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NOTES

New Way to Isolate Simian Virus 40 Nucleoprotein Complexes from Infected Cells: Use of a Thiol-Specific Reagent

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A new method for the isolation of simian virus 40 nucleoprotein complexes from nuclei of lytically infected cells is described. The method is based on the addition of a thiol-specific reagent, 5,5′-dithiobis(2-nitrobenzoic acid), to lysis and extraction buffers. By inhibiting an uncoating activity during simian virus 40 extraction, 5,5′-dithiobis(2-nitrobenzoic acid) allows the use of efficient extraction buffers, such as ones containing Triton X-100 and EDTA, for the isolation of native simian virus 40 minichromosomes and virion-type structures. Use of the method is illustrated by following encapsidation of simian virus 40 minichromosomes in a pulse-chase experiment. Since 5,5′-dithiobis(2-nitrobenzoic acid) is an inhibitor of many different enzymes, the 5,5′-dithiobis(2-nitrobenzoic acid) extraction technique may be useful for the isolation of not only papovaviruses but also other viruses and possibly cellular chromatin.

A variety of procedures have been developed for the selective extraction of simian virus 40 (SV40) and polyoma nucleoprotein complexes (NPCs) from lytically infected cells (1–5, 7–12). Earlier methods have made use of nonionic detergents, the chelating agent EDTA, and monovalent salts at 0.1 to 0.2 M as components of extraction buffers (8, 14). These procedures yielded ~70S SV40 minichromosomes containing supercoiled SV40 DNA I in a complex with cellular histones and smaller, variable amounts of other proteins. More recently developed extraction media allow isolation of significant amounts of both previrion and virion structures, in addition to 70S minichromosomes, from SV40-infected green monkey cells (4, 5, 7, 9). These approaches are based largely either on the omission of chelating agents from extraction buffers or on the use of detergentless buffers in the presence of divalent cations. By these methods it became clear that the apparent absence of heavier SV40 complexes from the conventional Triton X-100–EDTA extracts is an artifact due to at least a partial uncoating, disassembly of previrion and virion structures, in the course of extraction in vitro (7).

In this note we describe the use of a thiol-specific reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (16), as a new way to prevent artifactual disassembly of SV40 NPCs during extraction. This method makes it possible to use various media, including both Triton X-100- and EDTA-containing ones, for gentle extraction of SV40 NPCs.

When nuclei of SV40-infected CV1 cells were isolated at 40 to 45 h postinfection and extracted with Triton X-100–EDTA or Nonidet P-40–EDTA buffers, the major SV40 NPC in the extract was a ~70S particle, a minichromosome (Fig. 1D and F). Identical extractions, except in the presence of 0.1 or 0.5 mM DTNB, resulted in a strong reduction in the relative amount of the ~70S minichromosomal peak, with a concomitant appearance of the ~180S peak (cf. Fig. 1A and D). Variation of the DTNB concentration between 0.1 and 0.5 mM did not result in a significant change in sedimentation patterns of extracted SV40 NPCs (cf. Fig. 1B and C). On the other hand, an increase in pH of the DTNB-containing extraction buffer from 7.5 to 8.0 resulted in a significant lowering of the relative proportion of the 180S peak (cf. Fig. 1B and A). Use of the DTNB-containing extraction buffer at pH 7.0 yielded results similar to those obtained at pH 7.5 (Fig. 1A), but the total yield of the SV40 NPCs was lower (data not shown). Addition of DTNB to only the lysis buffer yielded the same result as having DTNB in the lysis, rinse, and extraction buffers.

One set of optimal conditions for extraction of

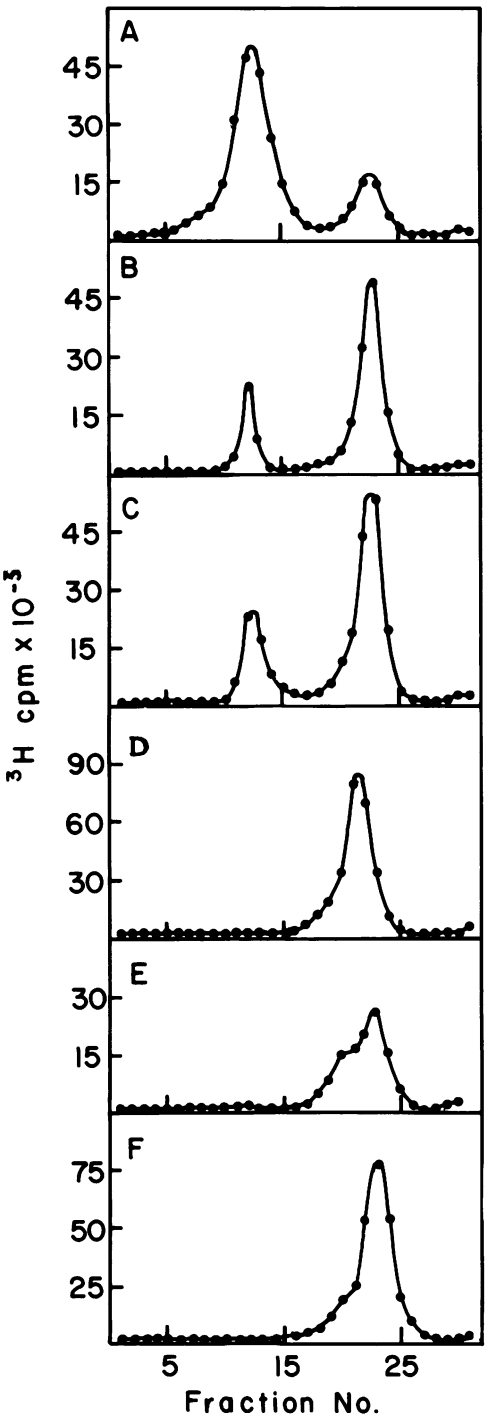
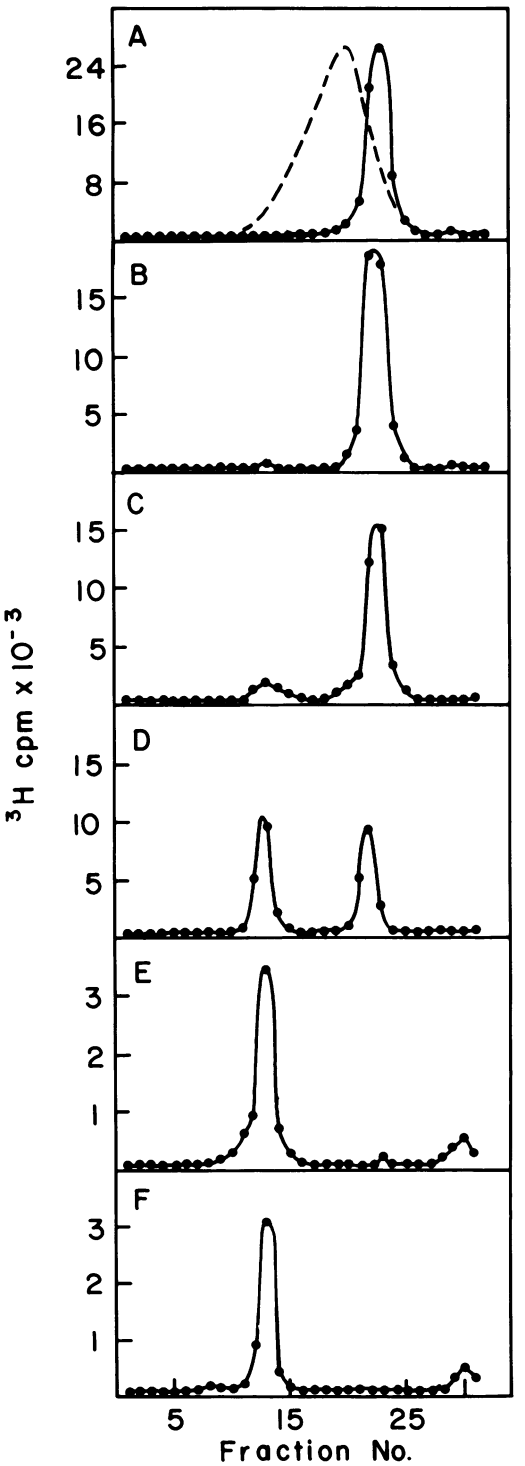


FIG. 1. Sucrose gradient sedimentation of SV40 NPCs. Cells were labeled with [*methyl*-^{3}H]thymidine from 28 to 42 h postinfection (13–15). (A) Cell monolayer rinsed with 0.14 M NaCl–5 mM Tris-hydrochloride, pH 7.5, followed by addition of cold lysis buffer (0.25% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTNB, 10 mM sodium EDTA, 10 mM Tris-hydrochloride, pH 6.8). DTNB (Sigma Chemical Co.) was added shortly before use from a freshly prepared 50 mM solution, the pH of which was adjusted to 7.5 with Tris base. The lysate was scraped from the plate with a rubber policeman, dispersed by siphoning through a plastic 10-ml pipette, and centrifuged at 4,000 × *g* for 5 min. The pellet was washed once with the lysis buffer and then briefly washed once more in the lysis buffer plus 0.1 M NaCl. The pellet was suspended and extracted in 0.25% Triton X-100–0.12 M NaCl–0.1 mM phenylmethylsulfonyl fluoride–0.5 mM DTNB–10 mM sodium EDTA–10 mM sodium HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.5, for 3 h with intermittent mild blending in a Vortex mixer. The suspension was centrifuged at 5,000 × *g* for 15 min, and the supernatant was then centrifuged through a linear 15 to 30% sucrose gradient containing 0.1 M NaCl, 1 mM sodium EDTA, and 10 mM triethanolamine-hydrochloride, pH 7.5, in an SW40 rotor at 30,000 rpm for 150 min at 4°C. (B) Same as in (A) but 0.1 mM DTNB instead of 0.5 mM and pH 8.0 instead of 7.5. (C) Same as in (B) but 0.5 mM DTNB. (D) Same as in (B) but no DTNB. (E) Cell lysis and extraction carried out by the method of Coca-Prados and Hsu (4). The virtual absence of 180S previrions in (E) could be due to a relative lability of strain 777 virions as compared with strain 776 (unpublished data). (F) Same as in (D) but 0.5% Nonidet P-40 instead of 0.25% Triton X-100.

SV40 NPCs in the presence of DTNB is described in the legend to Fig. 1A. Although the total yield of SV40 NPCs was 10 to 20% less in the presence of DTNB as compared with conventional Triton X-100–EDTA procedures (1, 13–15), it was still at least twice as great as the yield with divalent cation-containing extraction buffers, such as the one shown in Fig. 1E.

Previous work in several laboratories (4, 5, 7, 9, 11) has shown that replicating SV40 minichromosomes, after segregation into monomeric SV40 DNA I-containing 70S NPCs (13), are gradually converted into mature virions. To illustrate the use of the DTNB–Triton X-100–EDTA extraction technique, we followed the path of SV40 virion assembly in a pulse-chase experiment (Fig. 2). Shortly after the pulse, the label was found in replicating SV40 minichromosomes which sedimented faster than 70S monomeric minichromosomes (dashed line in Fig. 2A). After 30 to 60 min the label was found almost exclusively in 70S minichromosomes (Fig. 2B). Eventually, the label appeared in 180S previrion particles (Fig. 2C to E), the relative proportion of which increased steadily until at 54 h postinfection most of the pulse-label was found associated with the 180S peak (Fig. 2E). The 180S SV40 NPCs cosedimented with mature, CsCl-purified SV40 virions (cf. Fig. 2E and F) were indistinguishable from them by electron microscopy (Fig. 3), but unlike mature virions



were sensitive to high salt (Fig. 4). It is not clear what features of capsid structure or composition distinguish salt-sensitive previrions from salt-resistant mature virions. One possibility is that a network of disulfide bridges between VP1 capsomers (6), which certainly contributes to the salt resistance of mature SV40 virions, is either absent or incomplete in the 180S previrion particles isolated at ~40 h postinfection. The sedimentation coefficient usually assigned to mature SV40 virions is 240S; in our gradients, however, using the 60S ribosomal subunit as a marker, we estimated the sedimentation coefficient of both SV40 virions and previrions to be approximately 180S, in agreement with other authors (11).

It is not yet known how the thiol-specific reagent DTNB acts to inhibit virion uncoating in nuclear extracts in vitro. Disulfide linkages between capsid subunits appear to help stabilize virion structure in adenoviruses as well as in SV40 and polyoma virus (6). It has also been shown that stability of capsids can be lowered in the presence of reducing agents such as dithiotreitol (2). DTNB is known to react with exposed SH groups of proteins, converting them to aryl disulfides (16). The kinetics of this reaction can vary between SH groups that are exposed in a protein molecule to differing degrees, and the reaction of DTNB with disulfide linkages is limited by their rate of dissociation to free sulfhydryls. DTNB is known in a number of cases to promote the formation of disulfides in proteins (16 and references therein). It remains to be determined whether the inhibitory action of DTNB on virion uncoating in vitro results from its action on sulfhydryls of the virion or on other components of the nuclear extract or perhaps on both. The possibility that DTNB acts by reacting with an uncoating factor(s) in the nuclear extract stems from the observation that Triton X-100–EDTA buffers by themselves are insufficient to cause dissociation of SV40 virions, but Triton X-100–EDTA extracts of either infected or uninfected nuclei will carry out virion uncoating (7).

Whatever its mode of action, DTNB is a mild and selective agent useful in preserving a labile SV40 virion structure. It may also prove to be useful in the isolation of other types of labile virions and cellular chromatin.

FIG. 2. Maturation of SV40 NPCs as observed by the DTNB extraction method. Cells were labeled with [*methyl*-^{3}H]thymidine for 15 min at 28 h postinfection and then chased. SV40 NPCs were extracted as in Fig. 1A. (A) Pulse for 15 min and chase for 30 min. Dashed curve indicates the sedimentation pattern in the absence of a chase. (B) Same but chase for 1 h. (C) Chase for 4 h. (D) Chase for 15 h. (E) Chase for 24 h. (F) CsCl-purified mature SV40 virions (a marker).

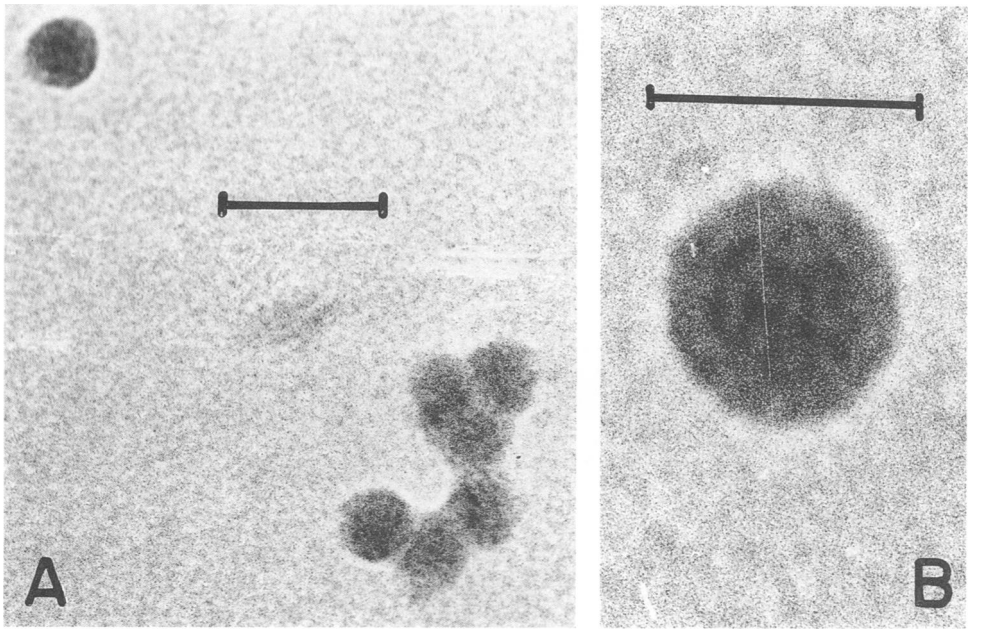


FIG. 3. Electron microscopic appearance of 180S SV40 NPCs. HCHO-fixed 180S SV40 NPCs (a larger peak in Fig. 1A) were negatively stained as described previously (14). The bars correspond to 100 and 50 nm in (A) and (B), respectively.

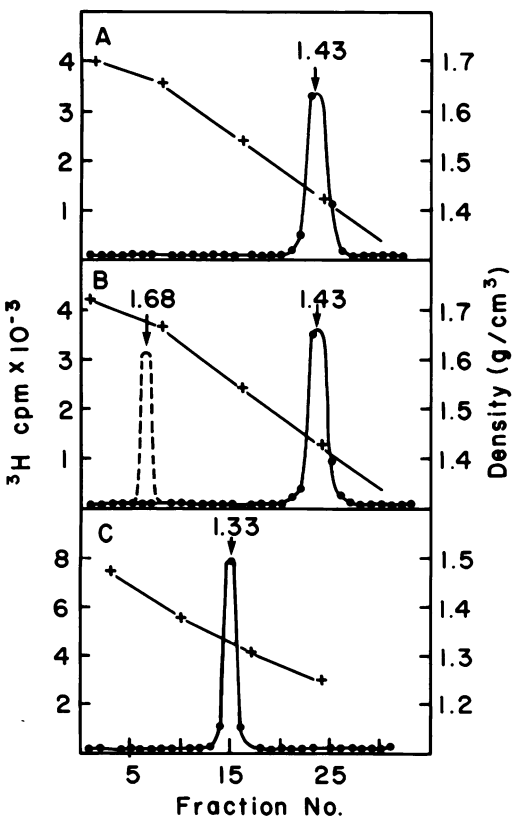


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FIG. 4. Isopycnic banding of SV40 NPCs in CsCl gradients. SV40 NPCs were fixed with HCHO (14, 15) and centrifuged to equilibrium in CsCl gradients containing 0.25% Sarkosyl NL95 in an SW50.1 rotor (14, 15). (A) Fixed 70S minichromosomes obtained by Triton X-100–EDTA extraction (see Fig. 1D). (B) Fixed 70S minichromosomes obtained in the presence of DTNB (see Fig. 1A). (C) Fixed 180S previrions (see Fig. 1A). Dashed curve in (B) denotes the position of the unfixed 70S minichromosomes banded in a separate tube in the absence of Sarkosyl.

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