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Simian Virus 40 Associates with Nuclear Superstructures at Early Times of Infection

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The association of infecting simian virus 40 with insoluble nuclear structures was assayed by disrupting infected nuclei and assaying insoluble fractions for virus. Three methods were used which lyse nuclei but maintain the insolubility of residual nuclear structures: sonication, high-salt–Triton–EDTA extraction, and low-salt–lithium diiodosalicylate extraction. After each type of nuclear extraction, infecting simian virus 40 remained associated with the residual nuclear structures. This association depended strictly on natural viral infections and on the use of buffers containing moderate amounts of salt and Mg^{2+} for the isolation of infected nuclei. These viral interactions exhibited behavior similar to host cell DNA interactions studied by analogous assays. Both viral DNA and coat proteins were found associated with the host cell nuclear superstructure. We conclude that at early times after infection the viral templates mimic the state of the host cell chromatin by attaching to the cellular nuclear matrix.

The DNA in higher eucaryotic cells is organized into loops of chromatin (6). The DNA is also intimately associated with nuclear protein structures, and it has been proposed that these associations are responsible for loop formation (7, 14, 28, 30, 38). The functional consequences of these associations are not yet clear, although numerous studies have proposed that they are important. These studies fall into two broad categories. In earlier studies, chromatin and nuclei were broken by enzymatic or physical means and the enrichment of active gene sequences within insoluble fractions was observed (see references 15, 17, 32, and 33, for example). More recently, the points of attachment of DNA to the insoluble nuclear matrix have been described and were often found to involve topoisomerase II (9, 18, 19) and to lie within noncoding DNA regions of potential regulatory function (13, 20, 26, 34, 37, 41). It has also been proposed that DNA replication is closely coupled to matrix attachment (see reference 38). Thus, there is a large body of indirect evidence that the ability to transcribe and replicate a gene may be influenced by its association with insoluble nuclear structures, which are often called the nuclear matrix.

If this phenomenon is important, it presents an interesting problem for an infecting virus. Since the virus is obviously unattached and inactive after infecting the host, the virus would need to either bypass a potential requirement for attachment to the host nuclear matrix or else evolve a mechanism for attachment. In the case of simian virus 40 (SV40), the early genes are known to be activated for transcription shortly after entering the nucleus of the host cell (36), and replication begins some hours later. It is not known whether the virus becomes associated with host insoluble nuclear structures during this early phase. The aim of this study is to determine whether such an association exists between the infecting virus and the host cell nuclear matrix.

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A potential problem in investigating the association of DNA with nuclear structures is that different methods use different salts and detergents that sometimes perturb protein-DNA associations. Moreover, these differences appear to vary among cell types. Therefore, it is critical to know how the host cell chromatin interactions are affected by procedures for preparation before studying the nature of virus-host interactions. In this study we show that under a variety of quite different experimental conditions, all of which preserve the association of host chromatin with insoluble nuclear structures, most or all of the infecting wild-type SV40 is found in an insoluble state within the host cell nucleus.

MATERIALS AND METHODS

Cell culture. CV-1 cells (22) were the host cells for all SV40 infections. Monolayers of CV-1 cells were grown on sterile petri plates and maintained in Dulbecco minimal essential medium supplemented with 5% calf serum. Cells were normally grown at a temperature of 37°C in an atmosphere containing 5% CO_2.

SV40. Strain 776 of SV40 was used as the wild-type strain (from M. Yaniv). The temperature-sensitive mutant *ts*D202 (12) was kindly provided by Robert G. Martin, National Institutes of Health.

In a typical preparation of radiolabeled virus, 40 10-cm confluent plates of CV-1 cells were infected with a common stock of wild-type SV40 (strain 776) at a multiplicity of infection (MOI) of 3 to 5 PFU/cell. To radiolabel viral DNA, [^{3}H]thymidine (40 to 50 Ci/mmol; New England Nuclear Corp.) was added to infected plates in Dulbecco minimal essential medium plus 1% calf serum 10 to 15 h after infection, at a specific radioactivity of 10 μCi/ml of medium. When protein-labeled virus was desired, L-[^{3}H]leucine (60 to 90 Ci/mmol; New England Nuclear) was added at 10 μCi/ml to infected plates of CV-1 cells maintained for 10 to 15 h in minimal essential medium devoid of L-leucine and containing 1% calf serum. Radiolabeled viral lysates were harvested 3 to 5 days after infection, when 50 to 75% of the cells showed a cytopathic effect.

Immediately after being harvested, radiolabeled SV40 lysates consisting of cells scraped off plates in medium were freeze-thawed twice, and then cell debris was clarified by low-speed centrifugation (17,000 × *g*) in a GS-3 rotor at 4°C for 20 min. The supernatant was saved, and the cell pellet was suspended in residual medium and freeze-thawed one more time. After the resuspended cell pellet was clarified in a second centrifugation, the supernatant was combined with the original viral supernatant.

Following removal of cell debris, radiolabeled virus was concentrated by centrifugation at 60,000 × *g* in a Beckman SW27 rotor for 3 h at 4°C. Virus which pelleted at this centrifugal force was then suspended overnight in sterile phosphate-buffered saline at 4°C.

Whole virions were separated from empty shells by sedimentation in CsCl by the method of Ozer (27). Final preparations of both DNA-labeled and protein-labeled virus contained 1×10^6 to 5×10^6 cpm/ml and 10^9 to 10^{10} PFU/ml.

Preparation of CV-1 nuclei. Nuclei were isolated from CV-1 cells growing on 6-cm plates. All procedures were carried out at 4°C. Confluent cell monolayers were washed twice with 1 ml of cold phosphate-buffered saline and then dislodged with a rubber policeman in 1 ml of the same buffer. Cells were then pelleted by spinning in plastic tubes (1.5 ml) for 3 min at 650 × *g* in an HB-4 swinging bucket rotor. Cell pellets were suspended in 0.5 ml of either an isotonic buffer or a hypotonic buffer by vortexing for 5 s. The isotonic buffer, which will be referred to as buffer A, contained 5 mM Tris hydrochloride, pH 7.5, 85 mM KCl, 5% sucrose, and 1 mM $MgCl_2$. This buffer is similar to a buffer used by Benyajati and Worcel to purify *Drosophila melanogaster* cell nuclei (6). The hypotonic buffer, termed buffer B, contained 1 mM Tris hydrochloride, pH 7.5, 1 mM KCl, and 1 mM $MgCl_2$. Cell lysis was initiated by the addition of an equal volume of either buffer A or B containing 1% Nonidet P-40 (NP40) to the resuspended cell pellets. Samples were vortexed vigorously for 20 s and incubated on ice for 3 min. Occasional vortexing during incubation helped to lyse the majority of the cells. Residual nuclei were pelleted by a low-speed spin at 100 × *g* in an HB-4 rotor for 3 min. At this centrifugal force, nuclei pellet away from cytoplasmic debris solubilized by NP40, but only barely. The nuclei which remained in the supernatant were similar in size and shape to the pelleted nuclei. Nuclei were then washed two additional times by low-speed centrifugation in buffer A or B containing 0.5% NP40. The last spin was for 5 min at 250 × *g*. Normally, nuclei purified from confluent 6-cm plates of cells were suspended in a final volume of 0.2 ml. When CV-1 nuclei were desired without Mg^{2+}, nuclei were washed in the final low-speed spin in buffer A or B containing 2 mM EDTA, and the final pellet was suspended in the same buffer.

Assay for the general insolubility of cell and viral chromatin. (i) CV-1 cell chromatin. Cell DNA was radiolabeled overnight by the addition of 2 to 5 μCi of [^{3}H]thymidine to preconfluent plates of CV-1 cells. At confluency, cells were harvested and their nuclei were isolated in buffer A or B as described in the previous section. The insolubility of CV-1 cell chromatin was measured by the following fractionation scheme. Samples (0.2 ml) of purified nuclei were incubated on ice for 30 min in plastic Eppendorf tubes (1.8 ml). Nuclei were then disrupted by sonication on ice with the microprobe of a Fisher sonic dismembrator (model 300) for 1 min at 0.4 to 0.6 relative output. Phase-contrast microscopy indicated that all nuclei were lysed under these conditions of sonication. After an additional 30 min on ice, samples containing fragmented chromatin were fractionated into an insoluble pellet and soluble supernatant by centrifugation at 5,000 × *g* in an HB-4 rotor. Both fractions of cell chromatin were solubilized to free DNA by incubation with proteinase K (100 μg/ml) and Sarkosyl (1%) at 56°C for 1 h (14, 15). Fractions were then counted for radioactivity in Hydrofluor (National Diagnostics). The percentage of total cell chromatin which was insoluble was defined as {[cpm in the pellet]/[total cpm in the nucleus (pellet + supernatant)]} × 100.

(ii) Viral chromatin. The insolubility of SV40 was determined in a manner similar to the assay described for cell chromatin. Confluent 6-cm plates of CV-1 cells were infected for 3 h with 10^4 cpm of purified virus. The MOI for each experiment was estimated to be 5 to 10 PFU/cell.

Nuclear matrix assay. (i) CV-1 cell chromatin. CV-1 nuclei were purified in the buffers used to determine the general insolubility of cell and viral chromatin. Nuclei were then lysed in high-salt or low-salt buffers containing detergent to yield a nuclear structure that was insoluble in these buffers and operationally defined as the nuclear matrix.

For high-salt lysis, 0.1 ml of nuclei prelabeled with [^{3}H]thymidine were apportioned into plastic Eppendorf tubes (1.8 ml) and incubated on ice for 15 min. Subsequently, nuclei were lysed by the addition of 0.3 ml of cold high-salt lysis mix containing 2.5 M NaCl, 2.7 mM Tris hydrochloride, pH 8.0, 67 mM EDTA, and 0.67% Triton detergent. Nuclear matrices were extracted at room temperature for 15 min after gently inverting the samples.

During the extraction period, samples were gently underlaid with 1.2 ml of 5% sucrose plus high salt (2 M NaCl, 2 mM Tris hydrochloride, pH 8.0, 10 mM EDTA, 0.2 μg of 4′,6′-diamidino-2-phenylindole dihydrochloride [DAPI] per ml and a cushion of 0.2 ml of 40% sucrose (5 mM Tris hydrochloride, pH 7.5, 85 mM KCl). Samples were centrifuged at 2,000 × *g* for 5 min and then at 8,000 × *g* for 20 min in an HB-4 swinging bucket rotor to sediment nuclear structures. The inclusion of DAPI (40) allowed the position of cell DNA free of soluble proteins to be monitored visually with a UV lamp. On the average, nuclei from 5×10^5 to 1×10^6 CV-1 cells were lysed by the high-salt mix and loaded on a gradient. Under these conditions of centrifugation, only large insoluble structures such as the nuclear matrix and residual nuclei penetrated the 5% sucrose–high-salt layer. Whole DNA which is stably associated with the nuclear matrix pellets as a dense white fluorescence in the presence of DAPI at the interface of the two sucrose layers. Nuclear matrix and attached DNA were removed in a volume of 0.3 ml with a 20-gauge needle attached to a 1-ml tuberculin syringe. Insoluble nuclear structures isolated by this procedure appeared similar to the "nucleoids" isolated by Cook et al. (14) when viewed with a phase-contrast microscope.

Matrix-attached DNA was solubilized by the addition of proteinase K (100 μg/ml) and Sarkosyl (1%) and incubation at 56°C for 1 h. The matrix sample (matrix) was then diluted to 1.5 ml with 5% sucrose containing high salt and counted for radioactivity along with the remaining 1.5 ml of the sucrose gradient (supernatant) in a total of 20 ml of Hydrofluor. The percentage of total DNA attached to the nuclear matrix was calculated as {[cpm in the matrix sample (matrix)]/[total number of counts (matrix + supernatant)]} × 100.

For low-salt lysis, the protocol for isolation of cell DNA attached to the nuclear matrix under low-salt conditions was similar to the procedure described above for high-salt lysis except for the contents of the lysis mix and the sucrose step gradient. The low-salt lysis buffer contained 5 mM Tris hydrochloride, pH 7.5, 2 mM KCl, 2 mM EDTA, 0.1%

TABLE 1. Insolubility of CV-1 cell chromatin[a]

Line no.	Nuclear isolation buffer	Sonication	Nuclear integrity (size)	% Insolubility
1	Isotonic	−	Slightly enlarged	97
2	Isotonic	+	Lysed	55
3	Isotonic (1 mM $MgCl_2$)	−	Normal	97
4	Isotonic (1 mM $MgCl_2$)	+	Lysed	97
5	Hypotonic	−	Swollen	97
6	Hypotonic	+	Lysed	6 (99% with 5 mM $MgCl_2$)
7	Hypotonic (1 mM $MgCl_2$)	−	Slightly enlarged	97
8	Hypotonic (1 mM $MgCl_2$)	+	Lysed	61

[a] The percentage of total CV-1 chromatin and SV40 which pelleted after sonication was measured. In line 6, the insolubility of chromatin in hypotonic buffer was also measured after the addition of 5 mM $MgCl_2$ to sonicated nuclei. The appearance of nuclei in various isolation buffers was determined from phase-contrast micrographs. In a typical experiment, 100% cell chromatin insolubility equaled 10^5 cpm of [^{3}H]thymidine-labeled cell DNA.

Triton detergent, and 25 mM 3,5-diiodosalicylate detergent (LIS; Eastman Kodak). The 5 and 40% sucrose layers contained the same ingredients without Triton detergent or DAPI.

(ii) SV40. The attachment of SV40 to the nuclear matrix was monitored by infecting confluent monolayers of CV-1 cells (6-cm plates) with CsCl-purified virus, labeled with either [^{3}H]thymidine or [^{3}H]leucine. Cells were infected with 10^4 to 10^5 cpm of radiolabeled virus at an MOI estimated to be 10 to 100 PFU/cell. The procedures for cell harvest, nuclei purification, and nuclear matrix extraction by high-salt and low-salt lysis were the same as the methods detailed above for cell DNA.

RESULTS

The appearance of nuclei and the integrity of chromatin can both depend on the solution in which they are suspended (1, 2, 5, 24). Studies of the nuclear matrix and its complexes with DNA sometimes include exposure of nuclei to low-ionic-strength buffers (8, 13, 18, 35), and the concentration of divalent cation often varies considerably among procedures (13, 26, 34); such conditions can be critical in determining the extent to which DNA matrix interactions are preserved in mouse 3T6 cells (21; J. D. Gralla and M. Yaniv, unpublished data). We chose to investigate these effects in CV-1 monkey kidney cells (22), which is a standard established cell line for SV40 infection.

Cells were incubated overnight with [^{3}H]thymidine to label cellular DNA, and nuclei were prepared in various ways. These nuclei were then lysed by sonication and separated into insoluble pellet and soluble supernatant fractions by low-speed centrifugation. A summary of the partitioning of DNA radioactivity into soluble and insoluble fractions of the sonicated nuclei is presented in Table 1. The results indicate that two variables are important in determining the degree of insolubility for lysed CV-1 cell DNA, ionic strength and MG^{2+} ions. First, as expected, the majority of the cell DNA was insoluble when the nuclei were not sonicated, and this effect was independent of the buffer used for nucleus preparation (lines 1, 3, 5, and 7). Secondly, the ionic strength of the isolation buffer governed the amount of cell chromatin solubilized by sonication. As shown in line 2 of Table 1, the majority of the sonicated chromatin remained insoluble when nuclei were prepared in isotonic buffer, whereas only 6% of the chromatin from hypotonic nuclei was insoluble (line 6). Most of the sonicated chromatin fragments were approximately 5 kilobases in size (not shown). Lastly, the addition of 1 mM $MgCl_2$ to either isotonic or hypotonic buffer protected chromatin against release by sonication (97% insolubility for isotonic [1 mM $MgCl_2$] in line 4, and 61% insolubility for hypotonic [1 mM $MgCl_2$] in line 8). However, since Mg^{2+} ions can precipitate chromatin in the range of 1.5 to 2 mM $MgCl_2$ (1, 2), the partial apparent protection may simply be due to artifactual precipitation. Indeed, broken chromatin released by sonication of hypotonically purified nuclei could be completely precipitated by 5 mM $MgCl_2$ (line 6, 5 mM $MgCl_2$ added after sonication of nuclei). Therefore, the observed 40% solubilization of chromatin obtained from hypotonic nuclei plus 1 mM $MgCl_2$ represents a minimum value, since some soluble chromatin may have precipitated.

Thus, the majority of the sonicated CV-1 chromatin was insoluble when nuclei were prepared in buffers of ionic strength resembling physiological conditions (isotonic plus $MgCl_2$). In striking contrast, incubation of CV-1 nuclei in hypotonic solutions sensitized cell chromatin to be released and solubilized by sonication. A similar finding was reported by Jackson and Cook (21), who observed that nascent cell DNA of HeLa cell nuclei prepared in a hypotonic solution was more sensitive to digestion by endonucleases. Also, DNA replication of HeLa cell DNA was reduced dramatically by treating nuclei with hypotonic buffer. These results confirm the importance of taking into account variations in methods for nuclei preparation when investigating the interactions of chromatin with insoluble nuclear structures.

Insolubility of early SV40. The observation that the relative insolubility of sonicated CV-1 cell chromatin varied with the buffer conditions chosen to isolate nuclei prompted a similar analysis of wild-type SV40 virions early after infection of CV-1 cells. Radiolabeled SV40 was purified from cell lysates and used to infect fresh monolayers of CV-1 cells. This was the only source of radioactive DNA in these experiments, and thus the state of viral DNA could be followed specifically. The procedures for purification of CV-1 nuclei and fractionation of chromatin by sonication and centrifugation were identical to those used in the previous experiments for cellular DNA. Initially, control experiments were performed in which the [^{3}H]thymidine-labeled virus was mixed on ice for 30 min with nuclei isolated from uninfected cells and then sonicated and assayed. Approximately 10% of the purified virus was rendered insoluble after sonication and centrifugation, independent of prior conditions for nuclei isolation (Fig. 1). The exogenous virus was not degraded by sonication, since the majority of sonicated virus sedimented with purified virions at a position of 240S on sucrose gradients (not shown). These results show that none of the conditions previously used to probe the solubility of fragmented CV-1 chromatin led to artifactual precipitation of SV40 even in the presence of lysed nuclei.

Next, radiolabeled virus was incubated with CV-1 cells for 3 h to allow infection to occur, and the infected nuclei were then purified. Under these conditions, it was observed that approximately 1% of the radioactive infecting virus entered the nucleus, consistent with the known low infectivity of SV40 particles (36). In this experiment, almost all of the virus pelleted with the nuclei, as expected (Fig. 1, not sonicated). Nuclei containing radiolabeled viruses were then

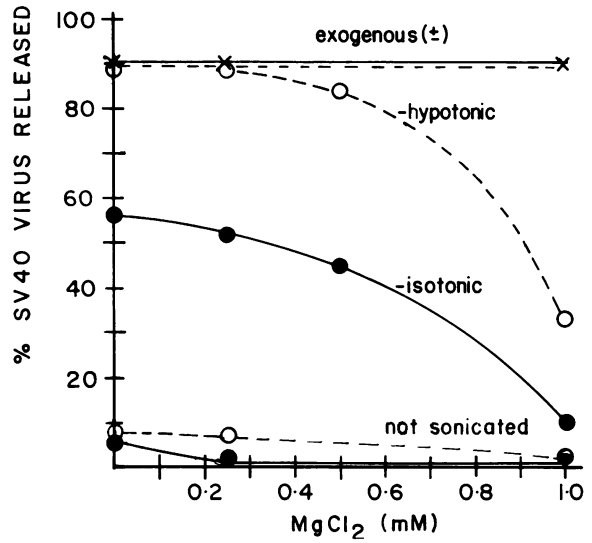

FIG. 1. Insolubility of early SV40. Confluent CV-1 cells (6-cm plates) were infected for 3 h with purified [^{3}H]thymidine-labeled SV40 776 (10^4 cpm). Nuclei were prepared in either isotonic (●) or hypotonic (○) buffers containing various concentrations of $MgCl_2$. All samples were then sonicated, except as indicated. The percentage of total nuclear SV40 which pelleted after sonication was measured by the fractionation scheme outlined in Materials and Methods. In control experiments (×), [^{3}H]thymidine-labeled virus (exogenous ±) was added to either isotonic or hypotonic nuclei isolated from mock-infected CV-1 cells (± refers to the presence or absence of sonication). Each point on the graph is based on approximately 100 to 200 cpm of total nuclear virus averaged from three different infections. Dashed lines refer to hypotonic conditions.

incubated in various solutions and broken by sonication. When nuclei were present in isotonic buffer with 1 mM $MgCl_2$, the viral DNA remained insoluble in the broken preparation of nuclei obtained by sonication (Fig. 1). As the concentration of $MgCl_2$ was lowered, the virus became partially sensitive to sonication-induced solubilization, although even in the absence of $MgCl_2$ about one-half of the virus remained insoluble after sonication (Fig. 1, isotonic). These properties are very similar to those of host cell chromatin (Table 1).

Figure 1 also shows that hypotonic conditions sensitized the viral DNA to be released by sonication, as was also observed for the release of cellular DNA in parallel experiments (Table 1). Again, Mg^{2+} provided partial protection, but at all test concentrations of $MgCl_2$ more virus was solubilized by sonication in hypotonic than in isotonic buffers (Fig. 1). Note again that the solubility properties of exogenous virus sonicated in the presence of nuclei were not at all affected by the presence of salt or $MgCl_2$, confirming that the observed effects were a consequence of infection (Fig. 1). Together these studies indicate that the majority of virus is driven into an insoluble state in the nucleus after infection and mimics the physical state of the host cell chromatin.

Association of cell DNA with the nuclear matrix. These results demonstrated that the DNA of the infecting virus was associated with the residual nuclear structures which remained after severe disruption by sonication. Cellular DNA is known to be resistant to even more severe nuclear extraction procedures. When nuclei from several cell types are challenged with 2 M salt, 50 mM EDTA, and Triton detergent, most of the chromosomal proteins are solubilized, yet the intact DNA remains insoluble (7, 14, 28, 34, 38). This is taken as evidence that the DNA is anchored to a residual insoluble nuclear protein structure, often called the nuclear matrix. We asked whether CV-1 cell DNA (and intranuclear viral DNA) also behaved in this manner.

CV-1 cells were prelabeled with [^{3}H]thymidine, and nuclei were prepared in buffers identical to those used to determine the general insolubility of sonicated CV-1 cell chromatin. Subsequently, nuclei were lysed in a high-salt buffer containing 1.9 M NaCl, 50 mM EDTA, and 0.5% Triton and then sedimented through a step gradient consisting of 5% sucrose plus the high-salt buffer and a dense cushion of 40% sucrose. These conditions separate the solubilized DNA remaining in the supernatant from large insoluble structures which penetrate the 5% sucrose–high-salt layer but not the 40% sucrose cushion (Gralla and Yaniv, unpublished; see also below). Clean isolations normally revealed a dense flocculent pellet at the interface of the two sucrose layers. The inclusion of DAPI, a fluorescent dye which binds DNA (40), allows the nuclear matrix-attached DNA and residual chromatin to be visualized as white fluorescent tangles. The fraction of the total radioactivity which resides at the interface is taken as the fraction of DNA associated with insoluble nuclear structures.

It was found that most of the host CV-1 cell DNA was attached to the nuclear matrix extracted with high-salt Triton-EDTA from either isotonic-Mg^{2+} or hypotonic-Mg^{2+} nuclei (Table 2). This association cannot be an artifact of Mg^{2+} precipitation, since the original 1 mM $MgCl_2$ in the nuclei was clearly quenched by the 50 mM EDTA present in the challenge solution. While more than 90% of the DNA in these nuclei containing Mg^{2+} was resistant to solubilization by high-salt challenge, hypotonic nuclei without Mg^{2+} yielded matrix structures with only 26% of the total DNA attached. This result argues against the chromatin being precipitated by the high salt in the challenge solution, since hypotonic and isotonic nuclei were subjected to the same high-salt challenge. Results obtained for isotonic nuclei without Mg^{2+} were not reproducible. We conclude that when CV-1 nuclei are prepared in $MgCl_2$-containing buffers, the DNA is attached to a nuclear structure which resists a severe solubilization challenge.

CV-1 nuclei were also extracted in a low-salt buffer containing the detergent LIS. LIS was included in the extraction buffers because it mimics the above procedure in its ability to extract nuclear proteins (13, 20, 26) but does not require the use of high salt. The low-salt-LIS extraction results led to the same conclusions as the high-salt extraction (Table 2). CV-1 DNA was stably attached (98%) to LIS-resistant, insoluble structures when nuclei were prepared in isotonic (1 mM $MgCl_2$) buffer. The attachment was again

TABLE 2. Attachment of CV-1 cell DNA to the nuclear matrix[a]

Nuclear isolation buffer	% Attachment to nuclear matrix	
	High-salt extraction	Low-salt-LIS extraction
Isotonic	25–85	93
Isotonic (1 mM $MgCl_2$)	95	98
Hypotonic	26	7
Hypotonic (1 mM $MgCl_2$)	92	35

[a] The percentage of total cell DNA which was resistant to high-salt and low-salt-LIS challenge was measured by the nuclear matrix assay outlined in Materials and Methods. On the average, 100% attachment equaled 10^5 cpm of [^{3}H]thymidine-labeled cell DNA.

disrupted by hypotonic conditions, with only 7% of the DNA attached to LIS-resistant matrices from hypotonically prepared nuclei. However, there were some differences in extractibility by the high-salt and low-salt-LIS buffers. Specifically, the addition of 1 mM $MgCl_2$ to hypotonic nuclei led to protection of only 35% of the DNA from the low-salt-LIS challenge, in contrast to 92% protection in the high-salt challenge. Together these two types of challenge experiments demonstrated that nuclei prepared in isotonic buffers with low amounts of $MgCl_2$ contained DNA which was stably attached to nuclear structures resistant to solubilization. These conditions, among those tested, most closely approximate physiological conditions.

Association of infecting virus with the nuclear matrix. The association of intranuclear SV40 with such insoluble nuclear structures was measured in an entirely analogous manner. Confluent cells were infected for 3 h with purified [^{3}H]thymidine-labeled SV40, and nuclei were then prepared in the various solutions described previously. In contrast to CV-1 cell DNA, which is nuclear, the extent of association of SV40 could include some contribution from virus in the small amount of contaminating cytoplasm. Thus, it was important to demonstrate that the exogenous virus would not artifactually associate with the nuclear matrix fraction in the high-salt challenge. Purified SV40 or late SV40 minichromosomes labeled with [^{3}H]thymidine were incubated with uninfected nuclei prepared in various buffers and then challenged with high-salt extraction buffer. Less than 10 to 15% of virus and minichromosome, respectively, became associated with the CV-1 nuclear matrix fraction in these control mixing experiments. Therefore, if contaminating cytoplasmic virus is present at the time of high-salt challenge, only up to 15% would be expected to pellet in the assay.

In CV-1 nuclei infected with radioactive SV40, more than 60% of the viral DNA was attached to the nuclear matrix at 3 h postinfection in isotonic or hypotonic buffer containing 1 mM $MgCl_2$ (61 and 63%, respectively, where 100 to 200 cpm represents 100% attachment in three separate experiments). It was critical that the nuclei be isolated in the presence of Mg^{2+}, since less than 30% of the infecting virus was attached to the nuclear matrix of nuclei prepared in the absence of $MgCl_2$ (26 and 29% for isotonic and hypotonic buffers, respectively). Recall that a high level of matrix attachment of cell DNA was possible in isotonic nuclei when Mg^{2+} was omitted. Apparently, there is a more stringent requirement for Mg^{2+} to maintain the association of virus with the cell matrix.

Comparison of CV-1 cell DNA matrix attachment (95%) with that of viral DNA (60%) indicates that one-third less viral DNA than cell DNA appears to be attached to the nuclear matrix. This could be explained if low amounts of cytoplasmic virus had survived the extensive nuclear washes. For example, since only approximately 1% of the total inoculum of radioactive virus entered the nuclei, cytoplasmic contamination of only 0.5% of the total input virus would account for a third of the viral radioactivity appearing in the high-salt supernatant. (Recall that the mixing experiments imply that > 85% of the cytoplasmic virus would stay in the supernatant.) Alternatively, the early SV40 may not be as firmly attached to the nuclear matrix as the host cell chromatin. In any case, the value of 60% attachment is very clearly a minimum estimate.

It is conceivable that the degree of attachment of the early SV40 to the host cell nuclear matrix changes temporally as the virus is activated for early transcription and then replication prior to the onset of late transcription. This possibility was addressed by measuring viral DNA attachment to the CV-1 nuclear matrix throughout the early phase of infection (not shown). We found a slight tendency for increased attachment with time, with 67% of the virus attached by 18 h.

TABLE 3. Attachment of [^{3}H]leucine-labeled SV40 to the nuclear matrix[a] at 15 h postinfection

Line no.	SV40 strain	Temp (°C)	% Attachment to nuclear matrix (high-salt extraction)
1	776	37	54
2	776 (ara-C)	37	53
3	776	33	53
4	776	41	58
5	*ts*D202	33	51
6	*ts*D202	41	52

[a] Confluent 6-cm plates of CV-1 cells were infected at different temperatures with purified SV40 viruses prelabeled with [^{3}H]leucine (10^4 cpm). After 15 h of infection at either 33, 37, or 41°C, the percentage of either wild-type (776) or mutant (*ts*D202) SV40 DNA resistant to high-salt extraction was measured in the nuclear matrix assay. The temperature-sensitive mutant *ts*D202 did not produce plaques at the restrictive temperature of 41°C. Arabinofuranosyl cytosine (ara-C) was added to the medium at a concentration of 50 μg/ml after the virus had adsorbed to the cell monolayer for 2 h (line 2).

Association of intact virus and coat proteins. The experiments above demonstrated that the SV40 DNA became attached to the nuclear matrix as early as 3 h postinfection, and this attachment continued for up to 18 h, which marks the beginning of the late phase of infection. Since the viral DNA is covered with coat proteins after entry into the cell, it seems possible that the coat proteins participate in mediating matrix attachment. It is known that sometime during the early phase of infection the virus is disrupted and some proteins are lost (4). [^{3}H]leucine-labeled virus was used to infect CV-1 cells, nuclei were purified, and soluble and insoluble nuclear matrix fractions were examined for viral protein at 14 to 15 h postinfection. At this time of infection, the infecting virus should be stripped of the majority of its coat proteins (4). The protein-labeled virus was attached to the nuclear matrix at a level of 50 to 60% (Table 3, line 1). This level of attachment was not altered by the presence of arabinofuranosyl cytosine, which prevents replication of the virus (11) and restricts it to the early phase (Table 3, line 2). This level of attachment was only slightly lower than the extent of matrix attachment found earlier with DNA-labeled virus (Fig. 1). Since both DNA and protein were attached to a comparable extent, these results raise the possibility that whole virus attaches initially via its exposed protein coat, and after partial uncoating both the protein and the DNA may remain on the matrix. However, we cannot be certain that labeled coat proteins do not associate with the matrix only after their dissociation from whole virus.

The existence of a temperature-sensitive SV40 mutant (*ts*D202) (3, 12, 31) allowed a further test of the proposal that the whole virus attaches to the nuclear matrix. This virus is defective in early transcription at the restrictive temperature, presumably due to a defect in uncoating. The attachment of [^{3}H]leucine-labeled *ts*D202 virus to the CV-1 nuclear matrix was measured at 15 h postinfection. There were no significant differences in the degree of attachment to the nuclear matrix between protein-labeled *ts*D202 virus at the permissive and restrictive temperatures and the wild-type virus at the restrictive temperature (Table 3). These results indicate that the majority of the *ts*D202 viruses attach to the nuclear matrix even when the virus cannot release its coat proteins. This observation supports the hypothesis that the

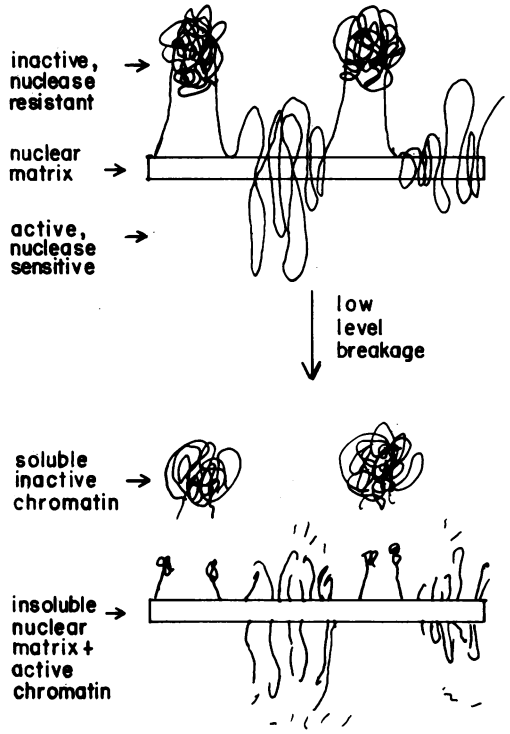

FIG. 2. Model for attachment of cell chromatin to the nuclear matrix. (Top) Loops of chromatin are drawn attached to an isolated nuclear matrix fiber. The condensed loops represent chromatin that is DNase-resistant and inactive for gene expression. These loops are attached to the matrix fiber at a minimal number of sites (two are shown). Expanded domains of DNase-sensitive active chromatin are shown as extended loops attached to the matrix fiber at a multiple number of sites. Therefore, this DNase-sensitive active chromatin would be strongly attached. (Bottom) After low-level breakage of chromatin (mechanical or nuclease), active chromatin is known to remain largely insoluble while inactive chromatin is more easily solubilized. The cartoon rationalizes this by showing that the condensed inactive chromatin is most easily released (solubilized) due to fewer sites of attachment to the matrix. By contrast, although the active chromatin is broken more easily, less of it is released (solubilized) because it is attached to the matrix at many points.

infecting wild-type virus attaches to the nuclear matrix partly through use of its protein coat.

DISCUSSION

These experiments used three different challenge assays, namely sonication, high-salt–Triton–EDTA extraction, and low-salt–LIS extraction, to demonstrate that SV40 becomes associated with insoluble host nuclear structures during the early phase of infection. When the infected nuclei were prepared in relatively physiological buffers, the virus was observed to be largely resistant to solubilization in all three assays. Insolubility was strictly dependent on infection, since virus simply mixed with nuclei remained soluble when the nuclei were disrupted by each of the three challenge methods. The virus could, however, be solubilized by some of these challenges when the infected nuclei were suspended in nonphysiological solutions containing little salt or lacking Mg^{2+}. The same nonphysiological solutions were also shown to sensitize the host cell chromatin to solubilization in the challenge assays. We conclude that the infecting virus becomes associated with insoluble nuclear structures and that such associations can be loosened in vitro by the lack of salt or Mg^{2+}.

What components of the virus mediate the attachment? The results show that the virus becomes associated with the nuclear structure as early as 3 h postinfection, which is before it is very active in early gene expression (36). This suggests that the virus particle may still be substantially intact after attachment; that is, it still may be covered with coat proteins. If so, these proteins may mediate the attachment, and this idea was tested by assaying the attachment of a mutant virus which is defective in uncoating and early transcription (*ts*D202) (3, 31). Since this mutant virus was also observed to be associated with the nuclear matrix, it seems that at least one type of attachment involves viral coat proteins. Recent studies with other systems (13, 26) have shown that specific DNA sequences can be selectively associated with matrix attachment sites. Our data cannot reveal whether certain SV40 DNA sequences are also selectively associated with the nuclear matrix. There could well be more than one type of attachment site for the virus, and below we suggest that this may indeed be true. It is clear that proteins are involved in the attachment of cellular DNA (9, 10, 18, 19, 25, 28, 29), and it would be interesting to learn whether the coat proteins of nuclear viruses mimic some of these cellular proteins in certain respects.

Although the attachment of chromatin to the nuclear matrix is often proposed to be of functional importance, this conclusion is made uncertain by apparent contradictions in the literature regarding the nature of attached DNA sequences (7, 15, 26, 34). Some of these may be reconciled by the present observations that DNA-matrix interactions may be loosened by incubating nuclei in buffers with low salt concentrations or lacking Mg^{2+} (21). Other problems, however, are clearly conceptual rather than experimental. Numerous studies have shown that active gene sequences are preferentially associated with insoluble structures (15, 17, 32, 33), yet numerous studies have also shown that active gene sequences are also more accessible to nuclease attack (23, 39). How can active genes be closely bound to insoluble protein structures and yet be more accessible to attack from solution? This is apparently true for SV40, since we show that the virus remains attached during the time it is known to become nuclease sensitive and activated for transcription and replication (16).

One simple way to reconcile these observations in the literature is to propose that after activation, attached preexisting stable loops of chromatin decondense and form additional associations between special sequences and the nuclear matrix. The chromatin would then be more accessible to attacking nucleases due to its expansion, but it would take many more DNA breaks to release the same amount of DNA since it is attached at additional points to the matrix (Fig. 2). In the case of SV40, one would argue by analogy that the inactive whole (condensed) virus first attaches via its coat proteins and then decondenses to allow the viral chromatin to form other associations with the matrix. The decondensed viral chromatin could then be available for the further binding of factors which normally associate with cellular chromatin-matrix templates. These might now interact with an analogous viral template to activate its transcription and replication.

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