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Analysis of Simian Virus 40 Chromosome–T-Antigen Complexes: T-Antigen Is Preferentially Associated with Early Replicating DNA Intermediates

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The fraction and DNA composition of simian virus 40 chromosomes that were complexed with large T-antigens (T-Ag) were determined at the peak of viral DNA replication. Simian virus 40 chromatin containing radiolabeled DNA was extracted by the hypotonic method of Su and DePamphilis (Proc. Natl. Acad. Sci. U.S.A. **73**:3466–3470, 1976) and then fractionated by sucrose gradient sedimentation into replicating (90S) and mature (70S) chromosomes. Viral chromosomes containing T-Ag were isolated by immunoprecipitation with saturating amounts of either an anti-T-Ag monoclonal antibody or an anti–T-Ag hamster serum under conditions that specifically precipitated T-Ag protein from cytosol extracts. An average of 10% of the uniformly labeled DNA in the 90S pool and 7.5% in the 70S pool was specifically precipitated, demonstrating that under these conditions immunologically reactive T-Ag was tightly bound to only 8% of the total viral chromosomes. In contrast, simian virus 40 replicating intermediates (RI) represented only 1.2% of the viral DNA, but most of these molecules were associated with T-Ag. At the shortest pulse-labeling periods, an average of 72 ± 18% of the radiolabeled DNA in 90S chromosomes could be immunoprecipitated, and this value rapidly decreased as the labeling period was increased. Electron microscopic analysis of the DNA before and after precipitation revealed that about 55% of the 90S chromosomal RI and 72% of the total RI from both pools were specifically bound to T-Ag. Comparison of the extent of replication with the fraction of RI precipitated revealed a strong selection for early replicating DNA intermediates. Essentially all of the RI in the 70S chromosomes were less than 30% replicated and were precipitated with anti–T-Ag monoclonal antibody or hamster antiserum. An average of 88% of the 90S chromosomal RI which were from 5 to 75% replicated were immunoprecipitated, but the proportion of RI associated with T-Ag rapidly decreased as replication proceeded beyond 70% completion. By the time sibling chromosomes had separated, only 3% of the newly replicated catenated dimers in the 90S pool (<1% of the dimers in both pools) were associated with T-Ag. Measurements of the fraction of radiolabeled DNA in each quarter of the genome confirmed that T-Ag was preferentially associated with newly initiated molecules in which the nascent DNA was nearest the origin of replication. These results are consistent with a specific requirement for the binding of T-Ag to viral chromosomes to initiate DNA replication, and they also demonstrate that T-Ag does not immediately dissociate from chromosomes once replication begins. The biphasic relationship between the fraction of T-Ag–containing RI and the extent of DNA replication suggests either that 1 or 2 molecules of T-Ag remain stably bound until replication is about 70% completed or that 4 to 6 molecules of T-Ag are randomly released from each RI at a uniform rate throughout replication.

Simian virus 40 (SV40) gene *A* encodes a 96,000-dalton (96K) polypeptide called large T-antigen (T-Ag) that is required for initiation of viral DNA replication and transformation of cells under normal growth control into cells with oncogenic characteristics (reviewed in references 8 and 28). Purified T-Ag binds specifically to nucleotide sequences in bare SV40 DNA that encompass the origin of DNA replication and the initiation sites for early gene transcripts, an interaction which appears critical to the function

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of T-Ag in the modulation of both of these viral activities (8, 28).

To examine the in situ association of T-Ag with viral chromosomes inside lytically infected cells, SV40 chromosomes have been characterized before and after reaction with tumor sera containing antibodies directed against T-Ag (21, 27, 30). These studies have indicated that 40 to 80% of replicating (90S) and 10 to 20% of mature (70S) SV40 chromosomes contained T-Ag (27, 30) and that T-Ag appeared to dissociate from chromosomes upon completion of viral DNA replication (30). This interpretation is complicated, however, by the facts that tumor sera can contain several antibody specificities and SV40 chromosomes contain many proteins (21, 24). Thus, precipitation may not have resulted from T-Ag alone. Furthermore, the previous methods of analysis have not permitted an accurate correlation between the presence of T-Ag and specific events in viral DNA replication. Therefore, we have extended these studies in three ways. First, immunoprecipitation of mature and replicating SV40 chromosomes was analyzed by comparing several tumor sera with monoclonal antibodies specifically directed against T-Ag (15). Second, DNA molecules from the anti–T-Ag precipitate, the anti–T-Ag supernatant, and the untreated chromosome fraction were quantitatively characterized by electron microscopy. In this way, circular and linear monomers, molecules containing replication forks replicated to various extents, and catenated and circular dimers were clearly resolved. All of these species are present in the 90S pool of replicating chromosomes (38), and several have been implicated as DNA replication intermediates (5, 8, 36, 39). Finally, the distribution of newly synthesized DNA in chromosomes containing T antigen was determined by restriction endonuclease mapping techniques. Our results demonstrate that immunologically reactive SV40 T-Ag is predominantly associated with early replicating DNA intermediates and is rapidly lost after 70% of the molecule has been replicated.

MATERIALS AND METHODS

Viruses and cells. SV40 derived from strain wt 800 (23) was grown at a low multiplicity of infection in an African green monkey kidney cell line, CV-1, as previously described (1). Experiments described below were done with cells infected with sufficient virus to give the maximum rate of viral DNA synthesis by 36 h postinfection (1, 35).

Preparation of SV40 chromosomes. SV40 DNA was radiolabeled by incubating infected cells at 37°C with [^{14}C]thymidine (Thd) (100 to 200 μCi per 150-mm dish; 50 mCi/mmol, Schwartz/Mann) from 24 to 36 h postinfection. At 36 h postinfection, [^{3}H]Thd (100 to 200 μCi per 150-mm dish; 50 Ci/mmol, New England Nuclear Corp.) was added to the culture medium, and incubation was continued for 2 to 120 min before floating the dishes on ice water to arrest DNA synthesis (38). DNA chromosomes were then prepared by the method of Su and DePamphilis (34, 35). Cells from 10 dishes were lysed in a hypotonic buffer consisting of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol at 0 to 2°C, and cytoplasmic and nuclear fractions were isolated. Nuclei were extracted with hypotonic buffer for 1 h in ice, and cytosol was immediately prepared by centrifugation at either 100,000 × *g* for 1 h or 30,000 × *g* for 30 min. About 40 to 50% of the total viral DNA (20 μg per 150-mm plate) that could be extracted by the method of Hirt (17) was recovered in the nuclear extract; a similar amount was recovered in the cytoplasmic fraction (35, 38). Viral chromosomes were then isolated by sedimentation of the nuclear extract through a 5 to 20% sucrose gradient (layered in four steps of 5, 10, 15, and 20% sucrose and allowed to diffuse for 1 h at 25°C and then for 2 h at 4°C) in the same buffered medium by using a Beckman SW27 rotor at 26,000 rpm for 195 min at 4°C. Radiolabeled DNA sedimented as two bands that represented replicating (90S) and mature (70S) SV40 chromosomes. Similar results (L. Tack and P. Beard, unpublished data) were obtained with a different CV-1 cell line, but extraction required longer times (8 to 10 h) with 1 mM disodium EDTA instead of 0.5 mM $MgCl_2$ to prevent cleavage of the viral chromosomes (2).

Immunoprecipitation. For analytical studies, 50 μl (10 μg of DNA/ml) of mature (70S) or 200 μl (2 μg of DNA/ml) of replicating (90S) SV40 chromosomes was incubated for 2 h at 0°C (unless stated otherwise) with increasing amounts of anti–T-Ag. All incubations contained final concentrations of 100 mM NaCl–10 mM EDTA–50 mM Tris-hydrochloride (pH 7.8)–0.1% Nonidet P-40 nonionic detergent (NET-NP40) in a final volume of 0.5 ml. Immunoadsorption was achieved by adding 100 μl of fixed *Staphylococcus aureus* suspension (The Enzyme Center, Boston, Mass., prepared according to the manufacturer, with NET-NP40 as the buffer) and continuing the incubation for 1 h at 0°C with occasional shaking. Immunoprecipitates were collected by centrifugation at 3,000 × *g* for 5 min at 0°C, and the pellets were then resuspended and washed twice with NET-NP40. Viral DNA was extracted from immunoprecipitates by incubating them at 65°C with 100 μl of 1% sodium dodecyl sulfate (SDS)–50 mM Tris-hydrochloride (pH 7.8)–1% β-mercaptoethanol for 2 h. *S. aureus* was removed by centrifugation at 10,000 × *g* for 5 min, and the radiolabeled DNA in the supernatant was quantitated either as total or as acid-precipitable radioactivity. Previous studies suggested using sheared CV-1 chromatin during the immunoprecipitation of viral chromosomes to reduce the background (30). In our hands, the background was reduced up to twofold, but the net radiolabeled DNA in the precipitate was reduced as much as 33%.

Three antibody preparations were tested. NIH-2 was an antiserum obtained from hamsters bearing tumors induced by an A2483-SV40-transformed cell line (National Institutes of Health). NIH-1 was a similar tumor serum generously provided by Valerie Setlow (National Cancer Institute, Bethesda, Md.). PAb101 was the medium produced by hybrid cell clone PAb101 (formerly clone 412 [15]) and generously pro-

vided by Elizabeth Gurney (University of Utah, Salt Lake City). Excess EDTA was necessary in immunoprecipitation reactions to prevent divalent cations normally present in monoclonal cell medium from precipitating viral nucleoprotein complexes in the absence of antibody. Control incubations consisted of substituting either preimmune hamster serum or medium from the parent hybridoma cell line, NS1, in place of NIH-2 or PAb101, respectively. Normal hamster serum or NS1 medium was subsequently used to adjust immunoprecipitation reactions to a constant volume.

For preparative studies, 2 ml of the 90S or 70S SV40 chromatin pools was adjusted to contain NET-NP40 and then incubated with either 40 μl of NIH-2 or 1 ml of PAb101 for 2 h at 0°C, followed by the addition of 200 μl of fixed *S. aureus* suspension. After gentle shaking for 2 h at 0°C, the immunoprecipitate was isolated by centrifugation and washed twice with NET-NP40, suspending the pellet by using a gentle vortex action. Viral DNA was extracted from the pellet by addition of 0.6% SDS–20 mM EDTA–10 mM Tris-hydrochloride (pH 7.8) followed by 1.1 M NaCl as described by Hirt (17). Viral DNA was purified as previously described (32, 38) from both the immunoprecipitate and the supernatant portion of the immunoprecipitation reaction and the original, untreated chromatin sample. After ethanol precipitation, the purified DNA was suspended in 100 μl of 10 mM Tris-hydrochloride (pH 7.8)–1 mM EDTA for analysis by gel electrophoresis, electron microscopy, and restriction endonuclease digestion. From 75 to 85% of the viral DNA in the immunoprecipitates was recovered in the Hirt supernatant, with about 80% of this DNA recovered after purification.

Viral chromatin in the cytoplasmic fraction released during the isolation of nuclei from infected cells was analyzed in the same way as was the chromatin prepared from nuclear extracts.

Analysis of immunoprecipitable proteins. Typically, 1 ml of cytosol obtained from one 150-mm-diameter dish of infected cells was immunoprecipitated with 500 μl of PAb-101 or 20 μl of NIH-2, followed by 200 μl of *S. aureus* suspension as described above. The pellets were washed once with NET-NP40. Proteins were then extracted with 100 μl of 2% SDS–50 mM Tris-hydrochloride (pH 7.8)–2% β-mercaptoethanol at 100°C for 5 min. *S. aureus* was removed by centrifugation, and 50 μl of the extract was analyzed by electrophoresis in a vertical SDS-containing 7.5% polyacrylamide gel slab (20) with an acrylamide/bisacrylamide ratio of 75:1. For total proteins, gels were stained overnight in 0.25% Coomassie blue in 50% methanol–10% acetic acid and then destained in 30% methanol–10% acetic acid before photography.

SV40-infected cells were also incubated with [^{35}S]methionine (13) followed by hypotonic lysis (34). The cytosol was immunoprecipitated as before, and the proteins were analyzed by gel electrophoresis followed by staining, destaining, and then fluorography (3). Pretreatment with iodoacetate (14) had no effect except to generate a slight increase in background.

Analysis of DNA. Purified SV40 DNA was fractionated by gel electrophoresis in vertical 2% agarose gel slabs as previously described (38). Electron microscopy of DNA was performed by the formamide spreading procedure (7), with the addition of 1 μg of ethidium bromide per ml to relax superhelical DNA (29). The extent of replication for each SV40 replicating intermediate DNA [SV40(RI) DNA] molecule was determined by measuring contour lengths with an Apple II computer and digitizing board. Restriction endonuclease digestion of DNA was performed first with *Taq*I for 1 h at 65°C, followed by *Msp*I and *Pst*I also for 1 h at 37°C under the conditions described by the manufacturer (New England Biolabs). Generally, 0.1 volume of 10× *Taq*I digestion buffer was added to the DNA sample, followed by 2 U of enzyme per μg of DNA. NaCl and KCl were added to give 100 and 5 mM, respectively, before addition of *Msp*I and *Pst*I. DNA concentrations were determined as previously described (38). Digestions were stopped with 20 mM EDTA–1% SDS, and DNA samples were analyzed by electrophoresis in 2% agarose gels (38). DNA bands stained by ethidium bromide were cut out of the gel and analyzed for radioactivity after incubation in Econofluor (National Diagnostics) containing 3% NCS (Amersham Corp.) at 37°C overnight in the dark.

RESULTS

Immunoprecipitation of unbound SV40 T-Ag with either monoclonal antibody or tumor serum. Three antibody preparations capable of reacting with SV40 T-Ag were tested for their specificity in an immunoprecipitation reaction with cytosol prepared from SV40-infected and mock-infected CV-1 cells. Cytosol was reacted with monoclonal antibody (PAb101), sera from hamsters bearing SV40-initiated tumors (NIH-1 or NIH-2), or preimmune hamster serum by using immunoprecipitation conditions given above. Proteins in the immunoprecipitates were analyzed by SDS-gel electrophoresis (Fig. 1). The only major proteins synthesized by virus-infected cells in the presence of [^{35}S]methionine that reacted with tumor sera were the 96K T-Ag and a 46K polypeptide that may be VP1 (lanes 8 to 11). When the gels were stained with Coomassie blue to reveal the total proteins present, the only viral polypeptide in the PAb101 immunoprecipitate (lanes 4 and 6) was T-Ag (96K); the other proteins were immunoglobulin G (IgG) heavy chain (50K) and IgG light chain (30K). In contrast, the NIH-2 immunoprecipitates (lanes 2, 3, and 5) contained many additional polypeptides. Some of these represent normal heterogeneity in serum IgG light chains (28 to 33K), whereas others may be viral polypeptides such as VP1 (46K), cross-reacting cellular proteins, or T-Ag breakdown products. For example, a dramatic increase in the amount of the 46K polypeptide was often observed when either NIH-2 (lane 2) or PAb101 (not shown) was used at 37°C. Under these conditions, T-Ag appeared to undergo degradation, releasing a major polypeptide of about 46K, because the same sample precipitated at 0, 25, and 37°C revealed increasing amounts of the 46K material with a corresponding decrease in the amounts of 96K T-Ag. NIH-1

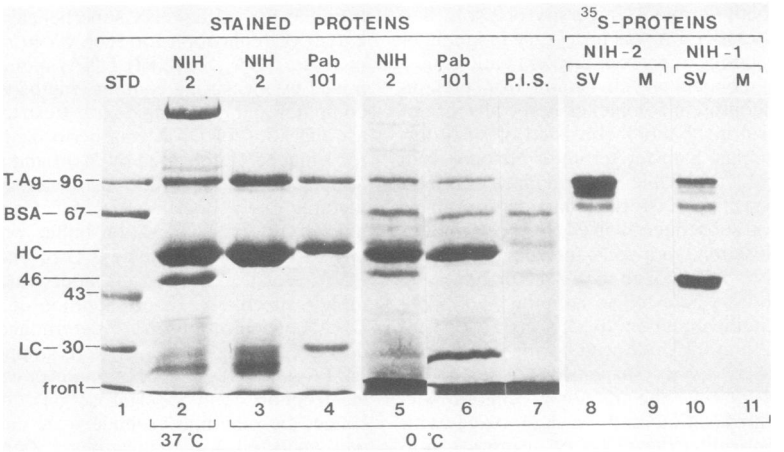

FIG. 1. Protein analysis of anti–T-Ag immunoprecipitates. Cytosol from SV40-infected CV-1 cells was immunoprecipitated at either 37°C (lane 2) or 0°C (lanes 3 to 11) with tumor serum (NIH-2), monoclonal anti–T-Ag (PAb101), or preimmune serum (P.I.S.) and then analyzed by SDS-gel electrophoresis. Lanes 1 to 7 were stained with Coomassie blue. Molecular weight standards (phosphorylase B, 96K; serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K [Pharmacia Fine Chemicals]) are shown in lane 1. Bovine serum albumin (BSA, 0.1%) was added to the precipitation reactions shown in lanes 5 to 7. The positions of T-Ag (T), IgG heavy chains (HC), IgG light chains (LC), and the bromphenol blue dye (front) are indicated. Cytosol from [^{35}S]methionine-labeled SV40-infected (SV) and mock-infected (M) CV-1 cells was immunoprecipitated with either NIH-1 or NIH-2 tumor serum at 0°C and then analyzed by SDS-gel electrophoresis. ^{35}S-labeled proteins were detected by fluorography (lanes 8 to 11).

serum always precipitated more of the 46K polypeptide (lane 10) than did NIH-2 (lane 8), even at 0°C, and therefore was not routinely used. Thus, optimal specificity of T-Ag precipitation was obtained at 0°C. Under the conditions used, saturating amounts of PAb101 removed at least 80 to 90% of the T-Ag that reacted with NIH-2 (consistent with the results of Gurney et al. [15]) without precipitating the 46K polypeptide.

Immunoprecipitation of SV40 chromosomes with anti–T-Ag. Both the monoclonal antibody, PAb101, and the tumor serum, NIH-2, were tested for their ability to immunoprecipitate SV40 chromosomes in conditions that were optimal for the immunoprecipitation of unbound SV40 T-Ag. Nuclear extracts containing SV40 chromosomes were prepared under hypotonic conditions that permit replicating SV40 chromosomes to faithfully continue DNA replication and chromatin assembly (33, 34) and that minimize disassembly of previrions back into species resembling mature viral chromosomes (8, 10, 12, 18). Extracts were then fractionated by sedimentation to separate replicating (90S) from mature (70S) viral chromosomes (Fig. 2). The 90S pool was enriched for DNA molecules rapidly labeled with [^{3}H]Thd, whereas the 70S pool contained molecules in which the [^{3}H]- or [^{14}C]Thd accumulated as the radiolabeling period was extended beyond that required for one round of DNA replication (about 20 min; reference 39). Electron microscopic analysis revealed that SV40(RI) DNA acounted for 6.2% of the DNA in the 90S pool, representing an enrichment of 21-fold over the concentration of replicating intermediates (RI) in the 70S pool (Table 1), in excellent agreement with our previous analysis of these chromosome fractions (38).

Conditions for precipitating the maximum amount of T-Ag–containing viral chromosomes were first determined by using 90S and 70S chromosomes that had been radiolabeled for 5 min. Samples containing about 0.5 μg of DNA were incubated with increasing amounts of PAb101, NIH-2, and control NS1 medium or preimmune serum. In this case, a maximum of 44% of the 90S chromosomes and 18% of the 70S chromosomes were precipitated by PAb101, whereas NIH-2 precipitated 54% of the 90S and 20% of the 70S chromosomes (Fig. 3). Control medium or serum precipitated 2 to 5% of the chromatin; these values were routinely subtracted from the amount of radioactivity in the corresponding anti–T-Ag precipitate before calculating the fraction of chromosomes associated with T-Ag. PAb101 consistently precipitated 83 ± 5% as much of the 90S chromosomes as did NIH-2; similar amounts of 70S chromosomes were precipitated by both antibodies. The fraction of 90S chromatin (labeled for 1 h) in the immunoprecipitate did not vary significantly from 1 h to 3 days of incubation with *S. aureus*. However, a similar experiment with 70S chromatin revealed

a threefold drop in immunoprecipitable radioactivity, suggesting that T-Ag may be less tightly bound to mature than to replicating SV40 chromosomes.

Since T-Ag is specifically involved in SV40 DNA replication and transcription, its association with viral chromosomes likely varies during the life cycle of the virus. Therefore, the fraction of SV40 chromosomes complexed with T-Ag was determined by using 90S and 70S chromosomes labeled with [^{3}H]Thd at 37°C in vivo for times ranging from 2 min to 12 h (Fig. 4). All samples were incubated with several different amounts of antibody to immune precipitate the maximum number of chromosomes. As illustrated in Fig. 3, a plateau was observed for each time point in Fig. 4. The fraction of both 90S and 70S chromosomes precipitable with anti-T-Ag rapidly diminished with increased time of radiolabeling. Approximately 80% of the 90S chromosome pool radiolabeled for the shortest times was precipitated by PAb101, and 95% was precipitated by NIH-2. However, the fraction of pulse-labeled chromosomes precipitated with either antibody was difficult to reproduce at the shortest labeling periods. Of the 10 samples radiolabeled for 3 to 4 min, an average of 72 ± 18% contained T-Ag. By 90 min of radiolabeling, this number had decreased to about 10%, at which level it remained even after 12 h of radiolabeling. Similarly, about 35% of the 70S chromosomes containing the shortest stretch of radiolabel reacted with anti-T-Ag, which rapidly decreased to a plateau of 7.5% after 1 h of labeling time. After correcting for the sixfold greater amount of DNA in the 70S pool, these data demonstrate that T-Ag is associated with only about 8% of the total viral chromosomes containing uniformly radiolabeled DNA: {[10(1) + 7.5(6)]/[100 + 100(6)]} × 100. However, most of the rapidly labeled 90S viral chromosomes (about 72%; Fig. 4A) contain T-Ag. That this number rapidly decreased with increasing times of radiolabel suggests that T-Ag is preferentially associated with SV40(RI) DNA early in replication.

Electron microscopic analysis of DNA from T-Ag–associated SV40 chromosomes. To accurately determine the structure of DNA molecules in T-Ag–containing SV40 chromosomes, DNA was purified from viral chromosomes before and after immunoprecipitation with either PAb101 or NIH-2 and analyzed by electron microscopy. Five distinct viral DNA structures were recognized in the electron microscope by their shape and size and identified as either SV40(RI) containing two replication forks, circular monomers that were either covalently sealed [SV40(I)] or contained an interruption in one strand [SV40(II)], full-length linear DNA [SV40(III)],

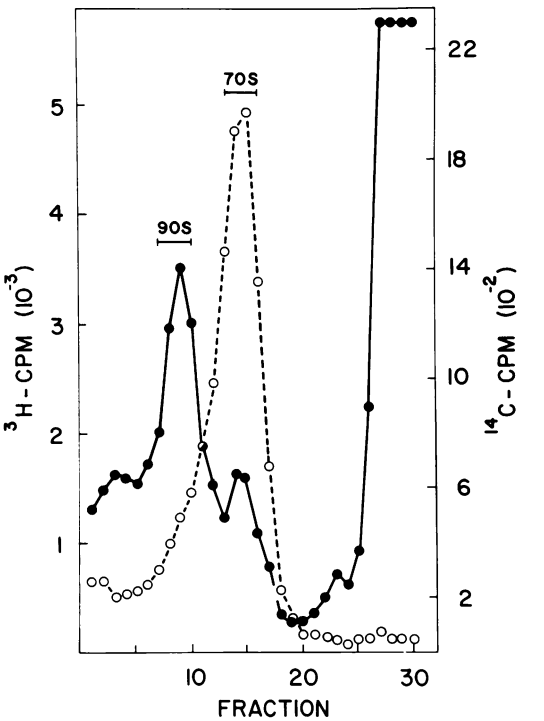

FIG. 2. Isolation of replicating and mature SV40 chromosomes. SV40-infected CV-1 cells were radiolabeled with either [^{3}H]Thd for 5 min or [^{14}C]Thd for 12 h. SV40 chromosomes were then extracted under hypotonic conditions and fractionated by sedimentation in sucrose gradients as described in the text. Total ^{3}H (●) and ^{14}C (○) label was measured in a sample of each fraction, and the fractions indicated were pooled to provide replicating (90S) and mature (70S) viral chromosomes.

two interlocked circular monomers (catenated dimers), or circular dimers (Fig. 5). The distribution of these molecules in the 90S and 70S pools before immunoprecipitation is shown in Table 1. A similar distribution was also inferred from the relative intensities of ethidium bromide-stained DNA bands after agarose gel electrophoresis of the same samples.

Before precipitation, SV40(RI) DNA in the 90S pool represented about 1.2% of the total viral DNA, a 21-fold enrichment over the RI in the 70S pool. In both chromosome pools, SV40(I and II) DNA accounted for at least 80% of the molecules. Catenated dimers were found in both pools at similar concentrations and represented 6% of the total DNA, but circular dimers were detected only in the 90S pool and accounted for 0.6% of the total DNA (see Appendix).

After immunoprecipitation, two changes in the DNA population were apparent. First, SV40(RI) DNA was preferentially precipitated. A 3.3-fold enrichment of RI was observed in the T-Ag–containing 90S chromosomes, and a ca.

TABLE 1. Distribution of SV40 DNA forms before and after immunoprecipitation with anti-T-Ag, as determined by electron microscopy[a]

DNA form	Distribution (%) of forms in chromosome fraction:					
	90S			70S		90S + 70S[b]
	Total	+T	−T	Total	+T	Total
RI[c]	6.2	20.3	1.8	0.3	5.6	1.2
Catenated dimers	8.3	1.4	5.3	5.5	<0.5	5.9
Circular dimers	4.1	5.4	2.5	<0.15	1.5	0.6
I, II, and III[d]	81.4	72.9	90.4	94.2	92.9	92.4
Molecules scored	1,549	1,254	2,125	670	197	

[a] Results were similar for three independent chromatin preparations. The fraction (%) of each form of SV40 DNA was determined with the total pool of chromosomes before immunoprecipitation (Total), with the anti-T-Ag immunoprecipitate (+T), and with the anti-T-Ag supernatant (−T). The amount of SV40 DNA in the 70S pool was sixfold greater than in the 90S pool, which allowed calculation of the total fraction of each DNA form (90S + 70S).

[b] See Appendix.

[c] Only molecules between 5 and 95% replicated were scored as RI.

[d] SV40(III) DNA represented only 2% of the total SV40 (I to III) DNA in the 90S chromosome pool and 6% in the 70S chromosome pool. It was most likely the result of endogenous endonuclease (8).

19-fold enrichment was found in T-Ag–containing 70S chromosomes. Since the RI in the 70S pool were 2- to 3-fold enriched for molecules less than 30% replicated, these data suggest a preferential association of T-Ag with chromosomes early in replication. Second, DNA molecules that had recently completed replication were preferentially free of T-Ag. Catenated SV40 dimers have been shown to be an immediate product of DNA replication which eventually dissociates into SV40(I and II) DNA (36, 37). The concentration of catenated dimers in the immunoprecipitate was about 6-fold less than in the total 90S pool and at least 11-fold less than in the total 70S pool. Thus, T-Ag appears preferentially associated with replicating intermediates, particularly those beginning replication, and T-Ag is preferentially lost by the time sibling chromosomes are separated.

The fraction of molecules containing T-Ag was estimated in two ways. The first method was to compare the DNA in the T-Ag immunoprecipitate with the DNA in the preprecipitated population. About 13% of all uniformly labeled 90S chromosomes were precipitated with anti–T-Ag; 10% were specific for anti–T-Ag serum or monoclonal antibody (Fig. 3 and 4). Table 1 provides the number of each molecular species present per 100 DNA molecules scored in the electron microscope. Assuming that nonspecifically precipitated DNA was representative of all molecules present, the fraction of RI in the 90S chromosome pool that contained T-Ag was ca. [20.3(0.1)/6.2] × 100 or 33%. The second method was to compare the DNA in the immunoprecipitate with the DNA in the immunosupernatant. Thus, the total RI present was 20.3(0.1) + 1.8(0.9) or 3.7 RI molecules per 100 molecules scored. Apparently, a significant number of RI were lost during the experiment. However, with this method, the fraction of RI containing T-Ag was [20.3(0.1)/3.7] × 100 or 55%. Although the reasons for this discrepancy are not clear, the second method seems more reliable since both the precipitate and supernatant were subjected to procedures from which the total chromosome population was spared.

Analysis of the uniformly labeled 70S chromosomes revealed a total of 10.5% of the chromosomes in the immunoprecipitate; 7.5% were specific for anti–T-Ag (Fig. 3 and 4). However, since the fraction of RI in the total chromosome pool was very small (only 2 RI were scored), greater than 100% of the RI appeared to have bound T-Ag. Assuming that the 5.6% RI in the 70S immunoprecipitate was a more accurate measure of the RI present and that essentially all of the RI had T-Ag, the 70S pool contained 5.6(0.105) or about 0.6% RI, as previously reported (38). Since the 70S pool also contained sixfold more DNA than did the 90S pool, the fraction of RI in both pools of chromosomes that was immunoprecipitated was ca. [6.2(0.55) + 0.6(6)/(6.2 + 3.6)] × 100 or 72%. By similar calculations, the fraction of catenated dimers containing T-Ag in the 90S chromosomes was estimated to be 3% in the 90S chromosomes and less than 1% in the total 90S plus 70S pools (see Appendix). The fraction of SV40(I and II) DNA associated with T-Ag was about 8.6% in the 90S pool, 7.4% in the 70S pool, and 7.6% of the total 90S plus 70S chromosomes (see Appendix).

The relationship between the extent of DNA replication and the presence of T-Ag was further analyzed by determining the relative fraction of SV40(RI) DNA at each stage in its replication

that appeared in the immunoprecipitate. Viral DNA was extracted, and only molecules between 5 and 95% replicated were scored by electron microscopy to avoid confusion of early SV40(RI) DNA with SV40(I and II) DNA and late SV40(RI) DNA with catenated dimers. In addition, both intact molecules and molecules cut once at a unique restriction site were analyzed to insure unambiguous identification of replicating DNA. Before immunoprecipitation, 90S chromosomes contained SV40(RI) DNA at all stages of replication, with 85- to 95%-replicated molecules 2- to 3-fold more prevalent than any equivalent sample of molecules earlier in replication (Fig. 6A). This was in excellent agreement with other analyses of either replicating chromosomes (31) or of the total SDS-extractable pool of SV40(RI) DNA purified by

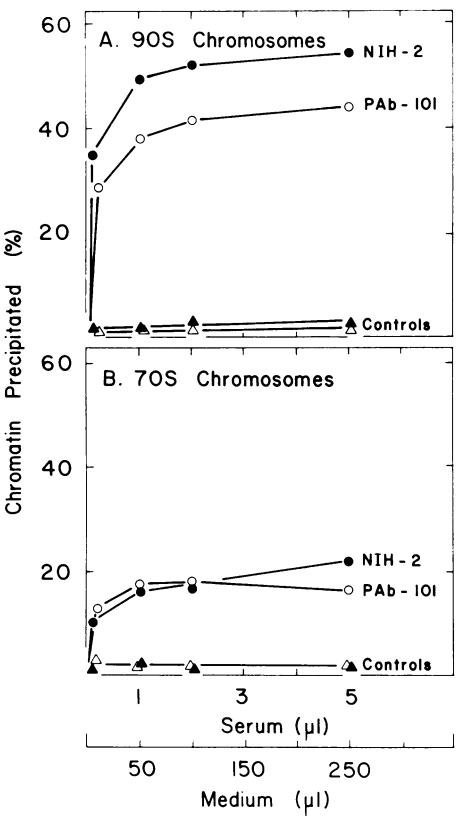

FIG. 3. Quantitative immunoprecipitation of T-Ag–containing SV40 chromosomes. Mature (70S) and replicating (90S) SV40 chromosome pools prepared from SV40-infected cells labeled for 5 min with [^{3}H]Thd were incubated with increasing amounts of monoclonal anti-T-Ag medium (PAb101 [○]), tumor serum (NIH-2 [●]), NS1 cell medium (△) or preimmune serum (▲). The fraction of labeled chromosomal DNA immunoprecipitated was measured as described in the text.

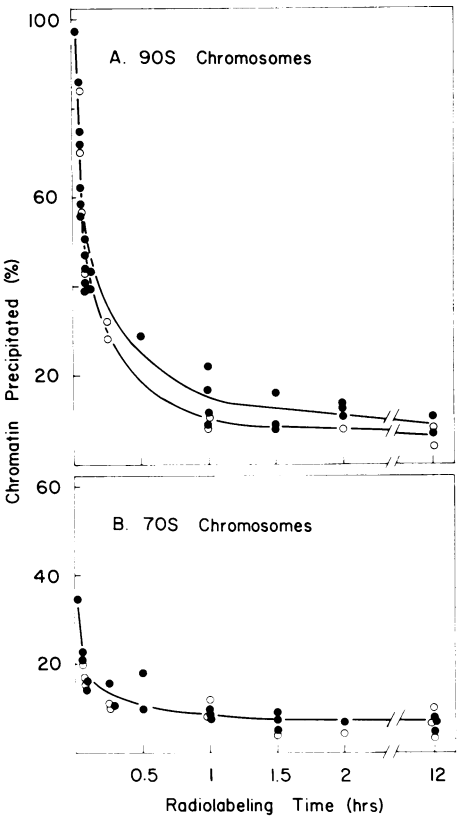

FIG. 4. Fraction of T-Ag–containing SV40 chromosomes as a function of time elapsed during and after DNA replication. Mature (70S) and replicating (90S) SV40 chromosome pools were prepared from SV40-infected cells incubated with [^{3}H]Thd for periods of time ranging from 2 min to 12 h. Samples were immunoprecipitated with saturating amounts of either monoclonal PAb101 (○) or NIH-2 (●). Data were corrected for the fraction of radioactivity precipitated by control medium or serum.

BND-cellulose chromatography (40, 41). After immunoprecipitation, SV40(RI) DNA was again found at all stages of replication, although the distribution of molecules had changed; the supernatant was enriched for late replicating molecules, and the precipitate was enriched for early replicating molecules (Fig. 6A).

To provide the relative number of RI bound to T-Ag at different stages in their replication, the fraction of RI found in the immunoprecipitate at each 10% increment in its replication was calculated from the data in Fig. 6A. The total fraction of RI in the anti-T-Ag immunoprecipitate from 90S chromosomes should be about 62%, based on the data in Table 1 ({20.3(0.13)/[20.3(0.13) + 1.8(0.87)]} × 100), and about 75%, based on the data in Fig. 4A (72% average precipitation + 3% nonspecific precipitation). Because of the quantitative variation among experiments, the num-

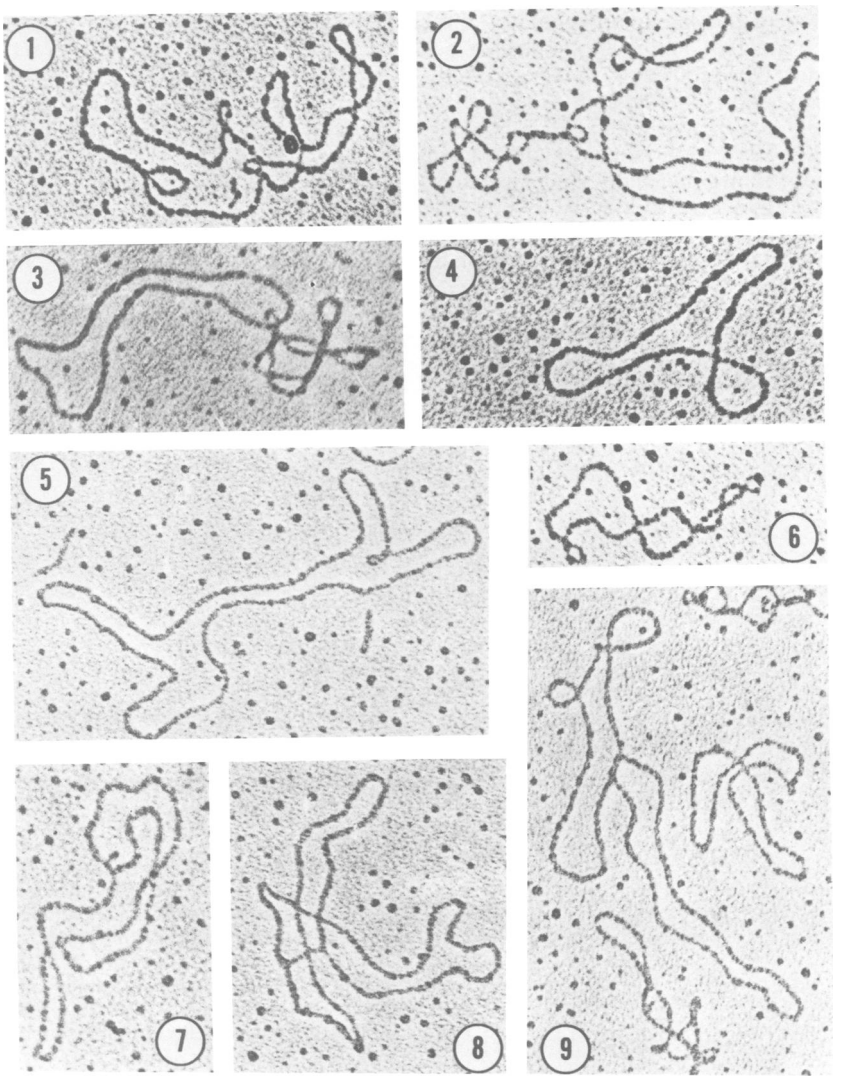

FIG. 5. Electron microscopic analysis of SV40 DNA prepared from 90S chromosomes. Superhelical (6 and 9) and relaxed (4 and 9) circular monomers are about 1.7 μm in circumference. Replication produces RI (7 to 9) which can generate catenated dimers (1 to 3) that may recombine into circular dimers (5). Identification and measurements were done on molecules enlarged about 35,000-fold.

ber of RI scored in each fraction was chosen to reflect the 76% of the total RI immunoprecipitated from both 90S and 70S pools ({[6.2(0.62) + 0.6(6)]/[6.2 + 0.6(6)]} × 100). The calculation revealed that about 88% of the RI that were less than 75% replicated were precipitated by anti–T-Ag. The data further indicated that about 96% of the earliest RI were bound to T-Ag (Fig. 6B). However, RI that had replicated more than an average of 70% rapidly lost their T-Ag, such that by 97% replication, only 17% of the RI were precipitated by anti–T-Ag. Since the total RI present in each fraction was included in these data, the total catenated dimers present in the 90S immunoprecipitate (Table 1) was calculated to be about 3.8% and included in Fig. 6B as molecules 100% replicated. Thus, at least 95% of the replicating chromosomes did not have T-Ag by the time sibling chromosomes were separated. Possible bases for this observation are discussed later.

Genomic location of nascent DNA in SV40 chromosomes. The electron microscopic analysis described above revealed the structures of SV40 DNA in chromosomes associated with T-Ag, regardless of whether or not these molecules were actively engaged in replication. The results suggested that T-Ag was preferentially associat-

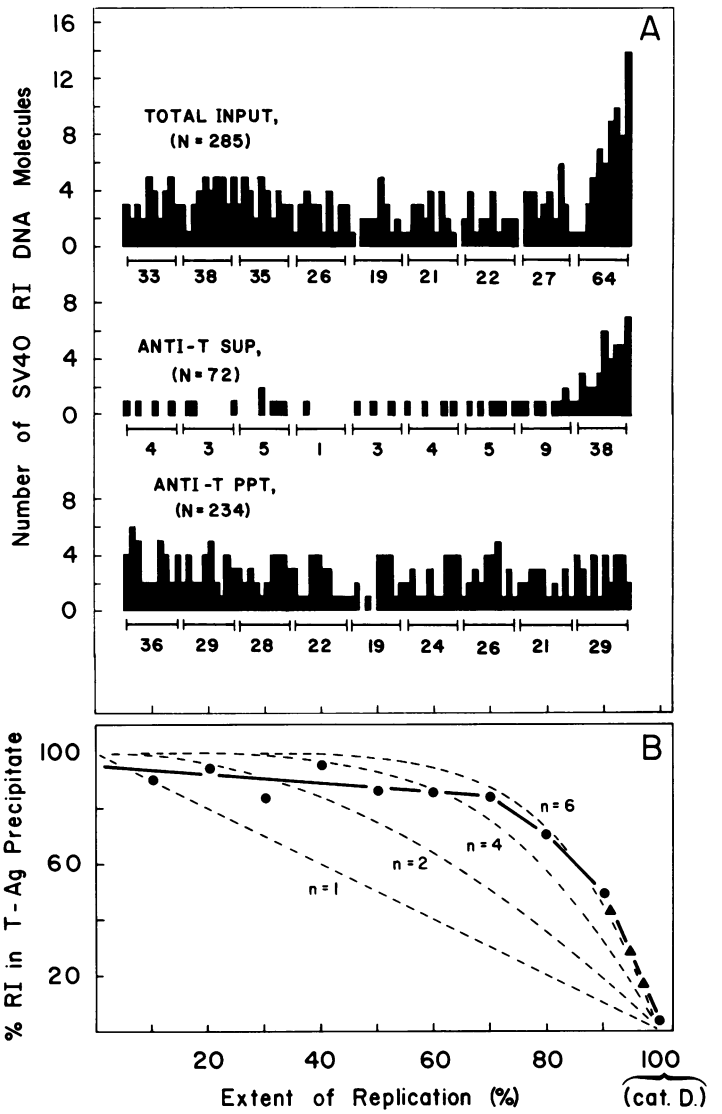

FIG. 6. Fraction of replicating SV40 chromosomes associated with T-Ag as a function of the extent of their DNA replication. DNA was purified from the 90S pool of SV40 chromosomes before and after immunoprecipitation with either PAb101 or NIH-2. (A) The number of SV40(RI) DNA molecules at each 1% increment in the extent of their replication was determined by electron microscopy (vertical bars) before immunoprecipitation (total input), for the immunoprecipitated DNA (anti-T ppt), and for the resulting supernatant fraction (anti-T sup). In addition, the number of molecules in each 10% increment of replication is indicated under the horizontal bars. The total number of molecules scored (N) was selected based on the relative number of RI in each sample (Table 1). (B) By using the data in (A) the fraction of replicating molecules containing T-Ag (●) was calculated for each 10% increment in the extent of their replication (i.e., 50% = 45 to 55% replication). To consider RI greater than 90% replicated, samples were digested with *BglI* restriction endonuclease to cut them once at the origin of replication, and molecules between 90 and 99% replicated were scored in 2% increments (▲). Finally, catenated dimers were scored as those molecules most recently completing replication and designated as 100% replicated. The total RI scored in the anti-T-Ag precipitate was 76% of the total RI in the precipitate plus the supernatant (A). Since this was similar to the total fraction of RI precipitated by anti-T (see text), the numbers indicated under the horizontal bars were used to calculate the fraction of T-Ag–containing RI at various extents of replication. The total fraction of catenated dimers in the anti–T-Ag immunoprecipitate of the 90S chromosomes was about $\{1.4(0.13)/[1.4(0.13) + 5.3(0.87)]\} \times 100 = 3.8\%$. The theoretical relationship between the fraction of RI bound to T-Ag and the extent of replication (dashed lines) was calculated from the expression $1 - X^n$ where X is the extent of replication, n is the number of T-Ag molecules per RI molecule, and the rate of loss of T-Ag is linear. Thus, X is the probability that a T-Ag will be released from the DNA. The presence of one T-Ag molecule per RI is assumed to be sufficient for precipitation of the RI.

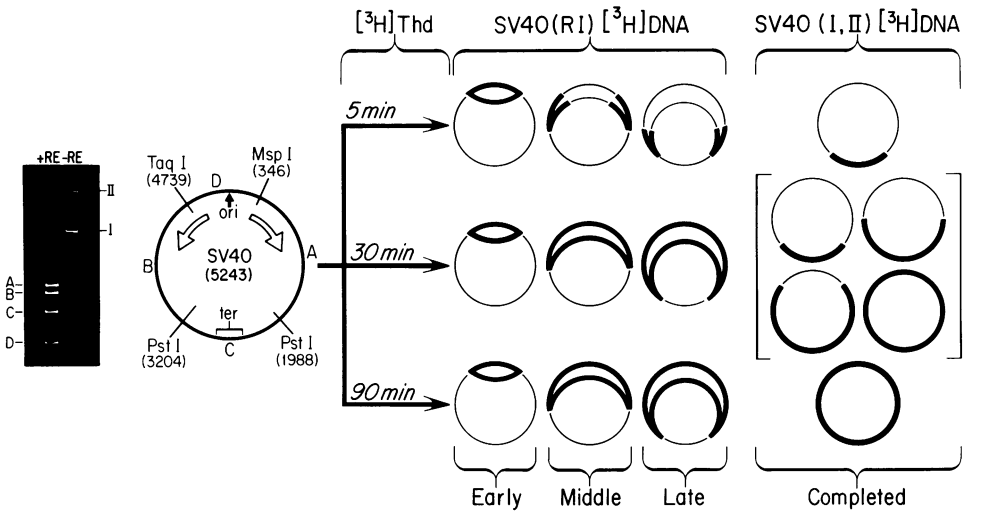

FIG. 7. Strategy for determining the genomic distribution of radiolabeled DNA in SV40 chromosomes. Viral DNA was isolated from replicating and mature SV40 chromosomes that had been labeled in cells with [^{3}H]Thd for various periods of time. The purified DNA was then completely digested with *Pst*I, *Msp*I, and *Taq*I to release four DNA fragments representing the origin region (*ori*, fragment D, 850 base pairs [bp]), the termination region (*ter*, fragment C, 1,216 bp), the early gene region (fragment B, 1,535 bp) and the late gene region (fragment A, 1,642 bp). These four fragments were fractionated by gel electrophoresis (see insert) and excised from the gel, and the amount of ^{3}H label per bp was measured. The expected regions of [^{3}H]DNA in early, middle and late RI [SV40(RI)], as well as molecules that have completed replication [SV40(I and II)], are indicated as darkened lines and are based on a replication rate of 145 bp per fork per min (39). Fragments A to D, which contain replication forks, are expected to run anomalously on agarose gels, resulting in a decrease in relative labeling for each fragment. However, since molecules replicated to different extents are equally present in the population, no qualitative difference in the results is predicted. Agarose gel analysis of DNA products purified from immune complexes before restriction enzyme digestion indicated that no conversion to linear molecules had occurred during the immunoprecipitation reaction.

ed with SV40(RI) DNA less than 70% replicated. This conclusion was tested further by determining whether or not T-Ag was preferentially associated with recently initiated DNA molecules. These molecules would contain newly synthesized DNA predominantly around the origin of replication. Thus, infected CV-1 cells were radiolabeled with [^{3}H]Thd for 5, 30, or 90 min or 12 h, and SV40 replicating (90S) and mature (70S) chromosomes were isolated as previously described (Fig. 2). DNA was purified from 70S and 90S chromosomes, and the genomic distribution of nascent [^{3}H]DNA was analyzed by the strategy outlined in Fig. 7. DNA samples were cleaved with *Taq*I, *Pst*I, and *Msp*I to generate four restriction fragments representing the origin region, the termination region, the early gene region, and the late gene region. These fragments were isolated by gel electrophoresis (Fig. 7) so that the amount of [^{3}H]DNA could be measured and then divided by the size of the respective restriction fragment to reveal the concentration of ^{3}H label in each genomic segment. About 20% of the SV40 genome becomes labeled during the relatively brief period of 5 min (39). In a steady-state population of bidirectionally replicating SV40(RI) DNA, this labeled region is found distributed throughout the genome, whereas in SV40(I and II) DNA, the [^{3}H]DNA is found only at the termination region in those molecules that just completed replication (26, 39). Radiolabeling cells for 30 min allows at least one complete round of replication to take place (39, 41). Because initiation of DNA replication continues during the 5- and 30-min periods, the concentration of [^{3}H]DNA in SV40(RI) DNA will be greatest at the origin and least at the termination region (26). Most of the RI are in the 90S pool. In contrast, recently completed SV40(I and II) DNA will contain a gradient of labeled nascent DNA that is greatest at the termination and least at the origin region. This occurs because molecules that were at earlier stages of replication at the beginning of the labeling period complete replication and join the SV40(I and II) DNA pool which is predominantly found in mature 70S chromosomes. Since bidirectional replication proceeds at similar rates in both directions (6, 11, 39), the amount of [^{3}H]DNA found on the early gene side should be

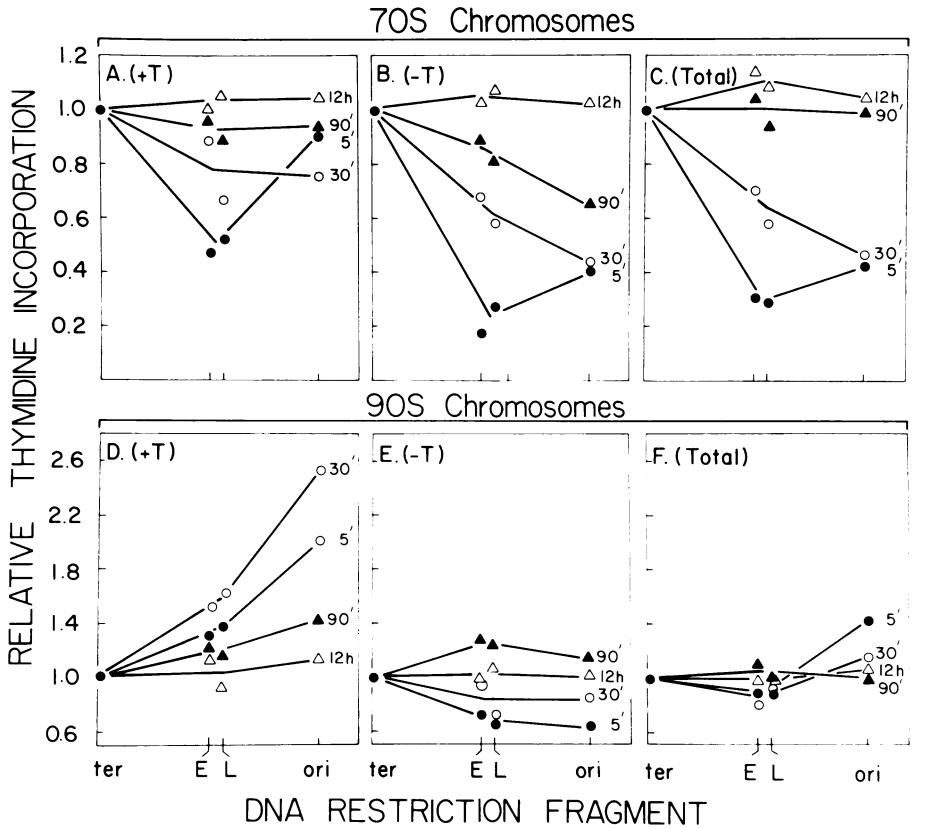

FIG. 8. The genomic distribution of radiolabeled nascent DNA in SV40 chromosomes with and without T-Ag present. Mature (70S) and replicating (90S) SV40 chromosomes were isolated from cells incubated with [^{3}H]Thd for indicated time periods. A portion of each of the total chromatin pools (C and F) was immunoprecipitated with NIH-2 to separate T-Ag–containing chromosomes (+T) (A and D) from non-T-Ag–containing chromosomes (−T) (B and E). The concentration of [^{3}H]DNA was determined in the regions where DNA replication is terminated (*ter*) and initiated (*ori*) as well as the early (E) and late (L) gene regions, as described in the legend to Fig. 7. The data were normalized relative to the termination region DNA restriction fragment (*ter*).

similar to that found on the late gene side of the origin of replication. As the labeling period is increased to 90 min and 12 h, the radiolabeled DNA becomes uniformly distributed throughout the genome.

The above analysis was carried out with viral DNA purified from both 90S and 70S chromosomes before and after immunoprecipitation of T-Ag–associated chromosomes. All data were normalized by comparing the [^{3}H]DNA concentrations in the replication origin, early gene, and late gene regions with that of the termination region (Fig. 8). Before immunoprecipitation, [^{3}H]DNA in mature (70S) chromosomes from 5- and 30-min labeling periods was preferentially located in the termination region (Fig. 8C), whereas replicating (90S) chromosomes from the same samples were enriched for [^{3}H]DNA in the origin region (Fig. 8F). The slight enrichment of label in the *ori* region of 70S chromosomes labeled for 5 min reflects the small amount of newly initiated RI previously observed in this sample (Table 1). After immunoprecipitation, both 70S and 90S T-Ag–containing chromosomes had from 2- to 2.5-fold more nascent [^{3}H]DNA in the origin region (Fig. 8A, D), relative to the total chromatin pools (Fig. 8C, F). Furthermore, non-T-Ag–containing 70S chromosomes remained preferentially ^{3}H labeled in the termination region (Fig. 8B), whereas the corresponding population of 90S chromosomes became enriched for DNA labeled in the termination region (Fig. 8E). As expected, chromosomes from cells labeled for 12 h with [^{3}H]Thd always contained uniformly labeled DNA, regardless of the presence or absence of T-Ag, demonstrating that the experimental procedures did not bias the results.

The genomic distribution of newly synthesized DNA in SV40 chromosomes precipitated with anti-T-Ag (Fig. 8) confirmed the preference of T-Ag for early replicating DNA intermediates

as observed by electron microscopy (Fig. 6). Immunoprecipitation of the 90S chromatin pool, which is highly enriched for actively replicating DNA (Fig. 2, Table 1), revealed that T-Ag–containing chromosomes were predominantly those which most recently initiated replication, whereas the non-T-Ag–containing chromosomes represented those that most recently completed replication. Immunoprecipitation of the 70S chromatin pool corroborated these results. Since only 20% or less of the pulse-labeled DNA in this pool contained T-Ag (Fig. 4), the distribution of label in the non-T-Ag–containing sample (Fig. 8B) resembled that in the total sample (Fig. 8C). The T-Ag–containing chromosomes include both the small number of early replicating molecules whose sedimentation properties are still similar to mature chromatin and the small fraction of recently completed DNA molecules that may still be associated with T-Ag. Comparison of pulse-labeled T-Ag–containing 70S chromosomes (Fig. 8A) with the total 70S pool (Fig. 8C) reveals a twofold enrichment for molecules that recently initiated replication. The data obtained for non-T-Ag–containing 70S chromatin (Fig. 8B) and T-Ag–containing 90S chromatin (Fig. 8D) are similar to those obtained from purified SV40(I) DNA (26, 39) and SV40(RI) DNA (26), respectively, when genomic fragments of the same size are analyzed.

DISCUSSION

Interaction of the large viral T-Ag with *ori* region DNA sequences has been demonstrated to be required for initiation of SV40 DNA replication (reviewed in reference 8). However, the normal association of T-Ag with viral chromosomes during a lytic infection has not been well defined. To this end, we prepared SV40 chromosomes from virus-infected CV-1 cells during the peak of viral DNA replication, using hypotonic conditions that permitted continued chromosome replication and minimized previrion breakdown to analyze the fraction and DNA composition of viral chromatin that was immuno-precipitated by anti–T-Ag. Viral chromatin was first fractionated into 90S chromosomes enriched for replicating molecules and 70S chromosomes enriched for mature, nonreplicating DNA. Conditions were selected to insure that precipitation of viral chromosomes resulted only from interaction of anti–T-Ag antibodies with chromosome-bound T-Ag. This procedure included using a monoclonal anti–T-Ag PAb101, which specifically recognizes the carboxyl terminus of T-Ag (9). This antibody consistently precipitated 83 ± 5% as much of the 90S chromosomes and essentially the same amount of 70S chromosomes as did NIH-2 tumor serum. Data from both anti–T-Ag preparations resulted in the same conclusions.

Based on the fraction of uniformly radiolabeled DNA that could be immunoprecipitated by saturating amounts of anti–T-Ag, T-Ag was tightly bound to only 8% of the total isolated viral chromosomes. However, 25 ± 5% of the chromosomal DNA in an unfractionated nuclear extract can be immunoprecipitated (Tack and Beard, unpublished data), indicating that T-Ag may be less tightly bound to an additional 17% of the chromosomes. Since the cytosol prepared from SV40-infected CV-1 cells contained about 100-fold more free T-Ag than was bound to isolated viral chromosomes (unpublished data), T-Ag binding must be limited either by the availability of DNA-binding sites or by a small active subpopulation of T-Ag. Since PAb101 preferentially binds mature rather than newly synthesized T-Ag (4) and was nearly as effective as tumor serum in precipitating viral chromosomes, T-Ag–containing viral chromosomes do not appear to prefer newly synthesized T-Ag. Electron microscopic analysis of SV40 DNA in a nuclear extract revealed that only 1.2% (Table 1) to 1.5% (38) of the molecules are RI. Therefore, the remaining 6 to 7% of T-Ag–containing chromosomes appear to contain nonreplicating DNA molecules that either had just completed or were about to begin replication. Alternatively, since T-Ag can repress transcription of early SV40 genes (16, 25), T-Ag–containing nonreplicating chromosomes may represent those DNA templates that are specifically available for late gene transcription.

T-Ag was preferentially bound to chromosomes containing early replicating DNA intermediates. The shorter the time that infected cells were incubated with [^{3}H]Thd, the greater the proportion of SV40 ^{3}H-labeled chromosomes that could be precipitated. Although 80 to 95% of the radiolabeled DNA could be precipitated at the shortest labeling periods, 72% was more typical. Variation in the fraction of ^{3}H-labeled chromosomes in the immunoprecipitate presumably reflects variation in the efficiency of precipitation and in the distribution of radiolabel among RI at various stages in their replication as a result of differences in their relative rates of fork movement (41). These differences may be enhanced by slight changes in the osmolarity of the culture medium (M. L. DePamphilis, L. E. Chalifour, M. F. Charette, M. E. Cusick, R. T. Hay, E. A. Hendrickson, C. G. Pritchard, L. C. Tack, P. M. Wassarman, D. T. Weaver, and D. O. Wirak, *in* N. R. Cozzarelli, ed., *Mechanisms of DNA Replication and Recombination*, in press). Nevertheless, pulse-labeled DNA was precipitated at least sevenfold more efficiently than was uniformly labeled DNA, demonstrating

that RI are preferentially associated with T-Ag. Electron microscopic analysis of the pre- and postprecipitated materials revealed that at least 55% of the RI in the 90S chromosome pool and 72% of the RI in both the 90S and 70S pools were specifically immunoprecipitated with anti–T-Ag. Since all of the RI in the 70S pool were less than 30% replicated and were concentrated in the anti–T-Ag immunoprecipitate, T-Ag appeared to be preferentially associated with early RI. This hypothesis was confirmed in two ways. First, a detailed analysis of the fraction of RI precipitated from 90S chromosomes as a function of the extent of its replication revealed a biphasic curve (Fig. 6B) that demonstrated that T-Ag was preferentially bound to RI which were less than 75% replicated. About 88% of the RI between 5 and 75% replicated contained T-Ag, but once these molecules had completed an average of 70% replication, T-Ag was rapidly lost. Only 17% of the RI at 97% replication and 3.8% of the catenated dimers (defined as 100% replication [36, 37]) were found in the anti–T-Ag immunoprecipitate; less than 1% of the total catenated dimers in both 90S and 70S pools specifically contained T-Ag. Second, analysis of the genomic distribution of newly synthesized DNA in molecules with and without bound T-Ag revealed a pronounced enrichment of newly replicated origin regions (16% of the genome) in T-Ag–containing chromosomes with a corresponding enrichment for newly replicated termination regions (23% of the genome) in the non-T-Ag–containing chromosomes. Thus, T-Ag binds to the *ori* region to initiate DNA replication, but once this event has occurred, T-Ag remains until about three-fourths of the DNA has replicated.

Theoretical plots were calculated (dashed lines, Fig. 6B) to show the fraction of RI that would be expected to precipitate, assuming different amounts of T-Ag per DNA molecule and a random but linear rate of T-Ag loss. Under these conditions, a biphasic curve will be observed only if 4 to 6 molecules of T-Ag are present on each RI. Alternatively, if 1 or 2 molecules of T-Ag per RI are present, then T-Ag dissociation would have to be delayed until about 70% of the RI have replicated. One complication is that the rate of DNA replication slows down after 70% of the molecule is completed (40, 41). Thus, if T-Ag is released at a linear rate throughout replication, the fraction of RI without T-Ag will rapidly increase as the rate of replication slows down, but the rate of T-Ag release remains constant. Possible mechanisms for T-Ag release include structural changes either in the T-Ag binding site or in T-Ag itself that normally occur during chromosome replication, or DNA sequences that promote T-Ag dissociation as they are reeled through a T-Ag–DNA replication complex that may involve both forks. The latter notion suggests that T-Ag may be released from *ori* sequences after initiation, but remains associated with the replication complex at replication forks. An alternative hypothesis to the dissociation of T-Ag from viral chromosomes during their replication is the blocking of T-Ag antigenic determinants either temporarily by masking with chromosomal protein rearrangements or permanently through chemical modifications. However, it seems unlikely that all antigenic sites would be masked from the seven different monoclonal antibodies tested so far (unpublished data) and the NIH-2 antiserum which presumably is directed against most, if not all, of the sites.

The loss of T-Ag from replicating chromosomes may have biological significance. The newly vacated T-Ag binding sites in recently replicated chromosomes may be more accessible to new T-Ag binding and thus explain why newly replicated SV40 DNA is a preferred template for initiation of DNA replication (43). Similarly, newly replicated chromosomes may be preferentially used for transcription of early viral genes, since these molecules can either bind T-Ag and initiate DNA replication while concomitantly blocking early gene transcription (16, 25) or bind RNA polymerase and initiate early gene transcription. The relative amount of T-Ag to DNA would determine which pathway is favored. In this way, DNA replication could continuously provide fresh templates both for new rounds of DNA replication and for synthesis of early gene products, despite the accumulation of T-Ag.

Several other observations are worth noting. About 1.5-fold more of the SV40 chromatin pulse-labeled for 5 min was immunoprecipitated from the cytoplasm fraction than from the nuclear extract fraction, suggesting a slight enrichment for early RI in the cytoplasm fraction. Otherwise, SV40 chromatin in the cytosol behaved the same as did SV40 chromatin in the nuclear extract. Electron microscopy of the 90S and 70S chromosomal DNA revealed that catenated dimers comprised 5.9% (Table 1) to 8.5% (38) of the viral DNA. The greater contribution of catenated dimers in hypotonically released chromosomes compared with SDS-salt extracts (41) is consistent with the observation that the SDS-salt extraction procedure (17) is inefficient at recovering oligomeric viral DNA (22). Finally, the pool of SV40 70S mature chromosomes, like that from polyoma infections (19), contains 0.3% (Table 1) to 0.6% (38) RI, most of it less than 30% replicated. The presence of these early RI could account for the apparent ability of mature polyoma chromosomes to initiate DNA

synthesis in the *ori* region when provided with the appropriate substrates and replication proteins (42).

APPENDIX

Calculations of the fraction of RI, catenated dimers, and circular dimers in the total population of SV40 chromosomes and the fraction of catenated dimers, SV40(I) and SV40(II), that contain T-Ag.

(a) % RI in 90S + 70S pools:
Table 1: $\{[6.2 + 0.3(6)]/[100 + 100(6)]\} \times 100 = 1.2\%$

Table 1 in reference 8: $\{[8.3 + 0.6(7.2)]/[100 + 100(7.2)]\} \times 100 = 1.5\%$.

(b) % Catenated dimers in 90S + 70S pools:
Table 1: $\{[8.3 + 5.5(6)]/[100 + 100(6)]\} \times 100 = 5.9\%$

Table 1 in reference 8: $\{[5.0 + 9.0(7.2)]/[100 + 100(7.2)]\} \times 100 = 8.5\%$.

(c) % Circular dimers in 90S + 70S pools:
Table 1: $\{[4.1 + 0]/[100 + 100(6)]\} \times 100 = 0.6\%$.

(d) % Catenated dimers that contain T-Ag:
90S pool: $\{1.4(0.1)/[1.4(0.1) + 5.3(0.9)]\} \times 100 = 2.9\%$

70S pool: $[0.5(0.075)/5.5] \times 100 = 0.68\%$

90S + 70S pool: $\{[2.9 + 0.68(6)]/[100 + 100(6)]\} = 1\%$.

The above is a maximum estimate because no catenated dimers were found (<0.5%) in the 70S immunoprecipitate (Table 1).

(e) % SV40(I) + SV40(II) containing T-Ag in the 90S pool: $\{[72.9(0.13) - 81.4(0.03)]/81.4\} \times 100 = 8.6\%$

70S pool: $\{[92.9(0.105) - 94.2(0.03)]/94.2\} \times 100 = 7.4\%$

90S + 70S pools: $\{[8.6 + 7.4(6)]/[100 + 100(6)]\} \times 100 = 7.6\%$.

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