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Structure and Function of the Polypeptides in Simian Virus 40

I. Existence of Subviral Deoxynucleoprotein Complexes

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Velocity sedimentation analysis of simian virus 40 degraded in alkaline buffers, *p*H 10.5, yields two components: soluble protein containing the largest polypeptides, VP1 and VP2, of the virion, and a deoxynucleoprotein complex (DNP-I) containing the viral deoxyribonucleic acid (DNA) and the small polypeptides, VP4, 5, and 6, and all or part of VP3. Dissociation of DNP-I by equilibrium centrifugation in CsCl yields a complex (DNP-II) of the viral DNA and residual, tightly bound polypeptide; VP4, 5, and 6, but not VP3, are recovered after separation from DNP-II. Treatment of the virus with β-mercaptoethanol and iodination experiments suggest that VP1 and VP2 might exist as compact structures cross-linked with disulfide bonds, perhaps forming the capsid. VP4, 5, and 6 form a relatively stable complex with the viral DNA and are supposed to be the internal proteins. The location of VP3 is not well defined; at least a portion of it is tightly bound to the viral DNA.

The simian virus 40 (SV40) virion is morphologically similar to the polyoma virus and has icosahedral symmetry of the T-7 type with a surface structure containing 72 capsomers (2). At least six different polypeptide chains with molecular weights ranging from 43,000 to 11,000 have been detected (5, 6).

Anderer et al. (1), Estes et al. (5), and Barban et al. (3) presented evidence that some of these polypeptides are associated with the viral deoxyribonucleic acid (DNA). The association could be factitious and nonspecific, arising upon degradation of the virus in vitro. However, three of the four DNA-associated polypeptides are reduced in quantity in empty capsids (5). Moreover, the apparent nonrandom binding of these polypeptides to the viral DNA suggests that they are truly nucleoproteins (8).

We present here structural analyses of the virion that point to the existence of subviral deoxynucleoprotein complexes in SV40. Their importance is indicated in experiments suggesting that the nucleoprotein is not merely a structural element, but may function to regulate the transcription of the viral DNA (8).

MATERIALS AND METHODS

Virus and cells. An SV40 large-plaque mutant and its propagation in MA-134 cells, a line of African green monkey kidney, have been described in previous reports (5, 15).

Purification of virus. Virus was concentrated by a method of precipitation with polyethylene glycol (15) with the following two modifications. The crude virus-containing fluids were precipitated overnight and eluted overnight instead of for 2 hr.

Purification was as before (15).

Labeled virus. Monolayer cultures of MA-134 cells in roller bottles (surface area, 1,000 cm^2) or in plastic flasks (75 cm^2) were infected with 30 to 50 plaque-forming units/cell. For labeling of the DNA, 0.05 μCi of ^{14}C-thymidine per ml (specific activity, 55 mCi/mmole) was added 2 hr after infection. For labeling of protein, 2 μCi of ^{3}H-mixed amino acids per ml (New England Nuclear) were added without amino acid deprivation 14 hr after infection.

Alkaline degradation of virus. Purified SV40 was degraded by dialysis at 4 C for 20 hr against three changes of one of the following buffers: (A) isotonic tris(hydroxymethyl)aminomethane (Tris)-ethanolamine buffer, *p*H 10.5, [0.15 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.02 M Tris-hydrochloride, *p*H 7.2; the *p*H was adjusted to 10.5 with undiluted ethanolamine, 16.1 M]. (B) Hypotonic Tris-ethanolamine buffer [0.04 M NaCl, 0.25 mM EDTA, 5 mM Tris-hydrochloride, *p*H 10.5, approximate concentrations, as described by Anderer et al. (1)]. (C) Carbonate-bicarbonate buffer, 0.15 M, *p*H 10.5 (16). The degradation products were separated by velocity centrifugation in a 5 to 20% sucrose gradient in buffer A or C, *p*H 9.0, in the Spinco SW-27 rotor at 25,000 rev/min for 6.5 hr. The rapidly sedimenting component (see Fig. 3) consisting of polypeptides and viral DNA is termed deoxynucleoprotein complex I

(DNP-I). After dialysis against Tris-buffered isotonic saline solution, DNP-I was stored at −70 C.

Degradation of DNP-I. DNP-I (see Fig. 3, from fractions 10–12) from the alkali-treated virus was dialyzed against buffer A or C, *p*H 9.0, and degraded further by equilibrium centrifugation in CsCl. DNP-I (2.5 ml) was layered on 3 ml of saturated CsCl solution, *p*H 9.0, and centrifuged in an SW-50 rotor at 42,000 rev/min for 20 hr at 4 C. The fractions were collected by puncturing a hole in the side wall near the bottom of the tube.

Iodination of intact or degraded virus. Purified whole virions or alkali-degraded virus were dialyzed against a 0.05 M phosphate buffer, *p*H 7.0 (14). Carrier-free ^{131}I (200 μCi in 0.1 ml) and 100 μg of Chloramine T (Eastman) in 0.1 ml of water were added to 0.2 ml of whole or alkali-degraded viruses [3–5 optical density at 260 nm (A_{260}) units/ml] and held in an ice bath for 10 min; 100 μg of sodium-metabisulfite in 0.1 ml of water was then added. The iodinated samples were dialyzed against three changes of phosphate-buffered saline solution and two changes of 0.075 M phosphate buffer, *p*H 7.2, for 2-hr periods. The reference for calculation of specific ^{131}I activities was virus labeled with ^{14}C-mixed amino acids; the protein content of this virus was determined by the Lowry method (11).

Electrophoresis in SDS-polyacrylamide gels. The procedure was the same as described before (5, 12). We used 14% polyacrylamide gels. Thirty microliters of virus, soluble virion protein, deoxynucleoprotein complex, and iodinated intact or unfractionated degraded virus were mixed with 50 mg of urea, 10 μliters of 10% β-mercaptoethanol, and 10 μliters of 10% sodium dodecyl sulfate (SDS) and held at 42 C overnight or at 60 C for 30 min before application to the gels.

For autoradiography, the gels were sliced longitudinally into four pieces and dried onto filter paper by suction and heat lamp radiation. Kodak X-ray film (RP/s X-o-mat) was used for the radioautographs.

Electron microscopy. The preparation of SV40 DNA and deoxynucleoprotein complexes for electron microscopy essentially followed the method of Kleinschmidt and Zahn (9), modified by staining with uranyl acetate.

RESULTS

Figure 1 illustrates analyses of iodinated virus, both intact and degraded in alkali, compared with noniodinated control virus labeled with ^{14}C-mixed amino acids (Fig. 1A). In the intact virus, the level of iodination of polypeptide VP1, the major polypeptide (Fig. 1B), is extremely low; VP1 (70% of the total virus protein) gained only 8.1% of the total iodine counts labeling the virus protein. The specific activity of VP1 and VP2 with intact virus was 257 and 1,310 counts per min per μg, respectively, whereas the specific activity of VP3, VP4 plus 5, and VP6 was higher, 5,800, 3,600, and 12,000 counts per min per μg, respectively. The low efficiency of iodination of VP1 and 2 suggested that these polypeptides had a low tyrosine content. However, when the same virus was first degraded in alkali and then iodinated (Fig. 1C), the specific activity of VP1 increased at least 15-fold to 5,430 counts per min per μg; iodination of VP2 also was enhanced two- to threefold to 4,000 counts per min per μg. In contrast, alkaline degradation had little effect on the iodination of VP3–6. Apparently the iodine reactive sites were accessible in VP3–6 in the intact virus, but in VP1 and 2 they were fully exposed only after degradation of the virus in alkali.

Influence of β-mercaptoethanol on the degradation of virus. The omission of β-mercaptoethanol caused: (i) disappearance of VP2, (ii) a decrease of radioactive counts in the VP1 peak, and (iii) the

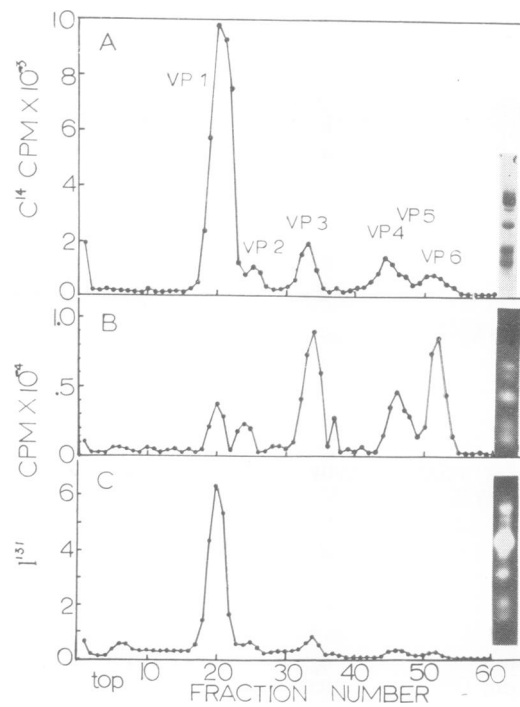

FIG. 1. *Polypeptide patterns of iodinated whole and degraded virus after electrophoresis in SDS-polyacrylamide gels. Intact virus and virus dissociated in alkali were iodinated with ^{131}I (see text). The viral samples were solubilized in urea, β-mercaptoethanol, and SDS and applied to 14% polyacrylamide gels. The running direction is from left to right. (A) Virus labeled with ^{14}C-mixed amino acids shows the expected high content of VP1 (70% of virion protein) (5). (B) When intact virus was iodinated with ^{131}I, VP1 was radioactively labeled to a relatively low degree compared with VP3, 4, 5, and 6. (C) Iodination of virus after it had been first degraded in alkali showed a large gain in the radioactive iodine content of VP1. VP2 also showed a relative increase in the iodine label. Specific activities are in the text.*

appearance of a broad, new biphasic peak with a molecular weight of about 70,000 to 75,000 (Fig. 2). The sum of the molecular weights of VP1 and VP2 (43,000 and 32,000, respectively) and the decrease in radioactive counts in the VP1 and VP2 peaks suggest that aggregation accounts for the changes and that a disulfide linkage might exist between VP1 and VP2. The peak at fraction 7 may consist of a dimer of VP1, and the peak at fraction 10, the missing VP2 plus one-third of the missing counts in VP1 (Fig. 2), but we have not recovered and subjected the material in these fractions to a second electrophoresis.

Products of dissociation of purified virions in alkali: isolation of DNP-I. Two components were resolved in sucrose gradients (Fig. 3): soluble protein and DNP-1 containing essentially all the viral DNA and some viral protein. The soluble protein contained VP1 and VP2 (Fig. 4). DNP-1 contained at least three polypeptides (VP4, 5, 6).

One of the viral structural proteins (VP3) was difficult to recover after degradation; some was bound to the viral DNA as shown later. In isotonic buffer A, there was a small amount of VP3 in the top fraction together with VP1 and VP2 (Fig. 4); with the carbonate buffer C (twice the ionic strength of buffer A), more VP3 was found in the soluble protein, but DNP-I still contained appreciable VP3.

With a buffer B of very low ionic strength (1), degradation was less complete. Figure 5 shows the distribution of soluble protein and DNP-I after degradation with the different buffers. The peak containing DNP-I from virus degraded in the hypotonic buffer B was broader than that resolved after degradation in either the isotonic buffer A or 0.15 M carbonate buffer C. The sedimentation value of DNP-I in neutral sucrose gradients is 35 to 40S. Electrophoresis of the polypeptides in DNP-I is shown in Fig. 6A and B.

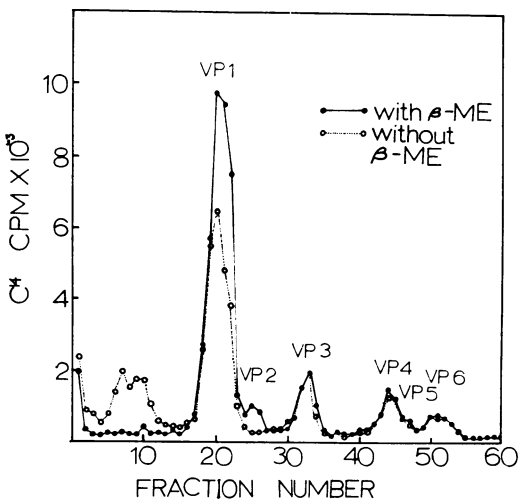

FIG. 2. *Influence of β-mercaptoethanol (β-ME) on the dissociation of SV40 virions. Virus labeled with a mixture of ^{14}C-amino acids (30 μliters) was solubilized in 50 mg of urea and 10 μliters of 10% SDS, and with (●——●) or without (○-----○) 10 μliters of 10% β-ME at 42 C overnight. When β-ME was omitted from the degradation mixture, a new peak with a molecular weight greater than that of VP1 appeared on electrophoresis (~70,000–75,000).*

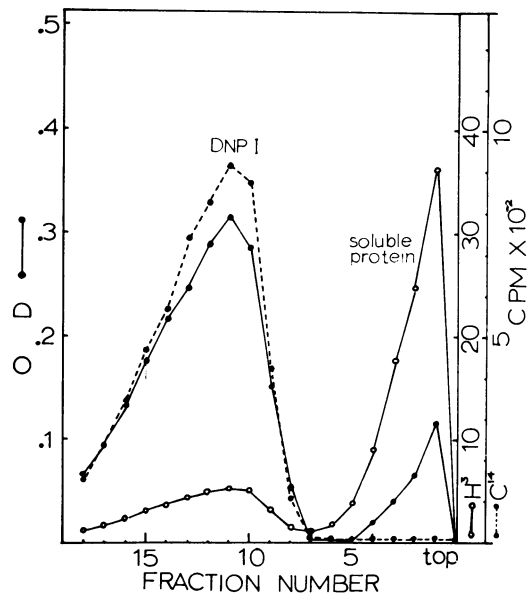

FIG. 3. *Velocity sedimentation analysis of alkali-degraded SV40. Purified SV40, doubly labeled with a mixture of ^{3}H-amino acids (○) and ^{14}C thymidine (●-----●), was degraded in isotonic (0.15 M NaCl) Tris-ethanolamine buffer A, pH 10.5, for 20 hr at 4 C. The sample was layered on a 5 to 20% sucrose gradient, pH 9.0, and centrifuged at 25,000 rev/min for 6.5 hr in an SW27 rotor at 4 C. Sedimentation was toward the left. The peak at the top of gradient contains only soluble protein and no DNA. The faster sedimenting component (DNP-I) contains the viral DNA and a remnant of viral protein. The polypeptide patterns are shown in Fig. 4. A_{260} (●——●).*

To determine whether the formation of DNP-I is due to nonspecific, random association of protein to viral DNA during dissociation of the virion in alkali, we mixed and degraded unlabeled SV40 and labeled SV40 DNA (Fig. 7). After velocity sedimentation of the mixture, no exchange of nucleoprotein during dissociation of the virion in alkali was apparent. The result suggests that the association of polypeptides and viral DNA preexisted in the virus particle.

Further dissociation of DNP-I: isolation of DNP-II. A pool of fractions 10, 11, and 12 (Fig. 3 and 5) containing DNP-I from virus degraded

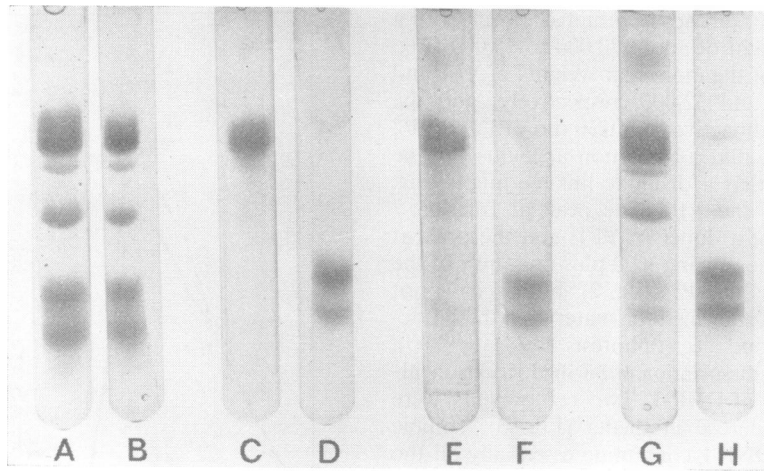

FIG. 4. *Polyacrylamide gel electrophoresis of whole SV40 virions, soluble protein, and DNP-I from virus dissociated in alkali. Gels A and B, whole virions dissociated with β-mercaptoethanol, SDS, and urea; the concentration of protein in gel B is three-fifths that of gel A. Gels C and D show the soluble protein and DNP-I, respectively, obtained from virus degraded with buffer B of low ionic strength (0.04 M NaCl). Gel E shows soluble protein from virus degraded with the isotonic buffer A. Gel F is of DNP-I. Gels G and H are of the soluble protein and DNP-I, respectively, from the virus degraded with 0.15 M carbonate buffer C. VP3 is only resolved with the carbonate buffer. The direction of electrophoresis is from top to bottom.*

in the isotonic buffer was dissociated by centrifugation to equilibrium in a CsCl density gradient (Fig. 8).

The minor component of lower density contained only ^{3}H-amino acid counts; the major component, termed deoxynucleoprotein complex II (DNP-II), contained mainly ^{14}C-thymidine counts and, after correction for quenching, a small amount of ^{3}H-amino acid counts. Some protein, therefore, probably persists in the major DNA-containing peak. With purified virus labeled only with ^{14}C-mixed amino acids, the result was similar: nearly 12% of the ^{14}C counts in DNP-I sedimented with the DNA. With virus labeled with ^{3}H-lysine, the result was again similar, which excludes the possibility that the mixed amino acid label enters DNA through de novo synthesis.

The density of the DNP-II peak ranges from 1.65 to 1.68 g/cm^{3}, but there is too little polypeptide to detect by staining the gels. The sedimentation value of DNP-II estimated in neutral sucrose gradients is approximately 20*S*.

Electrophoresis of DNP-II did not disclose its polypeptide content; the protein counts remained at the top of the gel, presumably because of adherence to the viral DNA (Fig. 6D). Even after deoxyribonuclease digestion, the polypeptide content of DNP-II was not resolved due to insufficient counts (not shown).

However, the polypeptides separated from DNP-I during the isolation of DNP-II in CsCl included VP4, VP6, and contaminating VP1; VP5 was presumably also in the separable protein, but it was not resolved here (Fig. 6C). The pattern corresponds closely to the analysis of the polypeptides of DNP-I, shown in Fig. 6A and B, except that VP3 is absent.

Electron microscopy confirmation of DNA in deoxynucleoprotein complexes. We are unable to define the precise conformational difference between the deoxynucleoprotein complexes and viral DNA by electron microscopy because of the limitation of showing the proteins bound to viral DNA when the cytochrome *c* membrane, uranyl acetate staining techniques were applied. SV40 DNA and DNP-II are indistinguishable (Fig. 9). In DNP-I, a large quantity of compact, tightly clumped single DNA molecules exist; this configuration of viral DNA is rare in DNP-II and purified SV40 DNA. There are about 10% open circular DNA molecules in SV40 DNA and in DNP-II and about 3% in DNP-I.

DISCUSSION

Four of the six viral polypeptides, VP4, 5, 6, and a portion of VP3, are associated with SV40 DNA in a deoxynucleoprotein complex (DNP-I) found after alkaline degradation of the virus. This complex is not the result of a random reassociation of viral DNA and protein taking place during degradation of the virus in vitro. A small amount of the protein in DNP-I cannot be separated from the viral DNA by exposure to high concentrations of salt during isopycnic centrifugation in CsCl; this persistent complex of protein and DNA is termed DNP-II. From deoxyribo-

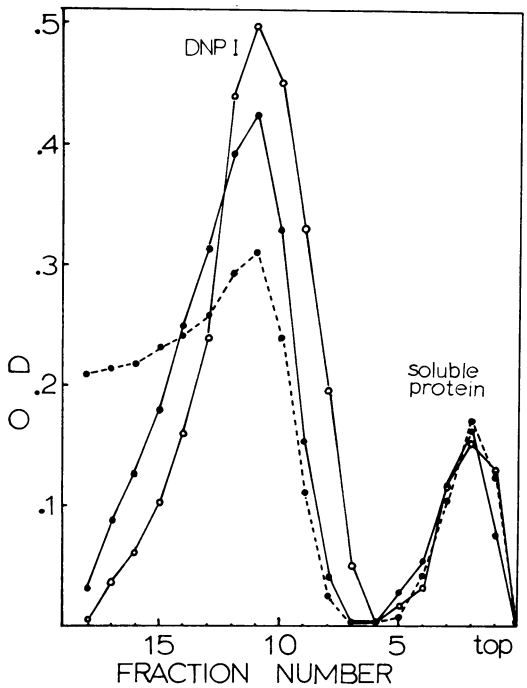

FIG. 5. *Velocity sedimentation analysis of SV40 degraded in different alkaline buffers. Purified SV40 virions were degraded by dialysis in 0.15 M carbonate-bicarbonate buffer C (○), isotonic (0.15 M NaCl) Tris-ethanolamine buffer A (●——●), and low ionic strength (hypotonic) Tris-ethanolamine buffer B (●-----●); all the buffers were adjusted to pH 10.5. The degraded samples were centrifuged on sucrose gradients as described in Fig. 3. We preferred the isotonic buffer A because of the better resolution and more complete dissociation of the virus.*

nuclease digestion experiments, VP3 or a portion of it is presumed to be the tightly binding polypeptide of DNP-I and DNP-II. This kind of tight interaction could be a hydrophobic binding, but the protein might be merely trapped by the viral DNA. However, the amount of at least three of the minor polypeptides in empty SV40 capsids is sharply reduced (5), which makes it unlikely that DNP-I is a factitious complex arising only in vitro.

Anderer et al. (1) also described an SV40 DNA-polypeptide complex; it contained one of the three polypeptides they detected in the virion. Since antiserum against this complex reacted with intact virus, the authors concluded that an antigen in the nucleoprotein complex was also on the surface of the virion (10). This conclusion may be faulty since nucleoprotein complexes isolated from virus degraded with their buffer are contaminated with VP1 (Fig. 4, 6). If VP1 is the main capsid polypeptide, present in large amounts and therefore difficult to remove entirely, it is probably a stronger antigen than the DNA-associated proteins, which are cationic polypeptides.

There is some evidence that the major structural proteins, VP1 and VP2, are viral coat proteins (2, 3, 6). These two polypeptides can be isolated as a soluble component from alkali-degraded virus. Alkaline degradation of the virus greatly enhanced the iodination of VP1 and 2 but not of VP3, 4, 5, and 6, presumably because the cryptic iodine reactive sites, viz., tyrosine residues, were exposed in VP1 and 2 by destruction of compact

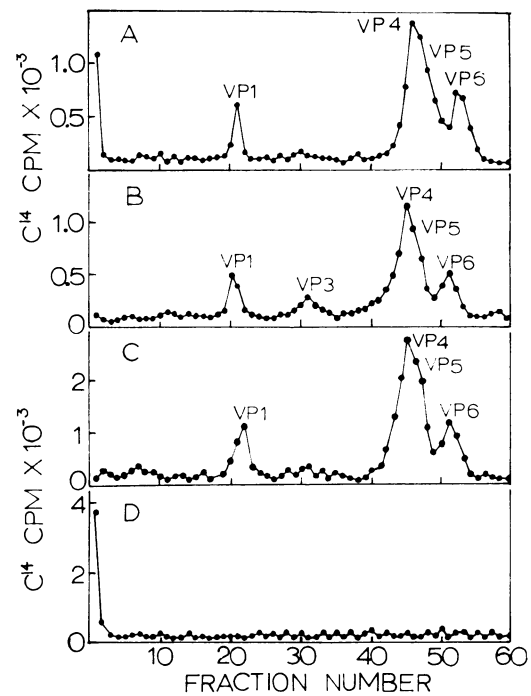

FIG. 6. *Polypeptide patterns in gels of DNP-I, DNP-II, and soluble viral protein. DNP-I labeled with ^{14}C-amino acids obtained from sedimentation in a sucrose gradient of virions degraded in buffer A was solubilized in urea, β-mercaptoethanol, and SDS as described in Fig. 1. Migration is toward the right. (A) Without deoxyribonuclease treatment of DNP-I, VP4, VP6, and probably VP5 was resolved along with relatively slight contamination by VP1; however, about 7% of the total ^{14}C counts remained in the top fraction of the gel. (B) After treatment of DNP-I with pancreatic deoxyribonuclease (20 μg/ml with 5 mM $MgCl_2$ for 3 hr at 37 C), the counts in the top fraction disappeared, and VP3 appeared.*

DNP-I was dissociated into DNP-II and viral protein by centrifugation in CsCl (see Fig. 8). (C) The low-density protein component, concentrated by lyophilization and subjected to electrophoresis, contains all the polypeptides of DNP-I except VP3. (D) After electrophoresis of DNP-II all of the protein counts remained in the top fraction of the gel with the viral DNA.

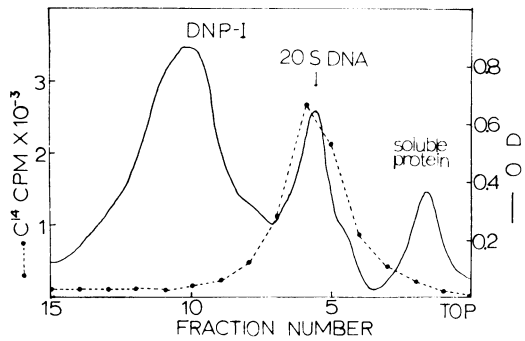

FIG. 7. *Lack of random association of viral protein and viral DNA during degradation of virus in alkali. Purified unlabeled SV40 (0.25 ml with six absorbancy units at 260 nm (A_{260})/ml) and ^{14}C-thymidine-labeled SV40 DNA (0.15 ml, 160 μg/ml, specific activity, 9,080 counts per min per μg) were mixed and dialyzed at 4 C for 20 hr with three changes of buffer A. The product was layered on a 5 to 20% sucrose gradient in buffer A, pH 9.0, and centrifuged at 42,000 rev/min for 2 hr in a Spinco SW50.1 rotor. Three components are resolved at A_{260} (——): soluble protein (at top), viral DNA (16 and 20S) and DNP-I (35-40S). There was no significant radioactivity (●-----●) associated with DNP-I.*

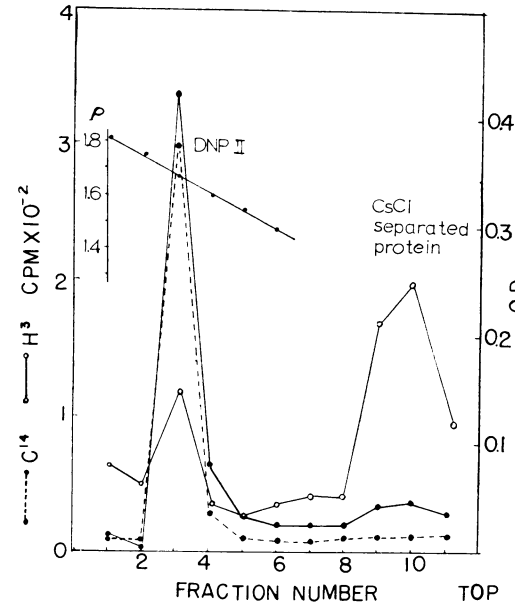

FIG. 8. *DNP-II: dissociation of DNP-I by centrifugation in CsCl. DNP-I labeled with ^{14}C-thymidine and a mixture of ^{3}H-amino acids (2.5 ml in buffer A, pH 9.0) was layered on top of 3 ml of saturated CsCl solution and centrifuged in a SW50 rotor at 42,000 rev/min for 20 hr at 4 C. The samples were collected from the bottom (left). The optical density (●——●) was measured at 260 nm in a Guilford 240 spectrophotometer. ^{14}C (●-----●); ^{3}H (○——○) radioactivity. The component of low density contains only ^{3}H-amino acid counts; the high-density component (ρ = 1.65–1.68) contains mainly ^{14}C-DNA counts and some ^{3}H-protein counts.*

structure, as if globular polypeptides were opened. Iodination with the lactoperoxidase method gave identical results (M. K. Estes, *unpublished data*). Also, the apparent aggregation of VP1 and 2 resulting from omission of β-mercaptoethanol

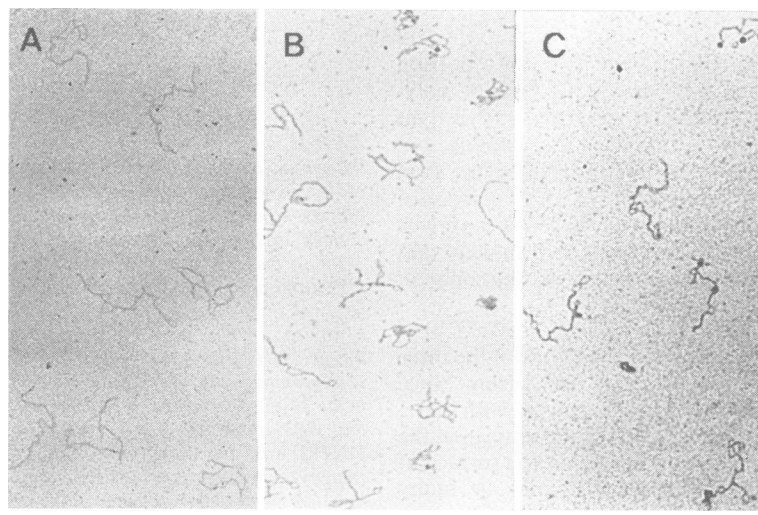

FIG. 9. *Electron micrographs of SV40 DNA and deoxynucleoprotein complexes I and II. SV40 DNA, DNP-I, or DNP-II was mixed with cytochrome c solution and spread over 0.15 M ammonium acetate. The films were picked up on grids and stained with uranyl acetate. Samples were examined in an AEI electron microscope: (A) SV40 DNA, (B) DNP-I, (C) DNP-II. The many compact, tightly clumped, single DNA molecules in the DNP-I preparation are not found in preparations of DNP-II or DNA-I.*

suggests that there is disulfide bonding between VP2 and VP1, which is important for the structural integrity of the polyoma virion (7).

The quantities of VP3, 4, 5, and 6 appearing in the gel are not affected by the omission of β-mercaptoethanol for virus degradation, and alkaline degradation of the virus did not enhance the iodination of VP3, 4, 5, and 6. It is possible to envision VP3, 4, 5, and 6 as existing as a linear helix intertwined with the viral DNA, favorable for packing the viral DNA and regulating gene transcription, rather than as a compact tertiary structure. These polypeptides have a pronounced effect on the transcription of the SV40 genome in vitro with both *Escherichia coli* and mammalian DNA-dependent ribonucleic acid polymerases (8). VP4, 5, and 6 appear to cell histones (P. M. Frearson and L. V. Crawford, *in press*; M. K. Estes, *unpublished data*).

VP3 is easy to detect upon electrophoresis of the whole virus, but it is only found in DNP-I after deoxyribonuclease treatment. This apparent discrepancy may be due to a tighter binding between VP3 and the viral DNA resulting once VP1 and VP2 have been removed, which is consistent with the idea that VP3 may serve as an intermediate structural element with affinity both for the capsid and internal elements. It is highly unlikely that DNP-I is an artifact because ratios of the polypeptides in DNP-I are consistent despite different conditions of degradation and because of the nonrandom limitation to transcription of DNP-I in vitro. The existence of DNP-II is less certain; it apparently contains small amounts of VP3, which is rich in arginine but unlike VP4, 5, and 6 also contains tryptophan (M. K. Estes, *unpublished data*), and possibly small amounts of other polypeptides.

Electron microscopy indicates that polyoma virus (13) and SV40 (2) contain 72 capsomers. Each virion may contain 60 hexamers (360 identical polypeptide chains) and 12 pentamers (60 identical polypeptide chains) for the viral capsid (4). The estimated number of polypeptide chains of VP1 and 2 was calculated as 320 and 54, respectively, close to the subunit values as postulated by the hypothesis (5). The data are consistent with the idea that VP1 might be the polypeptide for building the hexamers, and VP2 the polypeptide for the pentamers.

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