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Use of whole-cell fixation to visualize replicating and maturing simian virus 40: Identification of new viral gene product

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ABSTRACT **Formaldehyde fixation of simian virus 40 (SV40)-infected CV-1 cells at appropriate times after infection permits us to isolate crosslinked complexes of SV40 minichromosomes during the time of DNA replication and during packaging with viral proteins. Such crosslinked complexes can be separated on the basis of density on CsCl/guanidine·HCl density gradients. During the course of these studies we observed the presence of a low molecular weight protein in a region of the gradient much enriched with viral nucleoproteins. This protein is present only in infected cells and has a molecular weight and amino acid composition consistent with it being the product of the so-called SV40 agnogene.**

Productive infection by simian virus 40 (SV40) reflects phased expression of specific regions of its genome. At early times after infection, viral expression is concerned with establishing appropriate amounts of early proteins and initiating replication of the viral DNA which becomes associated with host histones (see reviews in refs. 1–3). At later stages in infection, synthesis of the early functioning proteins declines and is replaced by vigorous synthesis of the three viral coat proteins involved in packaging the viral chromosome, still in association with most of its histone complement, into mature virion particles. Essentially all of the SV40-encoded proteins involved in this process (VP1, VP2, and VP3) have been defined, and study of the open reading frames within the primary sequence of the SV40 DNA molecule does not indicate that any previously undescribed proteins of high molecular weight can be coded for by the DNA. However, in the leader sequence region of the late 16S mRNA there exists an initiation codon followed by a 61-codon open reading frame (4). At this time, such a product of this so-called agnogene has not been described in infected cells.*

Fixation methods that permit analysis of nucleoproteins without loss of critical components have recently been developed (5, 6). Thus, it is possible to separate replicating chromosomal DNA from bulk chromatin on the basis of its lesser density, presumably due to the increased amount of protein associated with replicating material. Because the replicating SV40 minichromosome is rapidly becoming a valuable model system for replicating chromosomes in general, it seemed appropriate to determine whether replicating and bulk minichromosomes also could be distinguished on the basis of density differences after fixation of infected cells. We reasoned further that it might be possible to visualize the density changes during maturation and packaging of the SV40 minichromosome as the virus becomes associated with more coat proteins. One of the strengths of this approach is that fixation takes place *in situ*, so that losses from critical structures are minimal; furthermore, after resolution on density gradients, the protein components can be recovered from their crosslinks and displayed on gels.

MATERIALS AND METHODS

Growth of Cells and Fixation of SV40 Minichromosomes. African green monkey kidney cells (CV-1) were grown in 150-cm^2 tissue culture flasks as monolayers in Dulbecco's minimal Eagle's medium (DME medium) supplemented with 5% calf serum and 5% fetal calf serum (supplemented DME medium). Confluent cultures were infected with SV40 from a large-plaque clone of strain 776 applied at 5–10 plaque-forming units per cell. Cells were harvested by trypsin treatment and resuspended in supplemented DME medium. After incubation at 37°C for 2 hr to inactivate the trypsin, the cells were labeled, in 3-ml aliquots, with radiolabeled thymidine or amino acids. The cells were then centrifuged at 3000 rpm for 2 min, suspended in DME medium containing 1% formaldehyde, and incubated for 18 hr at 4°C. The cells were then washed and resuspended in 2 ml of DME medium and sonicated twice (Heat Systems/Ultrasonics, Plainview, NY) at setting 3 for 30 sec at 4°C with a 10-min interval between sonications. The solution was centrifuged at 5000 rpm for 10 min (SS34 rotor). The supernatant contained the majority of the minichromosomes and virions as judged by the presence of supercoiled SV40. Fixation stabilized these complexes such that they were relatively resistent to shear in the absence of denaturants.

Centrifugation on CsCl/Guanidine·HCl (Gdn·HCl) Gradients and Analysis of DNA and Protein Distribution. The supernatant isolated by sonication of the fixed cells was incubated with RNase A (50 μg/ml) for 15 min at 25°C and then added to 1.7 g of Gdn·HCl (Heico, Delaware Gap, PA) and 1.2 g of CsCl, and the final volume was adjusted to 4.6 ml. This solution was sonicated twice at setting 6 for 20 sec with a 10-min interval for cooling. This material was then centrifuged at 35,000 rpm for 72 hr at 4°C in an SW 56 rotor. Equal fractions (200 μl) were collected and aliquots were assayed directly in Bray's solution for [^{3}H]thymidine. To analyze for protein distribution, 2-mercaptoethanol (30 μl) was added to each fraction and the samples were heated to 100°C for 30 min (5, 6). These samples were dialyzed against two changes of 10 mM 2-mercaptoethanol overnight and then adjusted to 5% (vol/vol) glycerol/0.1% $NaDodSO_4$/50 mM Tris/0.1% bromophenol blue, pH 7, by using a 5-fold concentrated stock solution. The electrophoresis conditions used for $NaDodSO_4$ gels were a modification of the Laemmli procedure (5–7).

Analysis on Two-Dimensional Gels. CV-1 cells, either control or SV40-infected, were harvested and labeled with [^{3}H]lysine as described in the text. The cell pellet was then sonicated in H_2O, adjusted to 0.2 M in H_2SO_4, and sonicated two more times. The solution was then centrifuged at 15,000 rpm for 10 min, and the acid-soluble proteins in the supernatant were pre-

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Abbreviations: SV40, simian virus 40; DME medium, Dulbecco's modified Eagle's medium; Gdn·HCl, guanidine·HCl.

* See *Note Added in Proof.*

cipitated with 10 vol of acetone at −20°C. The precipitated protein was electrophoresed on Triton/acetic acid/urea gels (9) in the first dimension and then gel strips were electrophoresed in the second dimension on $NaDodSO_4$ gels (5, 6).

RESULTS

Separation of Newly Replicated SV40 Chromatin. Because the replicating SV40 viral minichromosome is a useful model system we were interested in testing whether the replicating minichromosome could be separated on the basis of density after whole-cell fixation. At 40 hr after African green monkey kidney cells (CV-1 cells) were infected with SV40, the cells were pulsed with [^{3}H]thymidine for either 1 min to identify newly replicated material or for 15 min to label the bulk SV40 minichromosomes. The pulses were terminated by mixing with cold (4°C) medium containing formaldehyde which rapidly inhibits cellular replicative activities. The cells were then fixed for 18 hr at 4°C before being collected by centrifugation. The cells were disrupted by brief sonication, and the supernatant fraction, which contained SV40 material, was separated from the nuclear fragments and host chromatin (which sedimented into the pellet). The amount of host chromatin that was soluble in the presence of the divalent cations in the DME medium was minimal—in the range 5–15%. This approach yielded a supernatant fraction that contained 85–90% of the SV40 molecules as judged by the yield of supercoiled SV40 DNA molecules upon agarose gel electrophoresis (data not shown).

Analysis of the SV40 chromatin obtained in this manner gave the results shown in Fig. 1. DNA labeled during a 1-min pulse was distributed toward the light side of the gradient whereas DNA from the 15-min pulse was primarily in the denser region of bulk chromatin although, as expected, a fraction of this material had not yet matured and still showed the lower density characteristic of recently replicated DNA (5).

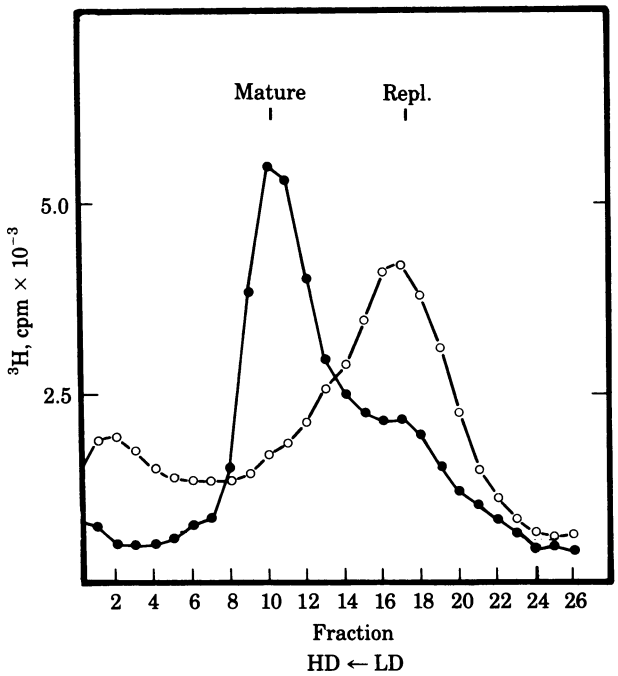

FIG. 1. Analysis of SV40 replicative chromatin on CsCl/Gdn·HCl density gradients. CV-1 cells infected with clone 776 SV40 (10 plaque-forming units per cell) were harvested after 40 hr and pulsed for 1 min with 0.5 mCi of [^{3}H]thymidine (○) or 15 min with 50 μCi of [^{3}H]thymidine (●). Cells were fixed with formaldehyde for 18 hr; the SV40 minichromosomes and virions were extracted and centrifuged to equilibrium in the CsCl/Gdn·HCl gradients. LD, low density; HD, high density; Repl., recently replicated.

Proteins Associated with SV40 Chromosomes. The density gradient analysis in Fig. 1 reveals only the positions of either newly replicated or relatively recently replicated DNA. There is no *a priori* reason why this isolation procedure should not also yield viral material involved in the packaging process. Indeed, because these constitute a major fraction of the total of SV40 DNA in these supernatants, we would expect that these materials should also be visualized on the density gradients. At 40 hr after infection, infected CV-1 cells were labeled by a 6-min exposure to [^{3}H]lysine. After fixation of whole cells as described above, the SV40 nucleoprotein supernatant was isolated and separated on the basis of density in CsCl/Gdn·HCl gradients. The proteins associated with different fractions are shown in Fig. 2. The lighter side of the gradient contained newly synthesized histones, a not unexpected result in view of our previous studies with replicating nuclear chromatin (5, 6). The denser region of the chromatin (the bulk viral chromatin) contained all five histones, but H1, H2A, and H2B were more highly labeled. This is also entirely consistent with previous reports that these three histones can deposit on old chromatin (5). The lighter region of the gradient also contained relatively small

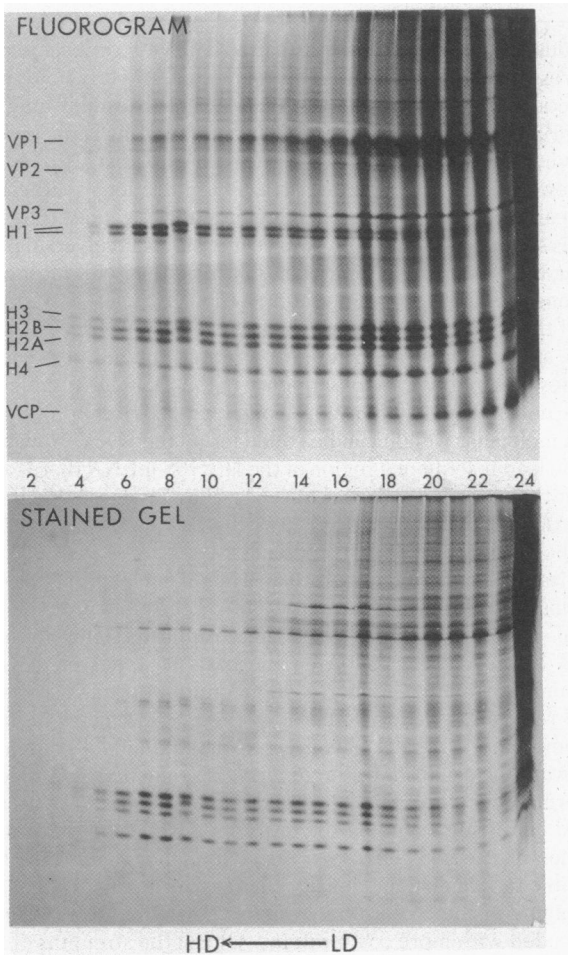

FIG. 2. Analysis of protein associated with the SV40 virions and minichromosomes. CV-1 cells were infected with 10 plaque-forming units per cell. At 40 hr, the cells were pulsed with [^{3}H]lysine for 6 min and then fixed directly. The SV40 virions and minichromosomes were extracted and centrifuged to equilibrium on CsCl/Gdn·HCl gradients. After reversal of fixation, the proteins were analyzed on $NaDodSO_4$ polyacrylamide gels both for amount (stained gel) and for radiolabel (fluorogram).

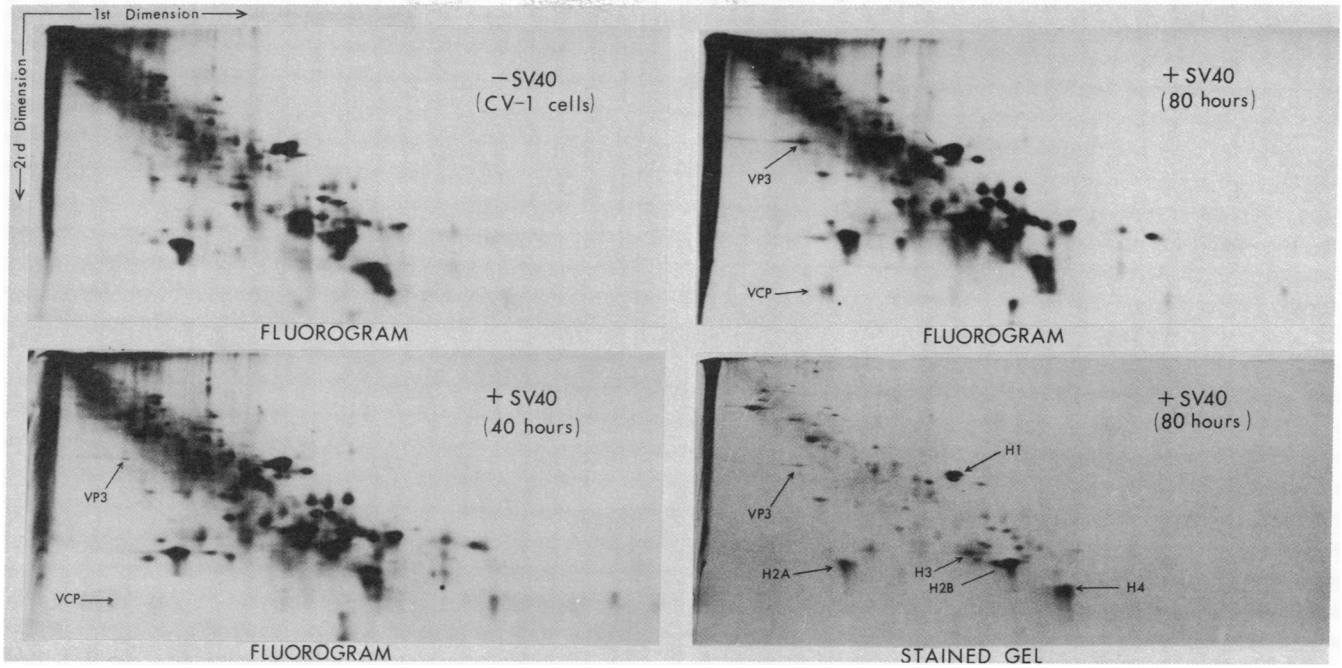

FIG. 3. Two-dimensional electrophoresis of the acid-soluble proteins from infected and uninfected cells. CV-1 cells were labeled for 15 min with [^{3}H]lysine. The acid-soluble proteins were isolated and separated. (*Upper Left*) Labeled protein distribution for uninfected CV-1 cells. (*Lower Left*) Early infection (40 hr). (*Upper Right*) Late infection (80 hr). (*Lower Right*) Stained gel of 80-hr fluorogram; positions of the histones and VP3 are indicated.

amounts of VP1, VP2, and VP3, the major SV40 packaging proteins. However, at this stage of infection these three proteins are obviously being made more rapidly than most other proteins (other than histone), as shown in the fluorogram.

Because a 6-min lysine pulse is not long enough to permit packaging into virus particles of those histones labeled in this time period, we conclude that the light region of the gradient contains at least two components: (*a*) newly replicated SV40 chromatin, and (*b*) SV40 chromatin molecules actually in the process of accumulating viral structural proteins and therefore showing decreased density. The largest amounts of VP1, VP2, and VP3 were found about five tubes into the gradient from the top, indicating that they are associated with nucleic acid yielding complexes of higher density and are not simply reflecting tailing from material at the top of the gradient. Also, because some of the viral proteins were found at the very top of the gradient, it is quite likely that we are also detecting newly synthesized viral proteins not yet associated with DNA.

Identification of a New Viral Specific SV40-Encoded Protein. The fluorogram in Fig. 2 reveals a single exception to the observation that the most rapidly synthesized proteins at this stage of infection are VP1, VP2, VP3, and histones. This exception is a rapidly migrating protein which we have designated VCP (virally coded protein). Either because the steady-state level of this protein is low or because it stains poorly as a consequence of its low molecular weight, we see only minimal staining by Coomassie dye. Thus, its rapid rate of synthesis at this stage of infection is a clearly distinguishing feature, leading us to wonder if it might be coded for by SV40 itself. The distribution of this protein toward the top of the gradient is different from that of VP1, VP2, and VP3 which peak before the top of the gradient. In contrast the yield of VCP increases towards the top of the gradient, indicating that much of the protein is probably not tightly bound to viral material. Nonetheless, because a significant fraction of this protein was found halfway down the gradient at $\rho = 1.43$ g/cm^3, it is likely that a portion of this material was associated with the viral material at the time fixation was initiated.

Initially we wondered if this protein were present only in virally infected cells. Control and infected CV-1 cells were exposed to 15-min pulses of [^{3}H]lysine at 40 and 80 hr after infection. The cells were then disrupted and extracted into acid. This single step removed most of the proteins. VCP previously had been found to be acid soluble. The entire acid-soluble pro-

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ATG	GTG	CTG	+ CGC	+ CGG	CTG	TCA	+ CGC	CAG	GCC	TCC	GTT	+ AAG	GTT	+ CGT	+ AGG
Met	Val	Leu	Arg	Arg	Leu	Ser	Arg	Gln	Ala	Ser	Val	Lys	Val	Arg	Arg
TCA	TGG	ACT	- GAA	AGT	+ AAA	+ AAA	ACA	GCT	CAA	+ CGC	CTT	TTT	GTG	TTT	GTT
Ser	Trp	Thr	Glu	Ser	Lys	Lys	Thr	Ala	Gln	Arg	Leu	Phe	Val	Phe	Val
TTA	- GAG	CTT	TTG	CTG	CAA	TTT	TGT	- GAA	GGG	- GAA	- GAT	ACT	GTT	- GAC	GGG
Leu	Glu	Leu	Leu	Leu	Gln	Phe	Cys	Glu	Gly	Glu	Asp	Thr	Val	Asp	Gly
+ AAA	+ CGC	+ AAA	+ AAA	CCA	- GAA	+ AGG	TTA	ACT	- GAA	+ AAA	CCA	- GAA	AGT	TAA	
Lys	Arg	Lys	Lys	Pro	Glu	Arg	Leu	Thr	Glu	Lys	Pro	Glu	Ser	Stop	
														443	

FIG. 4. Sequence of agnogene DNA and amino acid sequence of its putative product. The numbering system for the DNA sequence is taken from Reddy *et al.* (10). The amino acid sequence is derived from the genetic code and has been reported (11). The underlined regions indicate the hydrophobic amino acids; +, basic amino acids; −, acidic amino acids.

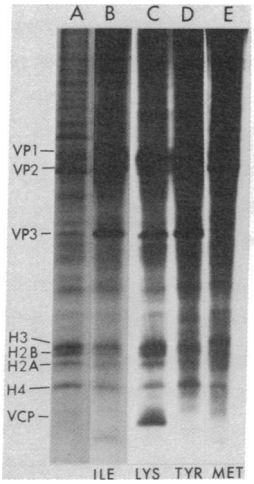

FIG. 5. Analysis of amino acid content of VCP in SV40-infected cells. CV-1 cells that had been infected for 2 days with SV40 were labeled with [^{3}H]isoleucine, [^{3}H]lysine, [^{3}H]tyrosine, or [^{35}S]methionine. The cells were then fixed and the SV40 virions and minichromosomes were extracted. After reversal of the fixation, the extract was then dialyzed against 50 mM 2-mercaptoethanol overnight and analyzed on $NaDodSO_4$ gels. The selective extraction of minichromosomes and virions by the fixation procedure is necessary to remove much of the background. Lanes B–E are fluorograms of the selective labeling. Lane A is a stained gel of the fluorogram in lane C, illustrating the bulk protein distribution.

tein fraction was then subjected to two-dimensional polyacrylamide gel separation. Because these are primarily basic proteins, instead of the system of O'Farrell we used a first dimension of Triton/acid/urea and a second dimension with $NaDodSO_4$ (Fig. 3). Of the SV40 coat proteins, only VP3 is acid soluble and can be identified only in infected cultures. It was particularly evident 80 hr after infection. The rate of synthesis for VCP was proportional to that of VP3, which would suggest that its synthesis occurs late in infection. In addition, VCP was found only in infected cultures. This indicates that VCP is coded for by the virus or is induced in the host cell late in infection. Fig. 3 *Upper Right* reveals that VCP is heterogeneous in the Triton/acid/urea dimension. This is due to phosphorylation (unpublished observations).

Evidence that VCP Is a Virus-Encoded Protein. The SV40 late mRNA leader sequence contains a translation initiation sequence followed by an open reading frame of 61 codons (4). The existence of a protein encoded by this agnogene has been postulated but never described (4). The predicted sequence of such a protein (Fig. 4), based upon the nucleotide sequence of Weissman and his colleagues (10), produces a small basic molecule which lacks isoleucine and tyrosine and has a single terminal methionine residue. Terminal methionine residues are often removed during posttranslational processing (refs. 12 and 13; for review, see ref. 14).

We have previously found that VCP contains lysine. We then assayed the VCP to determine if it contains isoleucine, tyrosine, or methionine. Pulses (20 min) of the appropriate amino acids were administered late in infection. The viral material was isolated and analyzed on $NaDodSO_4$ gels (Fig. 5). The VCP band contained lysine but lacked isoleucine, tyrosine, and methionine. These amino acids are incorporated into VP1, VP2, VP3, and the histones according to primary structural considerations. For example, histone H4 has only a single methionine at position 84 (15); yet this single methionine can be detected. Likewise, if methionine were present in the VCP we should have been able to detect it. This result is consistent with the conclusion that VCP is the product of the agnogene.

VCP Is not Present in Mature Virions. The data of Figs. 2 and 3 indicate that VCP is synthesized at approximately the same time as VP1, VP2, and VP3. In addition, from its position in the density gradients, it appears to be crosslinked to maturing SV40 particles, indicating a close association for at least a part of the lifetime of the developing virion. We wondered if the

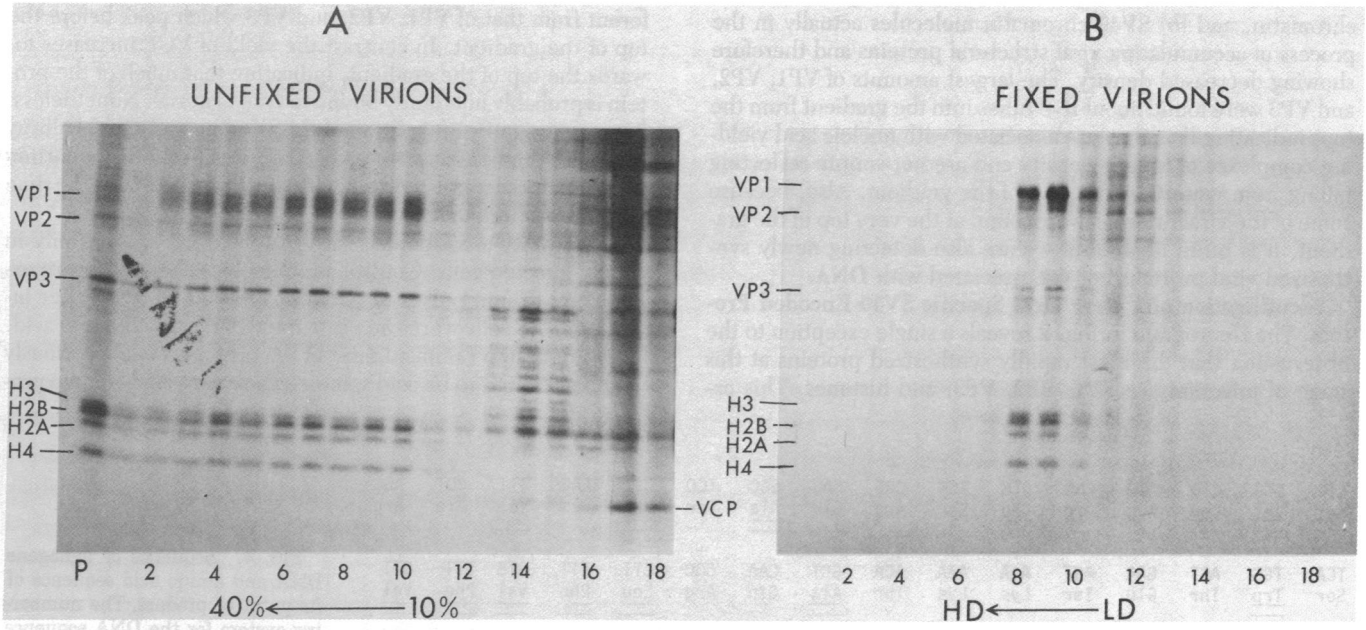

FIG. 6. Analysis of VCP association with mature virions. Infected CV-1 cells (plaque-forming units per cell) were grown for 1 week. (*A*) The cellular debris was collected and a portion was homogenized in 1 ml of 0.5% Triton X-100/10 mM Tris/0.1 M NaCl. The solution was centrifuged at 10,000 × *g* for 10 min. The supernatant was layered on 10–40% sucrose/10 mM Tris/0.1 mM EDTA/0.1 M NaCl and centrifuged at 35,000 rpm for 100 min in SW 41 rotor. Fractions were collected and analyzed on $NaDodSO_4$ gels. (*B*) A portion of the cellular debris was fixed for 18 hr in DME medium containing 1% formaldehyde at 4°C. The debris was then suspended in CsCl, ρ = 1.33 g/cm^3, and centrifuged to equilibrium in a SW 56 rotor at 33,000 rpm for 72 hr. Fractions were collected and adjusted to 4 M Gdn·HCl/0.5 M 2-mercaptoethanol for reversal at 100°C for 30 min. These samples were dialyzed and electrophoresed on $NaDodSO_4$ gels.

VCP were in fact packaged into the mature virus but had not been detected previously because of its characteristic failure to stain with Coomassie blue dye or to be labeled with [^{35}S]methionine. Accordingly, [^{3}H]lysine-labeled virions from a whole-cell lysate were sedimented through a 10–40% sucrose gradient to provide the distribution of DNA-associated proteins shown in Fig. 6A. The viral proteins VP1, VP2, and VP3 as well as the histones (except H1) are clearly in evidence. VCP also is present but at the top of the gradient, indicating that either it is not present in the mature virion or that it is only loosely bound so that it was removed during the homogenization and centrifugation process. This last point was obviated by an experiment in which we fixed the mature virion with formaldehyde before density analysis on CsCl gradients (Fig. 6B). Again, histones and VP1, VP2, and VP3 were associated with the mature virion; however VCP was not present, and we conclude that it does not form a part of the overall structure of mature SV40 virus.

DISCUSSION

Techniques previously developed to separate replicating and nonreplicating chromatin have been applied to the study of SV40 replication and packaging in CV-1 cells. The approach involves fixation with formaldehyde *in situ* so that even weak protein–nucleic acid interactions are maintained. An additional advantage is that, after the fixed nucleoproteins have been resolved on a density gradient, the crosslinks can be reversed so that both DNA and protein components can be analyzed with ease. After such an approach we find we can separate 85–90% of the SV40 chromosomes free from the bulk of other cellular components. The SV40 material so obtained can then be displayed on density gradients in which the separation reflects the degree of association of proteins to SV40 DNA.

The material of highest density is nonreplicating SV40 minichromatin. It contains the four core histones, a measure, although possibly not a full complement of H1, and histone variants such as A24 protein. Only small amounts of VP1, VP2, and VP3 are found in this region. The lighter regions of the gradient in all likelihood contain several different species of SV40 molecules. A minor, although important, component is the replicating chromosome found in a relatively discrete region. The major component in terms of mass is a broad distribution of molecules containing larger amounts of the viral proteins VP1, VP2, and VP3. At later stages in infection these three proteins are major synthetic forms found in association with the SV40 DNA. In terms of the distribution of these proteins in the density gradient it appears that the virus is packaged by the sequential accumulation of all viral proteins, a result not inconsistent with previous reports (16–19).

The maturation process may also use another protein, VCP. This may be associated with the virus during the packaging process although it is not retained in the mature virion itself. VCP is synthesized only during virus infection, and we have presented evidence that supports the idea that it may be the product of the agnogene, a small coding region (61 codons) identified in the late 16S mRNA leader sequence. The VCP migrates anomolously slowly in Triton/acid/urea gels (Fig. 3). This is seen even though it is clear, from its migration in $NaDodSO_4$ gels, that it is a small protein. This could be due to at least two possibilities. Either (*a*) the overall positive charge on the protein at pH 3.2 (the pH of the electrophoretic system) is low or (*b*) the protein is binding a large amount of Triton X-100. This second possibility indicates the presence of sequential hydrophobic amino acid residues (9, 15) and accounts for the anomolously slow migration of histone H2A in Triton/acid/urea gel (Fig. 3). That the latter explanation may be correct can be inferred from the predicted amino acid sequence of the protein of the agnogene shown in Fig. 4. In the middle of the molecule is an extended array of 10 hydrophobic amino acids interrupted only by glutamic acid and glutamine. By analogy with the distribution of hydrophobic residues in H2A we might well expect the VCP to migrate anomolously slowly in the presence of Triton X-100.

The possibility that the agnogene might code for a viral protein was first raised by the work of Mertz and Berg (20) who showed that deletions in this region led to changes in overall growth rate of SV40. In the light of experiments reported in this paper it seems possible that the rate-determining step might be the maturation process, and we suspect that the rate of this process may be increased through the action of VCP.

Note Added in Proof. While this manuscript was being reviewed, Khoury and his colleagues (21) reported observations that identified the product of the agnogene.

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