Two-Dimensional Analysis of Proteins Sedimenting with Simian Virus 40 Chromosomes

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The nonhistone proteins sedimenting in low-salt glycerol gradients with simian virus 40 chromosomes were analyzed by two-dimensional gel electrophoresis, utilizing nonequilibrium pH gradients as the first dimension and sodium dodecyl sulfate-gel electrophoresis as the second dimension. By densitometric quantitation of the radiolabeled proteins present in each fraction of the gradients, it was possible to identify proteins sedimenting with all or a fraction of the simian virus 40 chromosomes. VP-1 sedimented with simian virus 40 chromosomes; additional evidence for its binding to chromosomes was obtained by immunochemical techniques. Four proteins (M_r 25,000, pI 6.0; M_r 32,000, pI 7.2; M_r 35,000, pI 8.5; and M_r 80,000, pI 7.2) sedimented with specific subsets of chromosomes.

One approach to the identification of proteins involved in eucaryotic DNA replication involves the isolation of replicating chromatin and physical or enzymatic characterization of the proteins found in association with the replicating DNA. Replicating papovavirus chromosomes may provide a useful model for the identification of proteins involved in cellular DNA replication because these chromosomes (i) are easily isolated in a form structurally similar to eucaryotic chromatin (2, 9, 11, 19, 20, 22-24, 28, 32-34, 36) and (ii) replicate like cellular chromosomes (12, 14-16, 18, 43, 49, 50).

The proteins sedimenting with partially purified chromosomes of simian virus 40 (SV40) (the papovavirus chosen for this study) have been analyzed by physical and enzymatic techniques with some success. By utilizing sodium dodecyl sulfate gel electrophoresis to identify proteins by molecular weight, SV40 chromosomes have been shown to sediment with VP-1 (10, 17, 23, 26, 28, 35, 38, 51, 52), VP-2 and VP-3 (28, 38, 52), cellular histone-H1 (6, 13, 23, 28, 31, 38, 54, 55), cellular histones H2a, H2b, H3, and H4 (6, 17, 22, 23, 26, 28, 31, 35, 37, 51, 52, 55), and T antigen (35, 45). In addition to these major proteins, a number of as-yet-unidentified minor proteins have also been observed on many of these gels (17, 28, 35, 38, 52). By comparison, SV40 virus contains only VP-1, VP-2, VP-3, and the histones H2A, H2b, H3, and H4 (1, 6, 7, 17, 21, 23, 28, 31, 35). The enzymatic activities sedimenting with SV40 chromosomes include DNA polymerases (13, 41, 42, 53), RNA polymerase (28), DNA relaxing enzyme (25, 28, 53), and a single-strand-specific DNA binding protein (42). The variable amounts of proteins and enzymes reported to copurify with SV40 chromosomes are probably

due to the different isolation and purification systems used in the different reports.

Unfortunately, the physical analyses have not unambiguously identified proteins functionally associated with SV40 chromosomes. This is in part due to an inherent limitation of the technique used. One-dimensional gel systems lack resolving capacity; thus, quantitation of protein bands is subject to the uncertainty that each band may contain more than one protein. Enzymatic techniques are not appropriate for all proteins; they require the availability of an appropriate assay. In addition, neither of these approaches allows proof of association beyond a demonstration of sedimentation of the proteins of interest with SV40 chromosomes.

One method of overcoming these difficulties is to use a two-step approach. The first step consists of two-dimensional gel electrophoresis (19) rather than the one-dimensional method previously used to assay across gradients or columns used to purify SV40 chromosomes. The increased resolution of two-dimensional gel electrophoresis allows recognition of proteins which would be overlooked by the one-dimensional method. In addition, because individual proteins are generally resolved from other proteins of similar molecular weight by two-dimensional electrophoresis, it is possible to legitimately quantitate the amount of each protein in each fraction of the gradient or column used for purifying chromosomes and thus determine unambiguously whether each protein comigrates with the chromosomes of interest.

The second step involves the use of the protein purified on the two-dimensional gel (or larger quantities of the protein purified from cells by using two-dimensional gel electrophoresis as an

assay) to induce the formation of antibodies in mice or rabbits. After appropriate demonstration of specificity, the antibodies could be used to test (i) whether the protein really is bound to SV40 chromosomes and (ii) what the role of the protein might be in the various functions carried out by SV40 chromosomes.

In this report, we describe the identification of several proteins which do and do not sediment with SV40 chromosomes. In addition, we provide immunochemical evidence suggesting that one of these proteins, VP-1, is actually bound to SV40 chromosomes.

MATERIALS AND METHODS

Viruses and cells. SV40 was a generous gift from Peter Tegtmeier and was propagated at a low multiplicity of infection in CV-1 cells (a gift from M. L. DePamphilis). Cells were grown at 37°C in a 5% CO₂ atmosphere in Eagle modified minimal essential medium (GIBCO Laboratories) supplemented with 5% fetal calf serum (GIBCO Laboratories). Virus was plaque assayed on BSC-1 cells. The virus had a titer of 10⁸ PFU/ml.

Infection of cells. CV-1 cells were seeded into 10-cm plastic petri dishes and infected during logarithmic growth with 2 ml of medium containing 2% fetal calf serum and 50 PFU of virus per cell or no virus in the case of mock-infected cells. After 2 h of adsorption with shaking at 15-min intervals, 8 ml of medium containing 2% serum was added, and incubation was continued at 37°C.

Radiolabeling of SV40 chromosomes. Plates of infected cells were incubated at 40 to 42 h postinfection with methionine-free medium for 15 min and then labeled for 10 to 15 min or 3 h with a mixture containing 0.5 to 1.0 mCi of [³⁵S]methionine (specific activity, 600 to 800 Ci/mmol) and 250 μCi of [³H]thymidine (specific activity, 400 Ci/mmol) in 2 ml of minimal essential medium lacking methionine. Identification of nonreplicating chromosomes was accomplished by labeling at 40 to 42 h with 0.2 μCi of [¹⁴C]thymidine (specific activity, 40.1 mCi/mmol) per plate for 3 h in 10 ml of medium. The medium was removed before cell harvesting, and, to identify replicating chromosomes, 100 μCi of [³H]thymidine (specific activity, 49.1 Ci/mmol) in 2 ml of fresh medium was added to each plate. After 10 min of incubation at 37°C, the medium was removed, and the cells were washed with ice-cold phosphate-buffered saline and kept on ice until harvested. The [¹⁴C]thymidine label was found incorporated primarily into nonreplicating chromosomes by this technique, whereas the [³H]thymidine was incorporated primarily into replicating chromosomes.

Isolation of nuclei. Cell monolayers were washed twice with 5 ml of cold phosphate-buffered saline per plate or flask, followed by washing with 5 ml of buffer A (5% glycerol, 2 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.5, 0.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After 5 min of swelling, the cells were suspended with a rubber policeman and centrifuged at 800 × *g* for 10 min. The packed cells were

suspended in 0.2 ml of buffer A per plate, mixed with 0.2 ml of 0.5% Triton X-100 per plate, layered onto 8 volumes of buffer A, and centrifuged at 800 × *g* for 10 min. The pellet was washed by suspension in 0.4 ml of buffer A per plate and centrifuged through buffer A, as described above.

Extraction of nuclei and glycerol gradient sedimentation. The nuclear pellets were extracted with 1 ml of buffer B (10 mM HEPES, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) for 2 h on ice. After the removal of the nuclear debris by centrifugation at 10,000 × *g* for 15 min, the supernatant SV40 chromosome extract was layered onto 10 ml of a 10 to 30% (vol/vol) glycerol gradient in buffer C (10 mM HEPES, pH 7.5, 5 mM KCl, 1 mM EDTA, 0.2 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) and centrifuged at 40,000 rpm and 4°C for 135 min in an SW41 rotor. After centrifugation, gradients were fractionated, and samples were analyzed by liquid scintillation counting.

Two-dimensional electrophoresis. Samples for electrophoresis were prepared by adding to each gradient fraction 20 μg of bovine serum albumin, 20 μg of RNase A (Worthington Biochemicals Corp.) and, after a 10 min incubation at 4°C, cold trichloroacetic acid (final concentration, 10%); this was followed by centrifugation at 10,000 × *g* for 10 min. Precipitates were washed once with cold acetone and centrifuged at 10,000 × *g* for 10 min. Electrophoresis conditions were essentially the same as described previously (40), with minor exceptions. Nonequilibrium pH gradient electrophoresis was in a Bio-Rad model 220 apparatus at 400 V for 3.25 h in slab gels containing 2% ampholines (LKB), pH 3.5 to 10. After electrophoresis, lanes were cut out and transferred to the second-dimension sodium dodecyl sulfate-gel (10% acrylamide) in a Bio-Rad model 221 apparatus and electrophoresed at 80 V overnight. The acrylamide stock solution contained 30% acrylamide and 2.25% diallyltartardiamide. Gels were dried on a Bio-Rad model 224 slab gel dryer and exposed for varying periods of time to Kodak XR-1 X-ray film. Development was in a Kodak model MCA-N RPX-Omat processor.

Identification of proteins. Anti-SV40 and preimmune sera were purchased from Flow Laboratories and were used at 1:10 dilutions in 0.67% agarose in an immune overlay (47). Actin was purchased from Sigma Chemical Co. Tubulin was a gift from Joanna B. Olmsted, and CV-1 nuclear matrix was prepared by Ronald Berezny (3). Digestions were performed by the method of Cleveland et al. (8).

Electron microscopy. Samples were prepared for electron microscopy by fixation with formaldehyde and glutaraldehyde, using the method of Griffith and Christiansen (24). Before adsorption to carbon-coated, glow-discharged grids, the samples were made 0.15 M with NaCl. Grids were then washed, dried, and rotary shadowed at an 8° angle with tungsten. Microscopy was performed with a Hitachi HU-11A electron microscope at 50 kV.

RNase A digestions. RNase A (Worthington Biochemicals) was heated to 95°C for 10 min and then spun at 10,000 × *g* for 15 min. Crude chromosomes were divided into two fractions, and one of the frac-

tions was digested at 0°C for 10 min with 1 µg of RNase per ml. The other fraction was incubated without RNase. This RNase concentration (1 µg/ml) digested labeled cellular RNA from SV40-infected cells but not DNA. The samples were centrifuged on glycerol gradients, as described above.

Quantitation of proteins. For quantitation of labeled proteins, all samples from a single gradient were prepared at the same time, as described above. Electrophoresis conditions were kept constant. When all fractions had been electrophoresed, the dried gels were exposed to the same lot of X-ray film for the same period of time and developed at the same time in an automatic developer. Protein spots of interest were scanned on a Corning model 750 scanning densitometer, sequentially on each gel. Scanning was done linearly through the center of each protein spot. Molecular weight calibrations were carried out by electrophoresis of protein markers (bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c) in a lane adjacent to the first dimension during electrophoresis in the second dimension or by identification of the positions within the two-dimensional gel of bovine serum albumin (added as carrier), tubulin, and actin.

Effect of antibody on the sedimentation of SV40 chromosomes. Pooled nonreplicating SV40 chromosomes were divided into three portions and then incubated with or without anti-SV40 horse serum for 10 min at 0°C. One control was incubated without additions, and the second was incubated with 10 µl of preimmune horse serum (Flow Laboratories). The third portion was incubated with 10 µl of anti-SV40 horse serum (Flow Laboratories). The portions were centrifuged as described above, and the position of the DNA label in each gradient was determined.

RESULTS

Extraction and partial purification of SV40 chromosomes. SV40 chromosomes were isolated from infected CV-1 cells by a procedure which maximized the yield of SV40 chromosomes capable of *in vitro* DNA synthesis (53). Infected cells were treated with low-ionic-strength buffer containing Mg^{2+} . After a brief Triton X-100 treatment to release nuclei and after washing of the nuclei with buffer, the chromosomes were extracted from the nuclei without Dounce homogenization by leaching into low-ionic-strength buffer containing EDTA. The SV40 chromosomes were separated from soluble proteins and cellular debris by centrifugation on low-ionic-strength, 10 to 30% glycerol gradients. As previously described, this method also produces a partial separation of replicating SV40 chromosomes from nonreplicating SV40 chromosomes (10, 13, 16, 17, 42, 50, 53, 54).

[³⁵S]methionine-labeled SV40 chromosomes. To characterize the proteins cosedimenting with SV40 chromosomes and also to monitor contaminating cellular proteins, it was necessary to radiolabel infected cells. The best

labeling conditions consisted of starving cells with methionine-free medium for 15 min, followed by labeling with 0.5 to 1.0 mCi of [³⁵S]-methionine per plate of infected cells in sufficient medium to keep the cells moist. With this procedure it was possible to obtain proteins labeled sufficiently for analysis of individual gradient fractions. When nuclear extracts of infected cells labeled for 15 min or 3 h or mock-infected cells labeled for 3 h with [³⁵S]methionine and [³H]thymidine were sedimented on 10 to 30% glycerol gradients, the patterns of radioactivity shown in Fig. 1A, B, and C, respectively, were obtained. DNA which was labeled for 15 min usually showed a biphasic distribution (Fig. 1A). Because 15 min is approximately the time necessary for one round of replication by SV40 chromosomes, the two peaks corresponded to replicating chromosomes and to newly completed chromosomes. There was much less DNA in the mock-infected nuclear extracts, and the little DNA present sedimented more slowly (Fig. 1C). The patterns of labeled protein were substantially the same in all cases. There was a peak at the top of the gradient, a small peak sedimenting slightly more slowly than the SV40 chromosomes, and an accumulation at the bottom.

Two-dimensional electrophoresis of proteins sedimenting with SV40 chromosomes. Individual fractions from glycerol gradients like those shown in Fig. 1 were analyzed by two-dimensional gel electrophoresis, utilizing a nonequilibrium pH gradient as the first dimension and sodium dodecyl sulfate-gel electrophoresis as the second dimension (40). The nonequilibrium pH gradient was chosen for the first dimension in order to observe basic proteins as well as acidic proteins. The conditions which we used were optimized for the separation of non-histone proteins (that is, acidic to moderately basic proteins with molecular weights between 15,000 and 100,000). Figure 2 shows autoradiograms of the proteins from fractions containing replicating and nonreplicating chromosomes which were obtained after labeling with [³⁵S]-methionine for 3 h (Fig. 2A) or for 10 min (Fig. 2B). Figure 2C shows a comparable fraction from a mock-infected, gradient-purified nuclear extract labeled for 3 h. It is apparent that all three autoradiograms contained a number of proteins in common (for example, actin; see below for protein identifications). The autoradiograms from SV40-infected cells contained a group of proteins greatly enhanced in intensity relative to the proteins in mock-infected cells, including VP-1, P-1 through P-4 (easily identifiable proteins which were quantitated and shown to sediment with SV40 chromosomes), and a large

group of other proteins less intensely labeled. In the shorter pulse, these proteins were less intensely labeled relative to actin and tubulin. Although the pattern of labeled proteins and the relative intensity of label in each protein were similar in various preparations of SV40 chromosomes, the absolute intensity of each spot and its absolute position depended on the labeling conditions used and on the batch of reagents used for electrophoresis. Small variations in position (compare actin and VP-1 in Fig. 2A and B) and in intensity were observed frequently. Many of the less intensely labeled proteins were not apparent in the photographic reproductions. To observe them, it was necessary to inspect the original autoradiograms. Although Fig. 2 in-

cludes the histone region of the autoradiogram, individual histones are not apparent. This was probably due to a selective loss of histones during sample preparation in 10% trichloroacetic acid, a relative absence of methionine in histones, and a lack of resolution at the very basic low-molecular-weight range of the gel.

When chromosomes were prepared from infected BSC-1 cells instead of CV-1 cells, the same protein spots were found as in Fig. 2 (data not shown).

Identification of proteins. VP-1 was identified by the following three criteria: (i) presence in infected cellular extracts and absence in mock-infected extracts; (ii) presence in fractions of partially purified virus; and (iii) positive re-

FIG. 1. Glycerol gradient sedimentation of nuclear extracts obtained from [^{35}S]methionine- and [^3H]thymidine-labeled CV-1 cells. Nuclear extracts were obtained from infected cells labeled for 15 min (A) and 3 h (B) and from mock-infected cells labeled for 3 h (C) and purified as described in the text. Samples (25 μl) from each fraction were counted. Symbols: $\bullet$, [^{35}S]methionine; $\blacksquare$, [^3H]thymidine. RC and NRC indicate the position of replicating and nonreplicating chromosomes, respectively.

FIG. 2. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins sedimenting with SV40 chromosomes. Samples were prepared and analyzed as described in the text. (A) 3-h label, infected cells. (B) 10-min label, infected cells. (C) 3-h label, mock-infected cells. The numbers on the left sides of the gels are molecular weights.

action in the immune overlay technique (47) when anti-SV40 serum was used. Proteins reacting with the antiserum showed a halo around the original position of the tested protein in the two-dimensional gel (Fig. 3). Two forms of VP-1 were observed, namely, VP-1, the major form, and VP-1*, a minor form whose intensity varied from preparation to preparation. No reaction was observed with preimmune serum. Actin and tubulin were identified by comigration of unlabeled authentic samples with labeled SV40 chromosome-containing gradient fractions, followed by protease digestion of the individual spots, using the method of Cleveland et al. (8). Although tubulin contains two subunits, only one large spot was observed in this system. This was perhaps due to a lack of resolution at the very acidic end of the gel. The nuclear matrix protein MTXa was identified by comigration with authentic samples prepared by the method of Berzney and Coffey (3). Figure 4 shows a schematic representation of some of the proteins sedimenting with SV40 chromosomes. The identified proteins (VP-1, actin, tubulin, and MTX-a), as well as additional proteins sedimenting with SV40 chromosomes (P-1 through P-4), were labeled.

FIG. 3. Immune overlay identification of VP-1. Two-dimensional gels of SV40 chromosomes were obtained, and the region of interest was enclosed in a rectangular form (4 by 8 cm) (47). Then, anti-SV40 serum (1:10 dilution) (in 0.67% agarose) at 56°C was poured into the form. The gel and overlay were incubated at 37°C in a moist atmosphere for 4 h, and the agarose overlay was removed and washed in a 0.1 M phosphate buffer (pH 7) containing 0.1 M NaCl for 18 h with buffer changes. The agarose overlays were then dried and autoradiographed. (A) Autoradiogram of duplicate of original gel. (B) Autoradiogram of immune overlay.

Electron microscopic analysis of gradient fractions. Fractions of gradients obtained during purification of SV40 chromosomes were analyzed by electron microscopy to determine the morphology of the SV40 chromosomes and to monitor the contamination of the chromosomes by cellular debris. As previously reported, nonreplicating chromosomes appeared as beads on strings (11) (Fig. 5C). In addition, there were other morphological forms similar to those obtained from mock-infected cells (Fig. 5D). There were no virus-like particles in the region of nonreplicating chromosomes or replicating chromosomes (Fig. 5B), but such particles appeared in the bottom fractions of the gradients (Fig. 5A). Although there were particles in fractions enriched for replicating chromosomes (Fig. 5B) which appeared similar in shape to compact chromosomes (9, 55), additional evidence would be needed to identify these particles as compact chromosomes. However, the lack of obvious beaded chromosomes in the region of replicating chromosomes suggests that some of the larger particles present were compact chromosomes. We can deduce from the specific activities and distributions of ^{14}C and ^3H in the gradients labeled for nonreplicating and replicating SV40 chromosomes, respectively, that even in gradient regions enriched for replicating chromosomes

FIG. 4. Schematic representation of the most intensely labeled proteins sedimenting with SV40 chromosomes. The identified proteins and proteins of interest are labeled. The numbers on the left are molecular weights.

FIG. 5. Analysis of glycerol gradient fractions by electron microscopy. (A) Fraction from bottom of gradient containing virus-like particles. (B) Gradient fraction containing replicating chromosomes. (C) Gradient fraction containing nonreplicating chromosomes. (D) Gradient fraction comparable to (C) from mock-infected cells. Bar = 150 nm.

(Fig. 5B), the majority of chromosomes present were not replicating.

RNase A digestion of SV40 chromosomes. Both the two-dimensional gels and the electron micrographs suggested that the chromosome-containing gradient fractions were contaminated with cellular material. Labeling with [35 S]methionine (Fig. 1) and with [3 H]uridine (data not shown) suggested that the contamination included proteins and RNAs, respectively. To exclude the possibility that the heavily labeled proteins detected on two-dimensional gels were associated with RNA, both crude nuclear extracts and partially purified SV40 chromosomes were treated with RNase A and further purified by glycerol gradient centrifugation. Analyses of the fractions containing SV40 chromosomes by two-dimensional gels showed that the heavily labeled proteins (with the exception of P-2 and P-3, which were detectably reduced) were not appreciably reduced and, in the case of VP-1, actin, and tubulin, actually increased (data not shown). Electron microscopy showed that the increase in the amount of VP-1 may have been due to the presence of virus-like particles in the fractions containing chromosomes, which were apparently freed by RNase from aggregates normally found at the bottom of the gradient. The increase in the structural proteins actin and tubulin was also probably a result of digestion of aggregates.

Resedimentation of SV40 chromosomes. In an attempt to purify SV40 chromosomes further and to remove contaminating proteins, individual chromosome-containing fractions which had been partially purified by gradient centrifugation were recentrifuged on 10 to 30% glycerol gradients. Interestingly, resedimentation had little effect on the S values of the

replicating and nonreplicating chromosomes (data not shown). Although there were minor losses of some protein, all of the major proteins which sedimented with replicating or nonreplicating chromosomes were found after resedimentation (data not shown).

Quantitation of proteins in gradient fractions. One way of testing for association of proteins with SV40 chromosomes is to measure the amount of a protein of interest and compare its sedimentation with that of the SV40 chromosomes. This procedure, which is analogous to an enzyme assay, does not require the identification of an enzymatic activity. The amount of each labeled protein of interest in each fraction of the gradients was measured by densitometry of the autoradiogram for that protein, normalized to the peak intensity of VP-1, and finally plotted against its fraction number. The calculations for normalized intensities were based on densitometric peak heights obtained by linear scans with a 5-mm slit through the centers of two-dimensional spots. Peak heights obtained in this way may not have been linearly proportional to the total density of label in the spot. Unfortunately, we did not have the equipment required to measure total density accurately (a microscanning densitometer). Proteins which were studied after a 3-h label and a 10-min label included structural proteins (actin, tubulin, and MTXa) and other proteins of interest (VP-1, P-1, P-2, P-3, and P-4). After a 3-h label (Fig. 6A), VP-1 was distributed in several peaks through the gradient; these were at the bottom, ahead of the replicating chromosomes, near the nonreplicating chromosomes, and near the top. The distribution of VP-1 after a 10-min label was similar (Fig. 6B), except that the peak near the top was much larger.

Of the proteins which sedimented in the chromosome region, P-1 (approximate molecular weight, 25,000; approximate pI, 6.0) sedimented in the region of replicating chromosomes after both 3-h and 10-min labels (Fig. 6A and B). Although proteins P-1 through P-4 did not appear as large peaks when normalized against the intensity of VP-1, the differences between their peak intensities and background were between one and two orders of magnitude. It was not possible to measure the amount of P-1 at the top of the gradient because of the complexity of the autoradiograms. In contrast, after a 3-h label, P-2 (molecular weight, 35,000; approximate pI, 8.5) and P-3 (molecular weight, 32,000; approximate pI, 7.2) sedimented near the nonreplicating chromosomes (Fig. 6A and C, respectively). In addition, there was a peak of each protein near the top of the gradients. After a 10-min label (Fig.

FIG. 6. Quantitation of radiolabeled proteins in glycerol gradients. The intensities of the proteins were measured as described in the text. (A) 3-h label. Symbols: ●, VP-1; ▲, P-1; ■, P-2. (B) 10-min label. Symbols: ●, VP-1; ▲, P-1; ■, P-2. (C) 3-h label. Symbols: ●, P-3; ▲, P-4. (D) 10-min label. Symbols: ●, P-3; ▲, P-4. RC and NRC indicate the positions of replicating and nonreplicating chromosomes, respectively.

6B and D) the pattern was similar, but the peak near the top was substantially increased in intensity relative to the peak cosedimenting with the chromosomes. A portion of P-4 (molecular weight, 80,000; approximate pI, 7.2) sedimented with SV40 chromosomes after a 3-h label (Fig. 6C), but after a 10-min label there was a peak only at the top of the gradient (Fig. 6D).

Although structural proteins were present in the same fractions as SV40 chromosomes after a 10-min or 3-h label (Fig. 7A and B), a 10- to 100-fold excess of each protein sedimented more slowly than the SV40 chromosomes. There was no indication of a specific association of any of these proteins with SV40 chromosomes. It is of interest that the patterns of sedimentation after 3-h and 10-min labels were very similar.

Antibody to VP-1 bound SV40 chromosomes. The evidence presented above suggests that VP-1 and several other proteins sedimented with SV40 chromosomes. To determine whether

VP-1 was actually bound to SV40 chromosomes, the effect of anti-SV40 serum on the sedimentation of SV40 chromosomes was tested. The antiserum utilized had been shown previously by immune overlay to react with VP-1 (Fig. 3). The effects of antiserum, preimmune serum, and no treatment on the sedimentation of nonreplicating chromosomes are shown in Fig. 8. Although the sedimentation of the chromosomes was affected slightly by preimmune serum, there was a much greater shift after treatment with anti-SV40 serum. The morphologies of the chromosomes which were reacted with either antiserum or preimmune serum were not appreciably different (data not shown) from those of the untreated control (Fig. 5). A similar effect was observed when replicating chromosomes were used. Thus, VP-1 is probably associated with at least a fraction of the nonreplicating and replicating SV40 chromosomes isolated at low ionic strength. Because the specificity of this antise-

FIG. 7. Quantitation of radiolabeled structural proteins in glycerol gradients. The intensities of the proteins were measured as described in the text. (A) 10-min label. Symbols: ●, actin; ▲, tubulin; ■, MTX-a. (B) 3-h label. Symbols: ●, actin; ▲, tubulin; ■, MTX-a. RC and NRC indicate the positions of replicating and nonreplicating chromosomes, respectively.

rum for VP-2 or VP-3 has not been determined, it is possible that a portion of the antibody may bind to these proteins also.

DISCUSSION

The nonhistone proteins cosedimenting with partially purified SV40 chromosomes, which have been isolated by a low-ionic-strength method that maximizes the yield of chromosomes capable of *in vitro* DNA replication (53), show a substantial heterogeneity when analyzed by two-dimensional gel electrophoresis. This is due partly to the increased resolution inherent in the two-dimensional system and partly to the increased number of proteins retained by SV40 chromosomes at low ionic strength.

FIG. 8. Effect of anti-SV40 antibody on sedimentation of nonreplicating SV40 chromosomes. Partially purified nonreplicating SV40 chromosomes labeled with [³H]thymidine for 3 h were incubated at 4°C with no antibody (▲), a 1:50 dilution of preimmune serum (■), or a 1:50 dilution of anti-SV40 serum (●) for 10 min and then sedimented on a 10 to 30% glycerol gradient.

Among the proteins identified as chromosome associated are two forms of VP-1. Multiple forms of VP-1 have been observed previously both by one-dimensional analysis (44) and by two-dimensional analysis (39). The second form of VP-1 (VP-1*) is found in variable amounts, and it is not clear whether it is a degradation product, as reported previously (44), or it has a functional significance.

By quantitating the amount of labeled protein in each gradient fraction, it was possible to demonstrate partial or complete overlap between peaks of nonreplicating or replicating SV40 chromosomes and peaks of VP-1 (major form) and induced proteins P-1 through P-4. In contrast, the structural proteins studied (actin, tubulin, and the principal matrix protein MTX-a) do not have separate peaks which sediment with SV40 chromosomes, suggesting the absence of association. The quantitation technique, although very useful, was not sufficiently sensitive in our hands to detect reproducibly minor changes in protein concentration. The maximum intensity of the proteins discussed here is 10- to 100-fold greater than the minimum intensity. The sedimentation positions of all of these proteins do not appear to be appreciably sensitive to RNase A treatment or to resedimentation. Although quantitation has not been utilized previously to show cosedimentation of VP-1 and SV40 chro-

mosomes, VP-1 has been observed previously in gradient fractions in which SV40 chromosomes are found (10, 17, 23, 26, 28, 35, 38, 51, 52). The pattern of VP-1 sedimentation observed here, with larger amounts of VP-1 cosedimenting with SV40 chromosomes of higher S value instead of with the nonreplicating chromosome peak, is consistent with the proposal that SV40 virus is formed by the gradual association of VP-1, VP-2, and VP-3 with SV40 chromosomes (9). Because of the potential for SV40 virus to be degraded during the isolation of SV40 chromosomes (46), it is not possible to exclude the possibility that some of the VP-1 observed was due to the presence of partially degraded virus or virus precursor particles. However, it is unlikely that degradation was a major contributor to the VP-1 sedimentation pattern because the pattern was observed after a 10-min pulse, a time too short for virus to be encapsidated and extensively degraded; and degradation was not a dynamic process after isolation since the relative amount of VP-1 and the position of chromosomes was not changed after resedimentation. Third, our isolation conditions yielded amounts of chromosomes and VP-1 which were very similar to those obtained by conditions which minimize virus disruption (17). It does seem clear from experiments with antiserum against VP-1 that VP-1 is actually associated with SV40 chromosomes and does not sediment with them for other reasons. The question of whether the VP-1 observed is due to maturing or degrading SV40 virus is under further investigation.

Of the other proteins which sediment with SV40 chromosomes, P-1 (molecular weight, 25,000; pI 6.0) may be associated with replicating chromosomes. In contrast, P-2 (molecular weight, 35,000; pI 8.5), P-3 (molecular weight, 32,000; pI 7.2), and P-4 (molecular weight, 80,000; pI 7.2) appear to sediment with nonreplicating chromosomes. The functions of these proteins have not yet been determined. However, the similarity among P-2, P-3, and the minor spots found between P-2 and P-3 in terms of both molecular weights and isoelectric points (Fig. 2) and the similarity of the peptides after protease digestion suggest that these proteins may belong to one of the principal groups of proteins associated with heterogeneous nuclear RNA (4). The fact that they cosediment with nonreplicating SV40 chromosomes is consistent with their association with newly transcribed RNA still linked to SV40 chromosomes (28; H. J. Edenberg, unpublished data). This hypothesis is also under investigation.

Just as enzyme assays have shown the induc-

tion of enzymes (29) and the sedimentation of DNA polymerases (13, 41, 42, 53) and DNA relaxing enzyme (25, 28, 53) with SV40 chromosomes, the methods described here demonstrate a similar induction and cosedimentation of proteins based on their physical characteristics. However, the following two problems remain to be solved: the identification of additional proteins which may be associated with SV40 chromosomes and characterization of the function of all associated proteins. The first problem may be made simpler by obtaining chromosomes of greater purity by using additional techniques for the purification of the isolated chromosomes, such as agarose gel electrophoresis (17, 55) or agarose gel filtration (53).

To test functional association between proteins of interest and SV40 chromosomes, we are utilizing three approaches. First, we are using mutants of SV40 temperature sensitive for replication or maturation, such as *tsA58* or *tsB11*. It should be possible to identify the proteins which are reduced after a temperature shift to restrictive temperature. These experiments are in progress. Second, immunological techniques also have potential for analyzing the functional associations of proteins (27, 48). Specific antibodies to particular histones have been used to show that particular histones are present in nucleosomes (5). Antibody to histone was shown to cause a shift in the sedimentation of nucleosomes (as reported here for anti-VP-1) and was also identified by electron microscopy on the nucleosome (5). The sedimentation shift which we observed with preimmune serum was also reported previously and was due to nonspecific binding (5). This was substantially reduced after purification of the immunoglobulin fraction. It is obvious that this approach requires highly purified, high-affinity immunoglobulin. The association of T antigen with SV40 chromosomes has also been successfully probed immunologically (45). We are presently preparing antibodies to proteins purified by two-dimensional gels. In addition, proteins cosedimenting with SV40 chromosomes are being used to obtain antibodies by the hybridoma technique (30). Finally, the proteins already identified are being purified to determine their effects on *in vitro* DNA replication.

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