Association of Simian Virus 40 T Antigen with Simian Virus 40 Nucleoprotein Complexes

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Viral nucleoprotein complexes were extracted from the nuclei of simian virus 40 (SV40)-infected TC7 cells by low-salt treatment in the absence of detergent, followed by sedimentation on neutral sucrose gradients. Two forms of SV40 nucleoprotein complexes, those containing SV40 replicative intermediate DNA and those containing SV40 (I) DNA, were separated from one another and were found to have sedimentation values of 125 and 93S, respectively. [³⁵S]methionine-labeled proteins in the nucleoprotein complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition to VP1, VP3, and histones, a protein with a molecular weight of 100,000 (100K) is present in the nucleoprotein complexes containing SV40 (I) DNA. The 100K protein was confirmed as SV40 100K T antigen, both by immunoprecipitation with SV40 anti-T serum and by tryptic peptide mapping. The 100K T antigen is predominantly associated with the SV40 (I) DNA-containing complexes. The 17K T antigen, however, is not associated with the SV40 (I) DNA-containing nucleoprotein complexes. The functional significance of the SV40 100K T antigen in the SV40 (I) DNA-containing nucleoprotein complexes was examined by immunoprecipitation of complexes from *tsA58*-infected TC7 cells. The 100K T antigen is present in nucleoprotein complexes extracted from cells grown at the permissive temperature but is clearly absent from complexes extracted from cells grown at the nonpermissive temperature and shifted up to the nonpermissive temperature for 1 h before extraction, suggesting that the association of the 100K T antigen with the SV40 nucleoprotein complexes is involved in the initiation of SV40 DNA synthesis.

All the viral DNA in simian virus 40 (SV40)- or polyoma virus-infected cells appears to be in the form of nucleoprotein complexes (11, 43). The majority of protein in these complexes is cellular histones, although there may be small amounts of the viral capsid proteins present (12, 15, 23). Viral nucleoprotein complexes isolated from SV40- or polyoma virus-infected cells with a nonionic detergent contain both replicating and mature supercoiled viral DNA (10, 11, 13, 43). Pulse-chase experiments indicate that the nucleoprotein complexes which contain replicative intermediate (RI) DNA are converted *in vivo* into nucleoprotein complexes containing mature supercoiled (I) DNA (13, 43). Viral nucleoprotein complexes extracted from the nuclei of SV40-infected cells by low-salt treatment have somewhat higher S values than detergent-extracted complexes and are capable of continued DNA replication *in vitro* (7, 8, 34). The (RI) DNA-containing complexes in this type of preparation are converted into SV40 (I) and SV40 (II) DNA-containing nucleoprotein complexes *in*

vitro. In contrast, the viral nucleoprotein complexes extracted with nonionic detergents appear to be defective in replicating DNA *in vitro* (34), perhaps due to removal of essential proteins by the detergent.

The early region of SV40 codes for a polypeptide with an apparent molecular weight of 85,000 to 100,000 (85 to 100K) (4, 21, 27, 30) and a small polypeptide of about 17K (27), both identified immunologically as SV40 tumor (T) antigens. Although the exact molecular weight of the large T antigen is probably near 85K (9), its apparent molecular weight estimated from its mobility in sodium dodecyl sulfate (SDS)-polyacrylamide gels changes considerably with the exact gel conditions (30). For the sake of consistency (21), we will call the large T antigen the 100K T antigen. This 100K protein is required for initiation of SV40 DNA synthesis (5, 35) and might, therefore, be expected to be a component of the viral nucleoprotein complexes, at least at some stage of the replication cycle. The precise function of the 17K protein is not known, although it may be essential for the establishment of transformation (2, 33).

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We have examined the SV40 nucleoprotein complexes extracted from infected cells in the absence of detergent for the presence of both the 100K and the 17K T antigens. In particular, we looked for the association of T antigen with either the SV40 (RI) DNA- or the SV40 (I) DNA-containing nucleoprotein complexes. Our results show that the 100K T antigen is predominantly associated with SV40 (I) DNA-containing complexes. The 17K T antigen, however, is not associated with either the SV40 (RI) DNA- or the SV40 (I) DNA-containing nucleoprotein complexes.

MATERIALS AND METHODS

Viruses and cells. Large-plaque, wild-type SV40 stock was prepared in TC7 cells grown in Dulbecco-modified Eagle minimum essential medium (DMEM) plus 10% fetal calf serum. SV40 *tsA58* (35) virus was obtained from Jim Robb; stock was prepared in TC7 cells grown at 32°C in DMEM plus 10% fetal calf serum. The wild-type SV40 stock had a titer of $>10^8$ PFU/ml, whereas the *tsA58* SV40 stock had a titer of 5×10^7 PFU/ml.

Infection and labeling of cells. TC7 cells were seeded on 9-cm plastic petri dishes and infected during the logarithmic phase of growth with either SV40 wild-type virus stock at a 1:10 dilution (multiplicity of 10 PFU/cell) or SV40 *tsA58* stock at a 1:5 dilution (multiplicity of 5 to 10 PFU/cell). After infection, all plates infected with SV40 *tsA58* were transferred to 32°C. Nucleoprotein complexes were extracted from wild-type-infected cells at approximately 38 h postinfection and from SV40 *tsA58*-infected cells at 72 h postinfection. Before extraction, plates of wild-type-infected cells were labeled for DNA either with 200 μ Ci of [3 H]thymidine per plate in 1 ml of Tris-buffered saline lacking Mg^{2+} and Ca^{2+} (specific activity, 6.7 Ci/mmol; New England Nuclear Corp.) for 3.5 min at 37°C or with 1 μ Ci of [14 C]thymidine per plate in 2.5 ml (specific activity, 49.7 mCi/mmol; New England Nuclear) for 21 h at 37°C. Other plates of wild-type-infected cells were labeled for protein in DMEM lacking methionine with 5% dialyzed fetal calf serum and containing 200 μ Ci of [35 S]methionine per plate in 2 ml (specific activity, >500 Ci/mmol; Amersham/Searle) for 5 h at 37°C. SV40 *tsA58*-infected cells were labeled with 200 μ Ci of [35 S]methionine per plate as described above, either for a total of 6 h at 32°C or for 5 h at 32°C, followed by a shift-up to 41°C for 1 h before extraction of the nucleoprotein complexes.

Extraction and sedimentation analysis of SV40 nucleoprotein complexes. SV40-infected cells were prepared for lysis essentially as described by DePamphilis et al. (6), except that the cells shifted up to 41°C were handled at 41°C until completion of trypsinization. The infected cells were suspended in hypotonic medium and lysed in a Dounce homogenizer (6). Viral nucleoprotein complexes were extracted by incubating the nuclei in 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol at 0°C for 1 h as described by Su and DePamphilis

(34). SV40 nucleoprotein complexes containing SV40 (RI) DNA were separated from those containing SV40 (I) DNA by sedimentation on linear 5 to 30% neutral sucrose gradients in 10 mM HEPES (pH 7.8), 5 mM KCl, and 0.5 mM $MgCl_2$ at 50,000 rpm for 50 min in a Beckman SW 50.1 rotor at 4°C (34). Portions (25 μ l each) of each fraction of the gradient were diluted with an equal volume of 2 $\times$ electrophoresis sample buffer (0.125 M Tris-hydrochloride, pH 6.8, 6% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue, 4 mM phenylmethylsulfonyl fluoride), and 10- μ l samples were applied per slot in an analytical SDS-polyacrylamide gel.

Sedimentation analysis and agarose gel electrophoresis of SV40 DNA. The nuclear extract containing the SV40 nucleoprotein complexes was treated with a final concentration of 1% SDS. SV40 DNA in the supernatant was analyzed by sedimentation on a linear 5 to 20% neutral sucrose gradient in 10 mM Tris (pH 7.5), 1 M NaCl, and 1 mM ethylenediaminetetraacetic acid. In addition, individual fractions in the peak of SV40 nucleoprotein complexes from the 5 to 30% neutral sucrose gradient were treated with 0.6% SDS and spun in a microcentrifuge at 10,000 rpm for 10 min at 4°C after precipitation of SDS in the cold. The SV40 DNA in the supernatants was then analyzed on 1.5% agarose gels run at 200 V for 3 h (36). Both nuclear extracts containing the SV40 nucleoprotein complexes and the residual nuclear contents were extracted by the method of Hirt (14) to determine the amount of SV40 DNA extracted from the nuclei and the amount of SV40 DNA still remaining in the nuclei. For this purpose, the nucleic acids present in the two fractions were recovered by phenol extraction and analyzed by sucrose gradient sedimentation as described above.

Immunoprecipitations. For the experiment shown in Fig. 2, 50- μ l portions of each fraction of the 5 to 30% neutral sucrose gradient were immunoprecipitated with SV40 anti-T serum (received from Jack Gruber, Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Md.) as described previously (21). The immunoprecipitates were dissolved in 50 μ l of sample buffer, and 10- μ l samples were applied per slot in an analytical SDS-polyacrylamide gel. For the experiment shown in Fig. 4, 75- μ l portions of the appropriate sucrose gradient fractions were made up to 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, and 10 mM sodium phosphate (pH 7), and 3 μ l of SV40 anti-T serum was added. After 1 h at 0°C, 750 μ g of fixed *Staphylococcus aureus* (17) was added, and the samples were left overnight at 4°C. The bacteria were pelleted at 2,000 rpm for 10 min at 4°C, before being washed as described (31). The immune complexes were eluted in 30 μ l of sample buffer, and 9- μ l samples were applied per slot. Anti-VP1 serum was a kind gift of Harumi Kasamatsu.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide (12.5%) slab gels were run as described by Laemmli (18) and Maizel (20) at a current of 12.5 mA. The radioactivity was detected by fluorography (19).

Tryptic peptide maps. The tryptic peptides of SV40 T antigen were prepared and analyzed on thin-layer cellulose plates as previously described (1).

RESULTS

Separation of SV40 (RI) DNA- from SV40 (I) DNA-containing nucleoprotein complexes. In parallel with the SV40 nucleoprotein complexes analyzed for protein content, we ran markers of nucleoprotein complexes containing labeled SV40 (RI) DNA and SV40 (I) DNA. For this purpose, SV40-infected cells were either pulse-labeled with [^3H]thymidine for 3.5 min to label SV40 (RI) DNA or labeled with [^{14}C]thymidine for 21 h to label SV40 (I) DNA. The two forms of SV40 nucleoprotein complexes extracted from these cells in the absence of detergent were separated from one another by sedimentation in a 5 to 30% neutral sucrose gradient (Fig. 1). The SV40 nucleoprotein complexes containing SV40 (RI) DNA sedimented as a 125S peak relative to ribosomal markers run in a parallel gradient, whereas the nucleoprotein complexes containing SV40 (I) DNA sedimented as a 93S peak. The sedimentation values of 125 and 93S for these two peaks differ from those of 90 and 70S obtained by Su and DePamphilis

FIG. 1. Sedimentation analysis of [^3H]- and [^{14}C]thymidine-labeled SV40 nucleoprotein complexes. Cells were infected with wild-type SV40 at 37°C and labeled, and nucleoprotein complexes were extracted as described in the text. Nucleoprotein complexes from approximately 4×10^6 cells were analyzed by sucrose gradient centrifugation as described in the text. A 75- μl amount of each fraction was analyzed for ^3H and ^{14}C by counting in an aqueous scintillator. In this and subsequent gradients, the first five fractions accounted for the dead volume of the collecting device and were discarded. The positions of 80S ribosomes and 60S and 40S ribosomal subunits run in a parallel gradient are shown.

(34). The reason for this discrepancy is not known, but different sedimentation markers were used.

Removal of the protein from the SV40 nucleoprotein complexes with SDS and sedimentation of the SV40 DNA in a 5 to 20% neutral sucrose gradient confirmed that all of the ^3H label was incorporated into SV40 (RI) DNA, whereas approximately 95% of the ^{14}C label was incorporated into SV40 (I) DNA, with the remainder incorporated into SV40 (II) DNA (data not shown). In addition, SV40 DNA fractions obtained by SDS treatment of nucleoprotein complexes from the individual ^{14}C peak fractions of the 5 to 30% neutral sucrose gradient were analyzed by agarose gel electrophoresis. The majority of the ^{14}C -labeled DNA was found to be SV40 (I) DNA with a minor fraction of SV40 (II) DNA in each of the individual fractions from the ^{14}C peak of the neutral sucrose gradient (data not shown).

Hirt extraction of the SV40 nucleoprotein complexes and of the nuclear contents remaining after extraction of the nucleoprotein complexes indicated that approximately 10% of the viral DNA was found in the nucleoprotein complexes, with 90% still remaining in the nuclei, either in unextracted nucleoprotein complexes or in assembled virions. The ratio of $^3\text{H}/^{14}\text{C}$ counts per minute in the Hirt extract of the nucleoprotein complexes was approximately 50% of the ratio of $^3\text{H}/^{14}\text{C}$ in the Hirt extract of the nuclei. It appears that nucleoprotein complexes containing SV40 (RI) DNA are somewhat less efficiently extracted than those containing SV40 (I) DNA, as has been noted by others (10). To ensure that our method for extracting nucleoprotein complexes yielded complexes capable of viral DNA replication *in vitro*, we incubated nuclear extracts containing nucleoprotein complexes from SV40-infected cells in an *in vitro* system containing cytosol and the four deoxyribonucleoside triphosphates, including [α - ^{32}P]deoxythymidine 5'-triphosphate. After incubation for 60 min, there was extensive incorporation of ^{32}P label into SV40 (I) DNA (data not shown).

Analysis of T antigen in SV40 nucleoprotein complexes. SV40 nucleoprotein complexes labeled with [^{35}S]methionine were analyzed by sedimentation in a 5 to 30% neutral sucrose gradient. A portion of each fraction of the gradient was subjected to SDS-polyacrylamide slab gel electrophoresis and fluorography to analyze the [^{35}S]methionine-labeled proteins present in the SV40 nucleoprotein complexes. In Fig. 2, fractions 11 through 14 correspond to the peak of SV40 nucleoprotein complexes which contain

FIG. 2. Sedimentation analysis of [^{35}S]methionine-labeled nucleoprotein complexes. Cells were infected with wild-type SV40 at 37°C and labeled with [^{35}S]methionine, and nucleoprotein complexes were extracted as described in the text. A part of each fraction was analyzed directly by SDS-polyacrylamide gel electrophoresis, and a further portion of each fraction was subjected to immunoprecipitation with anti-T serum before SDS gel electrophoresis. Shown are the results of analysis of fractions 6 through 28, the lower numbers referring to the bottom of the gradient as in Fig. 1; M refers to a marker track containing an immunoprecipitate of a pH 8 extract of [^{35}S]methionine-labeled SV40-infected cells. The positions of 100K T antigen, VP1, VP3, 17K T antigen, and the histones are indicated. The fluorogram in A was exposed for 16 h, whereas that in B was exposed for 160 h. (A) Fractions before immunoprecipitation; (B) fractions after precipitation with anti-T serum.

^{14}C -labeled SV40 (I) DNA, whereas fractions 8 through 10 correspond to the peak of nucleoprotein complexes which contain ^3H -labeled SV40 (RI) DNA. It is clear that there is a peak of [^{35}S]methionine-labeled proteins in fractions 11 through 15, whereas in fractions 8 through 10 there are relatively few labeled proteins. In the peak fractions, VP1, VP3, and three proteins of low molecular weight which correspond to histones, H2b, H3, and H4, can be seen. Histone H2a is detectable by staining in these fractions, but since it lacks methionine it is not seen in the autoradiogram. It should also be noted that because the histones contain very few methionine residues, whereas VP1 is rich in methionine, the absolute amount of histones present in the nucleoprotein complexes relative to VP1 is greatly underrepresented by the autoradiogram. In addition to VP1 and the histones, the nucleoprotein complex region of the gradient also contains a protein of 100K which migrates in the same position as SV40 T antigen immunoprecipitated from pH 8 extracts of SV40-infected cells (Fig. 2A).

The 100K protein present in the SV40 nucleoprotein complexes was analyzed further by immunoprecipitation of a portion of each fraction across the gradient with serum from hamsters with SV40-induced tumors (SV40 anti-T serum). In the immunoprecipitates of fractions 11 through 15, there is obvious enhancement of the 100K protein relative to the other proteins in the nucleoprotein complexes (Fig. 2B), and we conclude that this protein is the SV40 100K T antigen. It should be noted that VP1, VP3, and the histones are present in the immunoprecipitates of the nucleoprotein complexes (fractions 11 through 15). Although the majority of the 100K T antigen is found associated with the nucleoprotein complexes containing SV40 (I) DNA, there is some 100K T antigen in the region of the gradient where SV40 (RI) DNA-containing complexes sediment (fractions 8 through 10). In addition to enhancement of the 100 K protein, there is also enrichment of a protein with an apparent molecular weight of 85K, similar to one of the proteins found in the immunoprecipitate from extracts of SV40-infected cells. It is not known whether the 85K protein is generated from the 100K protein during extraction of nucleoprotein complexes, as has been found in pH 8 extracts of SV40-infected cells (37), or whether it is of functional significance in the nucleoprotein complexes. The majority of the 100K T antigen is present in the immunoprecipitates of the upper fractions of the neutral sucrose gradient (Fig. 2B). The peak of [^{35}S]methionine-labeled T antigen in fraction 23 sediments at

about 10 to 15S, similar to the values previously reported for T antigen extracted from lytically infected cells (24, 42). In the immunoprecipitates of these upper fractions, VP1 is present but VP3 and the histones are not. The lower-molecular-weight protein seen in the immunoprecipitates of this region is the 17K T antigen, which did not sediment into the gradient appreciably. Comparison of these fractions with the rest of the gradient indicates that the 17K T antigen is not present in the SV40 nucleoprotein complexes containing either SV40 (RI) DNA or SV40 (I) DNA.

The peak fractions containing the SV40 (I) DNA nucleoprotein complexes were pooled to prepare [^{35}S]methionine-labeled 100K T antigen. The tryptic peptides of the nucleoprotein complex-associated 100K T antigen were compared with those of the total SV40 100K T antigen immunoprecipitated from pH 8 extracts of SV40-infected cells. The methionine-containing tryptic peptides of both 100K T antigens were found to be very similar, with the exception of two additional peptides (X and Y) in the peptide map δf of the 100K T antigen from the nucleoprotein complex (Fig. 3). The relatedness of the two peptide maps was confirmed by analysis of a mixture of the two digests (data not shown). The peptide maps shown here are very similar to our previous maps of SV40 100K T antigen (21); the slight differences are due to the use of two different batches of trypsin.

Comparison of SV40 nucleoprotein complexes from *tsA58*-infected cells at permissive and nonpermissive temperatures. To determine the functional significance of the SV40 100K T antigen in SV40 nucleoprotein complexes containing SV40 (I) DNA, cells were infected with SV40 *tsA58*, a temperature-sensitive mutant in SV40 gene A, and [^{35}S]methionine-labeled nucleoprotein complexes extracted from cells grown at 32°C were compared with those extracted from cells shifted up to 41°C for 1 h by sucrose gradient analysis followed by immunoprecipitation. It was established by pulse-labeling with [^3H]thymidine that DNA synthesis had been completely shut off in the cells at 41°C after shift-up from 32°C for 1 h before extraction of the nucleoprotein complexes (data not shown). The positions of nucleoprotein complexes containing SV40 (I) and SV40 (RI) DNA from *tsA58*-infected cells grown at 32°C were determined in a parallel gradient and found to be identical to those for the wild-type nucleoprotein complexes (data not shown). Analysis of the nucleoprotein complexes before immunoprecipitation showed that the profiles of the other proteins (VP1, VP3, and four histones) from

FIG. 3. Tryptic peptide analysis of 100K T antigens. Tryptic digests of the 100K T antigen were prepared from the 100K band isolated from preparative gels of: (i) the immunoprecipitate of a pH 8 extract of [35 S]methionine-labeled wild-type SV40-infected cells and (ii) fractions 11 through 15 of the gradient shown in Fig. 2A. The digests were separated in two dimensions by electrophoresis at pH 4.7 from left to right towards the cathode and by chromatography from the bottom to the top. Approximately 5×10^3 cpm were applied in each case, and the peptide maps were exposed to Kodak NS5T X-ray film for 28 days. (A) Total 100K T antigen immunoprecipitated from a pH 8 extract of infected cells; (B) 100K T antigen associated with nucleoprotein complexes.

tsA58-infected cells grown at 32°C or grown at 32°C and shifted to 41°C were identical to those seen in the nucleoprotein complexes from wild-type-infected cells (data not shown). In addition, the amount of label associated with nucleoprotein complexes after labeling with [3 H]thymidine at 66 h postinfection for 6 h at 32°C was essentially the same as that found after labeling for 5 h at 32°C followed by 1 h at 41°C (data not shown). This indicates that the SV40 (I) DNA-containing nucleoprotein complexes in the *tsA58*-infected cells were still present and unaffected by the shift to 41°C, although presumably the SV40 (RI) DNA-containing complexes were absent.

The most significant result of our analysis is that 100K T antigen is present in the immunoprecipitates of the nucleoprotein complexes of the cells maintained at 32°C, but is not detectable in the nucleoprotein complexes of the cells shifted to 41°C (Fig. 4). It is important to note that the amounts of [35 S]methionine-labeled 100K T antigen and the other proteins at the top of the two gradients are the same, indicating that there had not been significant turnover of labeled 100K T antigen during the time the cells were at 41°C. In the cells grown at 32°C, most

of the 100K T antigen is associated with the nucleoprotein complexes containing SV40 (I) DNA (fractions 12 through 15), similar to the situation for the 100K T antigen from wild-type SV40-infected cells (Fig. 2). It can also be seen that VP1 and the histones are present in the immunoprecipitates of the nucleoprotein complexes from *tsA58*-infected cells grown at 32°C (Fig. 4B). We determined by densitometry that, in the nucleoprotein complexes of cells shifted to 41°C, the amount of VP1 decreased about 30%, whereas the amount of histones was reduced nearly fourfold (Fig. 4A). In contrast to the results with the *tsA58*-infected cells, the amount of 100K T antigen in the nucleoprotein complexes of wild-type SV40-infected cells was the same whether the cells were maintained at 32°C or shifted to 41°C (data not shown).

DISCUSSION

Our analysis of the viral nucleoprotein complexes extracted from SV40-infected cell nuclei by the method of Su and DePamphilis shows that there is a 100K protein associated with the nucleoprotein complexes. Immunoprecipitation with SV40 anti-T serum and tryptic peptide mapping indicate that this protein is essentially

FIG. 4. Comparison of [35 S]methionine-labeled nucleoprotein complexes in *tsA58*-infected cells grown at 32°C or grown at 32°C and shifted to 41°C. Cells were infected with *tsA58* at 32°C and labeled with [35 S]methionine as described in the text. At 71 h postinfection, half the plates were shifted to 41°C for 1 h before preparation of nucleoprotein complexes. The complexes from about 4×10^6 cells in each case were analyzed by sucrose gradient centrifugation. A portion of each gradient fraction was immunoprecipitated

identical to authentic SV40 100K T antigen. The association of the 100K T antigen with the nucleoprotein complexes appears to be specific and not due to trailing into the gradient, since there is a definite peak of 100K T antigen cosedimenting with the complexes containing SV40 (I) DNA (93S). Reconstruction experiments in which soluble [³⁵S]methionine-labeled T antigen, isolated from the top of a sucrose gradient of a low-salt nuclear extract, was mixed with gradient-purified nucleoprotein complexes and then reanalyzed by sucrose gradient centrifugation, did not show any specific association of the 100K T antigen with nucleoprotein complexes (data not shown). Thus, it appears unlikely that the 100K T antigen becomes associated with the nucleoprotein complexes during the extraction procedure. The lack of the 100K T antigen in nucleoprotein complexes in *tsA58*-infected cells shifted to the nonpermissive temperature also suggests that the association of the 100K T antigen with the complexes is a functional one.

The majority of the 100K T antigen in the nucleoprotein complex region of the gradient appears to be associated with complexes containing SV40 (I) DNA. There is, however, some 100K T antigen in the region of complexes containing SV40 (I) DNA. The SV40 (RI) DNA-containing complexes labeled in a short pulse contain primarily (RI) DNA at late stages of replication (22). Presumably there is a continuum of complexes at earlier stages of replication between this peak and the peak of complexes containing SV40 (I) DNA. Because the replicating molecules represent only a small fraction of the total pool of viral DNA, it is hard to decide whether the 100K T antigen in this region of the gradient is specifically associated with complexes containing SV40 (RI) DNA or is present for some other reason. Likewise, we cannot rule out that the 100K T antigen is also associated with viral transcriptional complexes.

The nucleoprotein complex region of the gradient appears to contain VP1, VP3, and the histones as well as the 100K T antigen and SV40 (I) DNA. This observation is substantiated by the results we obtained with immunoprecipita-

tion of gradient fractions with SV40 anti-T serum. Precipitation of the nucleoprotein complex fractions containing SV40 (I) DNA (fractions 11 through 14 in Fig. 2B) brought down the 100K T antigen, VP1, VP3, and histones, whereas precipitation of fractions from the top of the gradient only brought down the 100K and 17K T antigens and VP1. VP3 and the histones were not precipitated, even though they are present in these fractions before immunoprecipitation. The precipitation of VP3 and histones by anti-T serum in fractions from the nucleoprotein complex region of the gradient indicates that VP3 and the histones are associated either directly or indirectly with molecules recognized by anti-T serum. It seems likely that this is due to association of VP3, histones, and 100K T antigen with viral DNA. The VP1 in the immunoprecipitates of the gradient fractions may be nonspecifically rather than specifically brought down by the anti-T serum. However, there is a peak of VP1 in the nucleoprotein complex region of the gradient. The association of VP1 with nucleoprotein complexes has been noted by others (15).

The finding that the 100K T antigen is associated with nucleoprotein complexes containing SV40 (I) DNA has two possible interpretations. The first is that these nucleoprotein complexes are on their way to being packaged into mature virions. If this were the case, then one would expect to find 100K T antigen in SV40 virions, although perhaps just one molecule per virion. To examine this possibility, we purified SV40 virions from SV40-infected cells labeled with [³⁵S]methionine. The virions were examined directly by SDS gel electrophoresis and also dissociated with ethyleneglycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid and dithiothreitol (3) before immunoprecipitation and subsequent electrophoresis. There was no evidence for the presence of the 100K T antigen in the virions by either technique (data not shown), even though we should have been able to detect a single molecule of T antigen per virion. This suggests that those nucleoprotein complexes with which the 100K T antigen is associated are not in the process of being assembled into virions.

with anti-T serum as described in the text and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Shown are the immunoprecipitates of fractions 6 through 26 in each case. T refers to an immunoprecipitate of the nucleoprotein complex preparation before sucrose gradient fractionation. M is a marker track as for Fig. 2. The residues of fractions 13, 14, and 15 from the gradient of the nucleoprotein complexes of cells kept at 32°C were pooled, and 75 μ l was immunoprecipitated with anti-VP1 serum. This immunoprecipitate is shown in the track labeled anti-VP1. The positions of 100K T antigen, VP1, 17K T antigen and the histones are indicated. The fluorograms were both exposed for 21 days. (A) Immunoprecipitates of gradient fractions of nucleoprotein complexes from *tsA58*-infected cells grown at 32°C and shifted to 41°C. (B) Immunoprecipitates of gradient fractions of nucleoprotein complexes from *tsA58*-infected cells grown at 32°C.

The second possible reason for the association of 100K T antigen with mature nucleoprotein complexes is that they are intermediates in the initiation of viral DNA synthesis. Suggestive evidence for a functional role of the 100K T antigen associated with the nucleoprotein complexes comes from our study of nucleoprotein complexes from SV40 *tsA58*-infected cells grown at 32°C and from cells shifted from 32°C up to 41°C for 1 h before the extraction of complexes. It is known that *tsA* mutants are temperature-sensitive for the initiation, but not for the completion, of SV40 DNA synthesis at 41°C (5, 35). The presence of 100K T antigen in nucleoprotein complexes from cells grown at 32°C contrasted with its absence from complexes derived from cells shifted up to 41°C implies that the association of the 100K T antigen with the nucleoprotein complexes is functional. The lack of binding to viral DNA at 41°C is consistent with the observation of Tegtmeyer et al. (38), who found, by immunofluorescent staining, that T antigen had a predominantly cytoplasmic location in *tsA58*-infected cells, as opposed to the normal nuclear location found in wild-type-infected cells. In addition, partially purified T antigen from *tsA*-transformed cells is more thermolabile than the wild-type T antigen in its ability to bind to SV40 (I) DNA in vitro (39).

Partially purified T antigen is known to bind to SV40 DNA in vitro at or near the origin of DNA replication (16, 28, 40) and also binds to SV40 chromatin in vitro (25). In this regard, it would be interesting to determine the exact location and stoichiometry of binding of the 100K T antigen associated with nucleoprotein complexes. It should be noted that a considerable amount of 100K T antigen is not associated with the nucleoprotein complexes, but is instead found at the top of the sucrose gradient. In addition, approximately equal amounts of 100K T antigen were found in the cytoplasmic extract, in the nucleoprotein complex preparation, and in the residual nuclear contents after extraction of the complexes. The role of the large quantity of 100K T antigen not bound to nucleoprotein complexes is not clear. Although the proteins are very similar (Fig. 3), the differences between the peptide maps of the total T antigen and the T antigen found in the nucleoprotein complexes may indicate that modification influences the ability of T antigen to bind to SV40 DNA. The stoichiometry of binding of the 100K T antigen to the SV40 (I) DNA-containing complexes can be estimated if one assumes that a complex with a molecule of 100K T antigen bound is immunoprecipitated intact without loss of histones. Then, knowing the methionine contents of the

100K T antigen and the histones, and the number of histones per DNA molecule (12), one can make an estimate of the number of 100K T antigen molecules bound per DNA molecule. Making the further assumption that the 100K T antigen and the histones found in the nucleoprotein complexes are newly synthesized, and using the data in Fig. 4B, such a calculation suggests that each SV40 (I) DNA-containing nucleoprotein complex may have as many as four molecules of 100K T antigen associated. If any histones are lost during the immunoprecipitation, or if presynthesized histones are utilized in the formation of the nucleoprotein complex, then this value will be an overestimate. If every molecule of SV40 DNA in an infected cell is associated with 100K T antigen, then the large amount of 100K T antigen unbound to DNA reflects a large excess of 100K T antigen over that required for initiation of viral DNA synthesis.

The 17K T antigen is not associated with the wild-type or the *tsA58* nucleoprotein complexes, either those containing SV40 (I) DNA or those containing SV40 (RI) DNA; all of the 17K T antigen is found at the top of the 5 to 30% sucrose gradient in the soluble protein fractions. This is in keeping with the observation that the 17K T antigen, unlike the 100K T antigen, is not a DNA-binding protein (26). Since the 17K T antigen does not bind to DNA and is not found associated with nucleoprotein complexes, it seems unlikely that it is involved in the initiation of SV40 DNA synthesis. This is consistent with the finding that mutants lacking the 17K T antigen grow only slightly less well than the wild-type virus in lytic infection (2, 32, 33).

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