phosphorylase B, 97,400; BSA, 67,000; and ovalbumin, 45,000. Photographic negatives were scanned, and the relative TAG peak heights were determined for each lane after subtraction of the NS1 control.

10% of the label) consist primarily of young mature viral chromosomes that have recently replicated (35). The last fraction, M_L , was the 70S peak from cells labeled for 12 h. The TAG-containing DNA in this fraction (accounting for 8% of the label) consisted of old uniformly labeled mature viral DNA (35). In this way, we obtained SV40 chromosome-TAG complexes of various ages to react with antibodies recognizing different regions in TAG.

Immunoprecipitation of labeled SV40 chromosomes with monoclonal anti-T and fixed *S. aureus*: protein A-dependent RIA. The relative reactivity of 10 monoclonal anti-Ts (PABs 100 through 109) and the anti-p53 (PAB 122) hybridoma supernatants with labeled SV40 replicating 90S and mature 70S chromosome fractions was measured as described in Materials and Methods. Each of the labeled SV40 70S and 90S chromosome fractions was immunoprecipitated with increasing amounts of the monoclonal antibody and a fixed amount of *S. aureus*. A second antibody, RAM, was added to reactions containing IgG1 subclass monoclonal antibodies. Polyclonal hamster tumor serum was used for compar-

ison. Some of the antibodies were slightly more reactive with viral chromosomes than tumor serum, precipitating up to 20% more of the labeled DNA.

The results from adding increasing amounts of six of the monoclonal antibodies to two different viral chromosome fractions are shown in Fig. 4 (5-min pulse-labeled 90S chromosome fraction in Fig. 4A, 60-min pulse-labeled 70S chromosomes in Fig. 4B). For both of these chromosome fractions, saturation was reached for all of the antibodies with about 40% of the 5-min label and 7% of the long label maximally precipitable. This agrees with previously reported values for these chromosome fractions (35). The results for 30-min compared with 60-min pulse-labeled mature DNA samples were very similar. Mature 70S chromosomes labeled for 12 h were also analyzed in this way (data not shown). Note that with increasing labeling time (compare Fig. 4B with 4A), the amount of labeled chromosomal DNA reactive with tumor serum decreased, consistent with loss of TAG from replicating chromosomes after initiation (30, 35).

The results (maximum reactivity for each labeling time)

FIG. 3. Isolation of replicating and mature SV40 chromosomes. Infected CV1 cells were radiolabeled with [^3H]thymidine for various times. SV40 chromosomes were extracted under hypotonic conditions and fractionated by sedimentation in sucrose gradients into mature 70S and replicating 90S peaks. Radiolabel was measured for a sample of each fraction, and the fractions indicated were pooled. The labeling times shown are 5 min (A), 30 min (B), and 12 h (C).

FIG. 4. Quantitative immunoprecipitation of labeled SV40 chromosome-Tag complexes with saturating anti-T and fixed *S. aureus* cells. Mature 70S and replicating 90S chromosome pools prepared from SV40-infected cells labeled with [^3H]thymidine for various times were each immunoprecipitated with increasing amounts of each monoclonal anti-T medium or tumor serum ($\blacklozenge$) as described in Materials and Methods. Fixed *S. aureus* cells were used as the immunosorbent. NS1 cell medium was used as a control. The total amount of labeled chromosomal DNA in each immunoprecipitate was measured. (A) 90S pool labeled for 5 min. The total input per assay point was 10,600 cpm. The NS1 control (200 μl) was 0.2% of the input, and maximum precipitation was 40% of the labeled DNA. (B) 70S peak labeled for 60 min. The input was 20,000 cpm. The NS1 control was 1.6%, and maximum precipitation was 7%. PABs 100 ($\bullet$), 122 ($\circ$), 101 ($\blacktriangle$), 106 ($\triangle$), 109 ($\blacksquare$), and 103 ($\square$) are shown. The upper and lower horizontal scales refer to the volumes of monoclonal supernatant and tumor serum used, respectively.

FIG. 5. Relative reactivity of SV40 chromosomes of various ages with monoclonal antibodies in a protein A-dependent RIA. Replicating 90S and mature 70S viral chromosomes were prepared from SV40-infected cells labeled for 5 min to 12 h with [³H]thymidine (see Materials and Methods). In this way, total replicating (R_{TOT}, 5-min-labeled 90S), early mature (M_E, recently replicated, 30-min- to 2-h-labeled 70S), and late mature (M_L, 12-h-labeled 70S) viral chromosomes were prepared. A fixed volume (50 μ l) of each chromosome pool was reacted with saturating amounts of antibody as shown in Fig. 4. RAM was added to all IgG1 subclass antibodies. The amount of radiolabeled DNA in each fraction is expressed as a percentage of the value determined for hamster tumor serum (100%) for that fraction. PABs 100 (●), 101 (▲), 102 (○), 103 (□), 104 (▼), 105 (▽), 106 (△), 107 (Φ), 108 (◇), 109 (■), 122 (○), and NS1 (◆) are shown.

for all of the antibodies incubated with replicating 90S chromosomes and both young and old mature SV40 70S chromosomes are shown in Fig. 5. The relative reactivity of each antibody is expressed as a percentage of the tumor serum value for that chromosome fraction. Most of the monoclonal antibodies were highly reactive toward total SV40 replicating chromosomes, with greater than 80% of the labeled DNA precipitated compared with polyclonal tumor serum). The exceptions were PABs 100 and 122 (anti-p53), which were both poorly reactive (less than 13% of the labeled DNA was precipitated compared to tumor serum). For mature chromosomes labeled for longer times (i.e., 30 min or more), the extent of precipitation for some of the monoclonal antibodies, compared with each other and with tumor serum, appeared to change significantly. For example, PAB 101, which precipitated 79% of the RI compared with tumor serum, now precipitated only 40% of the 60-min pulse-labeled mature chromosomes. With longer labeling times these differences became even more pronounced for some of the antibodies (PABs 101, 103, and 109). Thus, with increasing age, there appears to be up to a fourfold decrease in the reactivity of several determinants in TAG when bound to mature chromosomes compared with that when bound to replicating chromosomes. Besides PAB 101, this was also apparent for PABs 102 and 109 and, to a lesser extent, PAB 103. The antibodies specific for SV40 TAG which cross-react with BK virus TAG (PABs 108 and 106) were the least affected by increasing age of the viral chromosomes. PABs 100 and 122 reacted poorly with SV40 chromosomes of all ages. The results of measuring the reactivity of two of the chromosome fractions (replicating 90S and young mature 70S) are also summarized in Table 1.

Relative reactivity of free TAG and chromosome-bound TAG with each monoclonal antibody. The reactivity of each of the antibodies toward free TAG (cytosol) with that of TAG bound

to replicating (R_{TOT}) and mature (M_E) chromosomes can now be compared in Table 1. Several conclusions are indicated. Determinants located in the N and C termini of TAG (regions I and IV, respectively) are as reactive in free TAG as in that bound to replicating chromosomes. However, all region III determinants, except that recognized by PAB 100, became significantly more reactive in TAG bound to replicating molecules than in free TAG. Thus, the region encompassing map units 0.28 to 0.43 in TAG appears to be the most dramatically altered when bound to replicating chromosomes. All but one of the five determinants in this middle region of TAG appear to be more accessible compared with unbound TAG. Interestingly, this is also the region of the TAG molecule that is sensitive to SDS denaturation (16). When mature chromosomes are compared to replicating chromosomes (M_E/R_{TOT}), one each of the antibodies specific for regions I (PAB 109) and IV (PAB 101) became less reactive, and two of the five antibodies specific for region III (PABs 102 and 103) also became less reactive. These results suggest that there are changes in several of the determinants in all three antigenic regions of TAG bound to mature chromosomes compared with replicating chromosomes. Thus, the structure of TAG bound to mature SV40 DNA appears different from that bound to RI. PAB 100, which recognizes a region III determinant, was poorly reactive with free TAG as well as TAG bound to either replicating or mature chromosomes. Based on the reactivity with the different TAG-containing fractions and the cross-reactivity with BK virus TAG, at least nine different antigenic determinants are distinguishable.

PAB 122 was as reactive with viral chromosomes as with free TAG (about 10% of tumor serum). Thus, the proportion of TAG-p53 complexes associated with viral chromosomes did not appear to increase compared with the total TAG population. Although there does appear to be about three

FIG. 6. Immunoadsorption of labeled SV40 chromosome-TAG complexes with anti-T with PVC plates. Various amounts of replicating 90S chromosomes labeled for 5 min with [³H]thymidine were incubated with 50 μ l of monoclonal antibody in RAM-coated wells as described in Materials and Methods. Input counts were 2,000 cpm. The amount of radiolabeled DNA bound per well was measured. PABs 100 (●), 102 (○), 106 (△), 107 (◇), and NSI (◆) are shown.

times less p53 bound to replicating chromosomes compared with mature chromosomes, comparison of such small numbers may not be very significant.

Immunoadsorption of labeled SV40 chromosome-TAG complexes with anti-T and RAM bound to microtiter wells: PVC-dependent RIA. A second immunoassay using an antibody directed against murine IgG and immobilized on PVC plates as the immunosorbent was developed to measure the reactivity of labeled viral chromosomes toward each monoclonal antibody (see Materials and Methods). The purpose was to compare the reactivity of labeled SV40 chromosomes in a protein A-independent assay with those with fixed *S. aureus* (see above). Microtiter wells previously coated with saturating amounts of RAM were incubated with gradient-purified 70S and 90S SV40 chromosome fractions (labeled for various times up to 12 h with [³H]thymidine and prepared as described above) together with a fixed amount of monoclonal antibody supernatant. After washing, the amount of labeled chromosomal DNA bound to each well was measured. One advantage of this assay was the ease with which large numbers of samples could be quickly assayed.

In preliminary experiments, the linearity of the response for various TAG-chromatin concentrations was tested. Up to 2 μ g of mature chromosomal DNA per well was added (8% of this fraction is bound to TAG). With increasing amounts of chromatin but a fixed amount of anti-T, saturation was reached (i.e., the amount of labeled chromatin bound to the well did not increase with the addition of more chromatin). Concentrations were chosen so that the amount of labeled chromosomal DNA bound to the RAM-coated well was proportional to the amount of chromatin added, indicating

that the amounts of bound RAM and added anti-T were in excess. Adding more anti-T had no effect. Therefore, when a fixed amount of chromatin within this linear range is used, differences in the amount of bound chromatin comparing each antibody should be due to differences in reactivity of the antibody with TAG complexed to the labeled DNA. The results are shown for various amounts of 5-min-labeled 90S chromosomes reacted with several of the monoclonal PABs (Fig. 6). Each coated well adsorbed about the same amount of labeled chromosome-TAG complex as when similar amounts of the same labeled sample and anti-T were immunoprecipitated with fixed *S. aureus*. Thus, this immunoadsorption assay appears to be as efficient as the protein A-dependent assay under these conditions.

The results with fixed amounts of replicating and mature chromosomes labeled up to 12 h and saturating amounts of each monoclonal antibody are shown in Fig. 7. The reactivity of each antibody was normalized with respect to PAB 106, because it was one of three antibodies (the others were PABs 108 and 104) consistently reactive with chromosomes of all ages when compared with tumor serum in the protein A-dependent assay. Furthermore, PAB 106 was consistently one of the most reactive of the monoclonal antibodies in both the RIAs and the ELISA (see below). In this assay, similar to the protein A-dependent assay, most of the antibodies were highly reactive with replicating chromosomes. Again, the poorest-reacting antibodies were PABs 100 and 122. With increasing labeling time, several of the antibodies became significantly less reactive with viral chromosomes compared with PAB 106; these were PABs 101, 102, 107, and 109. This was especially apparent for very long times (12 h) for PAB 101, where a fivefold decrease was observed with increasing age of the viral chromosomes. In contrast, other antibodies (PABs 104, 105, 106, and 108) had the same relative reactivity with older, long-labeled mature chromosomes as with short-labeled replicating chromosomes. These results are similar to those described above, with the exception that PAB 107 here was less reactive with chromosomes of increasing age. Quantitatively, PAB 109 was more reactive (about 1.5-fold), whereas PAB 104 was about 2.5-fold less reactive, with total RI in this assay compared with immunoprecipitation with PAB 106; PABs 105 and 103 were also both slightly less reactive in this assay.

Dissociation time for TAG bound to mature and replicating chromosomes. SV40-infected CV1 cells were labeled in vivo for either 5 min or 2 h with [³H]thymidine to label replicating or mature viral chromosomes, respectively. Nuclear extracts were fractionated into 70S mature and 90S replicating SV40 chromosomes on sucrose gradients, removing free TAG, which sediments at 5S to 25S (not bound to chromosomes and present in nuclear extract). The chromosomal DNA concentration in the 70S and 90S fractions was diluted about 5- and 35-fold, respectively, compared with an equivalent volume of nuclear extract (comparative ethidium bromide-stained samples electrophoresed in parallel on agarose gels; data not shown). The purified chromosomes were stored in ice (0°C). These were reacted at various times (from 1 h to 14 days after purification) with polyclonal tumor serum or with monoclonal PABs 108 or 106, for comparison with the two RIAs. As mentioned above, PABs 106 and 108 are relatively unaffected by the age of the viral chromosomes. The amount of labeled DNA in each of the two chromosome samples reactive with anti-T was determined.

The results with hamster tumor serum and the immunoprecipitation assay, measuring the reactivity with anti-T with increasing time after purification on sucrose gradients, are

FIG. 7. Relative reactivity of SV40 chromosomes of various ages with monoclonal antibodies in a PVC-dependent RIA. Both 70S and 90S chromosomes labeled with thymidine for various times in vivo were prepared as described in the legend to Fig. 5. A fixed volume (50 μ l) of each chromosome fraction was reacted with saturating amounts (usually 50 μ l) of each monoclonal antibody in a RAM-coated well. The amount of radiolabeled DNA in each chromosome fraction bound to anti-T was determined and normalized with respect to the value measured for PAb 106 (1.0). Peaks: R_{TOT} , 5-min-labeled 90S; M_{E1} , 30-min-labeled 70S; M_{E2} , 60- or 120-min-labeled 70S; and M_L , 12-h-labeled 70S. In one experiment with PAb 106, R_{TOT} input was 2,500 cpm, and 986 cpm was specifically adsorbed, giving a reactivity of 39%; M_{E2} input was 4,950 cpm, and 247 cpm was specifically adsorbed, giving a reactivity of 5%. Data shown are averages of three to six experiments. See the legend to Fig. 5 for antibody symbol key.

shown in Fig. 8. The same results were observed with all three antibody preparations and with both RIAs. The rate of loss of TAG reactivity was about fourfold slower for short-labeled 90S replicating chromosomes than for long-labeled 70S mature molecules at early times after purification (less than 4 days) under these conditions. Thus, TAG bound to mature chromosomes is significantly different from that bound to replicating chromosomes. Since polyclonal anti-T was used, this result indicates that TAG dissociates more quickly from mature than from replicating chromosomes. The curve for the mature chromosome-TAG complexes appeared biphasic. Replicating chromosomes may give a similar dissociation pattern if analyzed after longer times, but other changes may be occurring. About half of the TAG appeared to be lost from mature chromosomes after about 24 h at 0°C. Thus, care must be taken when analyzing gradient-purified mature chromosome preparations to ensure that significant dissociation of bound TAG has not already occurred.

Relative TAG content in subcellular fractions from infected cells. We measured the amount of TAG in each subcellular fraction obtained from infected monkey kidney cells (Fig. 1) by using an ELISA. Because the preferred reactive form of the adsorbed TAG complexes (free or chromatin bound) might vary with a given anti-T, four monoclonal antibodies, PAb 101, 102, 106, and 108, were used. These antibodies recognize different regions of TAG; PAb 108 and 106 are not sensitive to whether TAG is chromatin bound or free. The TAG fractions were used to coat microtiter plates at a range of concentrations and exposed to a saturating amount of

each of the four antibodies. The minimum volume of each fraction (cytosol, nuclear extract, nuclear pellet, and mature and replicating viral chromosomes) needed to obtain a maximum response in the ELISA was determined. The proportion that this volume represented of each cellular fraction was calculated and used to determine the relative amount of TAG in each fraction as a percentage of the total TAG in the cells. The results of reacting four different antibodies with each cell fraction were averaged together; four separate cell preparations were analyzed. Thus, a total of 16 data points were generated for each TAG fraction. Each of the four antibodies gave similar results.

There was about five times more TAG in the cytosol fraction compared with the nuclear extract fraction from infected cells. Similar results were obtained when the amounts of TAG in cytosol and nuclear extracts were quantified by immunoprecipitation with tumor serum followed by SDS-gel electrophoresis and comparison of stained TAG bands (data not shown). The cells appeared to contain about 60- $\pm$ 10-fold more free TAG than TAG bound to total viral chromosomes. The amount of TAG in the 90S chromosome fraction was consistently 1.2- $\pm$ 0.2-fold greater than that in the 70S fraction.

DISCUSSION

SV40 TAG complexes, free and bound to mature and replicating SV40 chromosomes, were isolated from SV40-infected CV1 cells. The relative reactivity of the TAG fractions was measured with saturating amounts of either a monoclonal anti-p53 or 1 of 10 monoclonal anti-T hybridoma

FIG. 8. Reactivity of labeled SV40 chromosome-TAG complexes toward anti-T tumor serum with increasing times after removal of free TAG. Short-labeled (3 to 5 min) replicating 90S and long-labeled (2 to 4 h) mature 70S SV40 chromosomes were fractionated on sucrose gradients (see Materials and Methods). The two chromosome fractions were stored in ice for 1 h to 14 days and analyzed for the amount of labeled chromosomal DNA associated with TAG by immunoprecipitation. The results with tumor serum and fixed *S. aureus* cells are shown for mature (M, ●) and replicating (R, ○) chromosome fractions and are expressed as a percentage of the maximum amount of labeled DNA bound to TAG at the first time point ($t = 1$ h).

supernatants. The antibodies specific for TAG each recognized one of three separate regions in TAG, including the N-terminal region I at 0.62 to 0.65 map units, the midregion III at 0.28 to 0.44 map units, and the C-terminal region IV at 0.17 to 0.28 map units. SV40 chromosome-TAG complexes were labeled with [3 H]thymidine and analyzed by using two different immunoassays, one protein A dependent and the other PVC dependent, both of which measure only those TAG molecules bound to labeled DNA. Both assays gave similar results. Free TAG in cytosols was analyzed by using the protein A-dependent assay followed by SDS-gel electrophoresis. The assays were all performed at pH 8 and with low amounts of detergent (0.05% NP-40).

All antibodies directed against the N-terminal (PABs 105, 108, and 109) and the C-terminal (PABs 101 and 107) determinants in free TAG were found to react as efficiently as saturating amounts of polyclonal hamster tumor serum. However, all of the midregion III antibodies (PABs 100, 102, 103, 104, and 106) were significantly less reactive than the tumor serum. In contrast, when short-labeled replicating viral chromosomes were analyzed, all but one of the monoclonal antibodies specific for TAG were equally or more reactive than tumor serum. Thus, the determinants in region III are now accessible to their respective antibodies; those in regions I and IV of the TAG molecule continue to be recognized when TAG is bound to replicating DNA molecules. With termination and separation of replicating molecules into mature chromosomes and with increasing age of the mature viral chromosome, several of the determinants in each of three different regions of TAG became increasingly less reactive. These included a region I site (PAB 109) and both region IV sites (PABs 101 and 107) as well as two others directed against region III (PABs 102 and 103). The two antibodies which cross-react with BK virus TAG were equally reactive with chromosomes of all ages; one recognized region I (PAB 108), whereas the other recognized region III (PAB 106). Nine different determinants were

identified in SV40 TAG based on changes in reactivity (Table 1) and the cross-reactivity with BK TAG.

PAB 100 recognizes a determinant in region III of TAG (10) and is sensitive to a mutation at residue 153 (27), although this amino acid lies outside the region III coordinates. PAB 100 has been reported to react preferentially with TAG bound to SV40 DNA in vitro but not with free TAG (31). Here, PAB 100 was poorly reactive toward all TAG fractions, including free forms and that bound to SV40 chromosomes of all ages.

When free TAG in cell extracts was immunoprecipitated with PAB 122 (specific for p53), about 10% of the total TAG was reactive and thus appeared to be complexed with p53. PAB 122 reacted to similar extents with mature and replicating SV40 chromosomes of all ages in the two RIAs. This suggests that p53 is not preferentially associated with either replicating or mature SV40 chromosomes. Although p53 could bind to viral chromosomes in a manner not recognized by PAB 122, the poor reactivity of PAB 122 with viral chromosomes is consistent with other results indicating that TAG-p53 complexes, present as 20S to 23S forms in SV40-transformed or infected cells, do not preferentially bind to SV40 origin DNA in vitro compared with uncomplexed forms of TAG (5, 28).

We found a 60-fold excess of free TAG compared with total chromatin-bound TAG in lytically infected cells by using an ELISA to quantify the amount of TAG. We also estimated the relative amount of free and chromatin-bound TAG in another way. Free TAG was quantified after immunoprecipitation of SV40-infected cell cytosols with anti-T tumor serum and analysis of stained TAG and standard protein (known concentration) bands on an SDS gel. The average amount of free TAG per 10^6 cells (10 different determinations) was estimated to be 3.5 μ g or 35 pmol (data not shown). The amount of chromatin-bound TAG was estimated from the average amount of SV40 DNA per 10^6 cells (10 μ g or 3.3 pmol) and the number of mature and replicating forms associated with TAG (35, 36) (see Results). SV40 RIs were observed to represent just 1.2% of the total viral DNA in an infected cell; about 72% of RI and 7.5% of mature DNA molecules are bound to TAG. Assuming one molecule of TAG per viral DNA-TAG complex, there would be 0.083 TAG molecules for every SV40 DNA molecule $[(0.012 \times 0.72) + (0.988 \times 0.075)]$. Thus, $3.3 \text{ pmol} \times 0.083 = 0.274 \text{ pmol}$ of bound TAG, which is 128-fold less than the amount of free TAG. Based on the viral DNA content and the amount of viral DNA complexed with TAG in each of the 70S and 90S fractions, we can estimate the relative amount of TAG bound to replicating compared with that bound to mature chromosomes (Fig. 1) (35, 36). There is sevenfold less viral DNA, on the average, in the 90S peak compared with the 70S peak. The 70S peak consists of mature SV40 DNA forms I, II, and III (94%) and a few early RIs (0.45%) that cosediment. About 90% of early RIs are complexed with TAG. Thus, $7[(0.075 \times 0.9955) + (0.90 \times 0.0045)] = 0.55$. In the 90S fraction, there are about 10 mature DNA molecules per RI, with about 60% of the RIs and 8% of the mature DNA molecules complexed with TAG. Thus, $(0.60 \times 0.10) + (0.08 \times 0.90) = 0.13$. From this, the 70S fraction is estimated to contain fourfold more TAG than the 90S fraction if the two types of viral chromosomes have equal numbers of TAG molecules bound per DNA-TAG complex, on the average. To be consistent with the 1.2-fold-greater amount of TAG present in the 90S compared with the 70S fractions as measured by ELISA (see Results), we estimate that each RI-TAG complex contains 4.8 times more TAG, on the average, than each mature DNA-TAG complex. Therefore,

the amount of TAg bound to total viral chromosomes $[(4.8 \times 0.012 \times 0.72) + (1 \times 0.988 \times 0.075) = 0.12]$ is 0.12/0.083 or 1.5 molecules of TAg per DNA-TAg complex; the amount of free TAg is estimated to be 60- to 88-fold greater than that bound to DNA.

The predominant form of free TAg in infected cell extracts has a middle region that is poorly reactive with specific corresponding antibodies. Based on changes in the reactivity of these antibodies, this region appears to be significantly altered structurally when TAg is bound to replicating SV40 DNA. Antigenic sites in region III are sensitive to SDS denaturation (16). This region contains the nucleotide binding site(s) (3, 9) and an ATPase-active site (7, 8). All of these activities appear to be important for viral replication. On further comparing the reactivity of the different forms of TAg, the structure of mature DNA-TAg complexes is different from either free or RI-associated TAg complexes. That TAg-RI complexes are different from TAg-mature DNA complexes is consistent with the four- to fivefold differential rate of TAg loss. TAg is more stable when bound to replicating chromosomes than with mature chromosomes. In addition, we estimated there is almost five times more TAg complexed with replicating molecules than with mature molecules by ELISA. Other results have also shown that TAg bound to replicating versus mature SV40 chromosomes is different and requires high (1.5 M or greater) salt for its extraction (32); 90% of the TAg bound to mature DNA is extracted by 0.3 to 0.4 M salt (32, 34). TAg bound to mature chromosomes may function predominantly in repressing early transcription, since its removal results in activation of early RNA synthesis with no effect on late RNA synthesis from viral chromosome templates (34). Together, these changes observed for the different TAg-containing species appear to reflect structural alterations in certain regions of TAg and presumably relate to the different functional states of chromatin-bound TAg during viral replication and, perhaps, transcription. The increased accessibility of TAg bound to replicating DNA molecules could also reflect facilitated recognition of TAg by enzymes such as DNA polymerase or other replication factors.

Possibilities accounting for changes in TAg of the reactivity of antigenic determinants for their respective antibodies are as follows: (i) alterations in some but not other regions of TAg after DNA binding due to conformational changes, covalent modification, or proteolysis; (ii) shielding of reactive sites in TAg due to binding of other molecules, for example, self-association of TAg; (iii) preferential binding of different structural subclasses of free TAg to SV40 DNA. Cytosol TAg from lytically infected cells is predominantly tetrameric (16S) in structure (2, 12), and the TAg units within this complex may be arranged in a way that shields a particular region. Thus, region III sites in free TAg may be poorly reactive with their corresponding antibodies because the determinants are inaccessible. TAg bound to replicating DNA may become more reactive through exposure of previously inaccessible sites, although it appears that monomer or dimer 5S to 7S forms (5, 14), rather than tetrameric or oligomeric forms, of TAg preferentially bind to bare SV40 DNA in vitro. Since TAg-DNA complexes represent a very small amount of the total TAg in a cell (35) (this paper), different structural subclasses of TAg could exist and associate with viral chromosomes. For TAg bound to mature DNA, two sites each in regions III and IV and one site in region I are less reactive. It is not clear whether TAg bound to mature chromosomes is a separate class or a precursor to that bound to replicating chromosomes.

That there appears to be more TAg bound to RI compared with mature DNA may reflect possible oligomerization of the TAg (different from that in cytosol) or that TAg is located at several sites on replicating DNA, i.e., replication forks and *ori* (31), compared with mature DNA. Recent analysis indicates that the stable association of TAg to origin DNA sequences requires interactions between several bound protein subunits to initiate DNA replication (20). Stepwise binding to DNA of TAg monomers which then interact with each other was proposed rather than binding of preformed dimers or tetramers of TAg to DNA. Regardless of the mechanism by which TAg binds and initiates viral DNA synthesis, our results clearly indicate that there are major structural differences in the three forms of TAg (free, replicating chromatin-bound, and mature chromatin-bound TAg) analyzed here.

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