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The structure of the nucleoprotein core of adenovirus has been investigated. Using a combination of biophysical techniques and nuclease digestion I have determined that the adenovirus core, as isolated with pyridine, shares several physical properties with cellular chromatin. The most notable difference is the lack of a nucleosome repeat in the staph nuclease digestion intermediates from adenovirus cores. I have exploited this difference to study the fate of the nucleoprotein structure of adenovirus DNA early during productive infection. When nuclei from cells infected with ^{32}P-labeled adenovirus are digested with staph nuclease the pattern of labeled digestion intermediates more closely resembles the pattern of cellular intermediates. This result indicates that virus core proteins are replaced, early during infection, with cellular histones. The newly assembled adenovirus nucleosomes differ from the bulk of the cellular chromatin by having a shorter repeat (160 vs. 185 base pairs)

and more labile linker and core regions. Experiments with inhibitors of cellular DNA synthesis indicate that adenovirus nucleosomes are assembled independently of cellular DNA synthesis. Using the Southern blotting technique it is shown that newly synthesized adenovirus DNA is not in a nucleosome repeat late in infection. At this time of infection, however, the input adenovirus genomes persist in a nucleosome structure. The results of these experiments are discussed in terms of the template requirements for eucaryotic transcription and replication.

Nucleoprotein Structure of Adenovirus

by

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NUCLEOPROTEIN STRUCTURE OF ADENOVIRUS

PREFACE

Interactions between proteins and DNA are of unquestioned importance in the facilitation and modulation of information transfer in living systems. Two classes of protein-DNA interactions can be described. Some proteins recognize and bind specific DNA sequences while others bind non-specifically or show only a weak base composition selectivity. Histones are among the class of proteins that bind non-specifically to DNA. In the last five years the structure of histone-DNA complexes in nucleosomes, chromatin, and chromosomes has become well understood but the function of histones remains an enigma. The phylogenetic diversity and evolutionary conservation of histones suggests they play a fundamental role in eucaryotic cellular processes. Early experiments suggested that histones function to repress transcription (Huang and Bonner, 1962). Recent experiments (Lacy and Axel, 1975; Axel et al., 1975; Kuo et al., 1976) have shown, however, that actively transcribed DNA sequences in eucaryotic cells are complexed with histones to form nucleosomes.

Three functional roles for histones can be imagined. First, histones might be functionally inert, acting only to compact DNA and allowing RNA and DNA polymerase free access to DNA. Second, histones might facilitate transcription and replication. For example,

histones could provide some of the energy, through conformational changes, necessary to unwind DNA. A third possibility is that histones repress transcription. Through post-translational modification of histones or selective transcription of histone sequence variants, nucleosomes could be constructed which deny RNA polymerase access to certain sequences. These three general functions are not mutually exclusive. Indeed, the number of different nucleosomes which can be constructed from the known histone modifications and sequence variants makes a variety of control mechanisms possible.

The study of viruses has yielded important information about the cells they infect as well as the molecules of which they are comprised. The papova viruses are a particularly promising group of viruses from the standpoint of elucidating the functional role of histones. These viruses are small, non-enveloped, icosahedral viruses containing circular double-stranded DNA. The most studied of this group are the monkey virus SV40 and the mouse virus polyoma. The genomes of these viruses are large enough to code for only three or four gene products, two of which are capsid proteins and one which is necessary for the initiation of virus DNA replication. The DNA of SV40 and polyoma is complexed with cellular histones to form nucleosomes (Louie, 1974; Griffith, 1975). This packaging of DNA exists not only in the mature virion but intracellularly where the

virus minichromosome utilizes the host RNA polymerase, DNA polymerase, and protein synthesizing system. Studies of SV40 and polyoma virus transcription and replication are thus bound to yield important information about the functional role of histones.

The adenoviruses are double-stranded DNA containing viruses with a genome about seven times larger than SV40 and polyoma. Rather than cellular histones, adenovirus DNA is complexed, in the virion, with a highly basic, virus-coded protein. In adenovirus type-2 this protein has been identified as virus protein VII. Lischwe and Sung (1977) have shown that the N-terminal sequence of protein VII contains clusters of basic residues positioned similarly to those in the N-terminal region of histone H4. This similarity suggests that protein VII might perform the same function as histones. The existence of chromatin lacking histones would present a novel situation. Can the cellular transcription and replication machinery utilize a template consisting of a protein VII-DNA complex? The aim of this study is to determine to what extent protein VII behaves in a fashion functionally similar to histones.

My thesis examines the nucleoprotein structure of adenovirus DNA both from virions and in infected cells. Part I examines the structure of the virus core isolated from virions. This complex, containing virus proteins V and VII, has some physical properties in common with cellular chromatin. Nuclease digestion experiments,

however, show that the virus core proteins do not protect DNA in repeating size classes as seen in digestion of cellular chromatin. This difference has been exploited in Part II to analyze the nucleoprotein structure of intranuclear adenovirus DNA during productive infection. The pattern of nuclease digestion intermediates of intranuclear adenovirus DNA shows that virus protein VII is replaced by cellular histones during infection. The implication of these results is that protein VII serves exclusively as a virus packaging protein and cannot substitute for histones during the virus growth cycle. While the present data does not show that nucleosomal adenovirus DNA serves as template for early virus transcription, nucleosomal templates are available to serve in this capacity. Further experiments have sought to determine the mode of assembly of adenovirus chromatin and its fate late in infection. Experiments on a temperature sensitive mutant of adenovirus type-2 have shown that protein VII is involved in the virus uncoating process.

PART I: NUCLEOPROTEIN STRUCTURE OF ADENOVIRUS CORES

Introduction

Adenoviruses are non-enveloped icosahedral viruses containing linear double-stranded DNA. Each virus particle contains one DNA molecule of 20-25 x 10^6 daltons which comprises 12-14 percent of the virion by weight. The 14 virus structural proteins are located externally, forming the virus capsid, or internally, forming the virus core. The adenovirus core is comprised of two virus-coded proteins complexed with the virus DNA. The major adenovirus core protein, virus protein VII, is an arginine rich (Laver, 1970; Prage and Petterson, 1970; Russell et al., 1971; Sung et al., 1977) 18,000 dalton protein present in 1070 copies per virion, approximately the same total weight as viral DNA (Everitt et al., 1973). Recent sequence analysis has shown that the arrangement of basic amino acids in the N-terminal region of protein VII is similar to the N-terminal sequence of histone H4 (Lischwe and Sung, 1977). The second major core protein, protein V, is moderately arginine rich (Laver, 1970) and present in 180 copies per virion (Everitt et al., 1973). On the basis of core protein stoichiometry and the protection of virus DNA from nuclease digestion we earlier proposed a chromatin-like structure for the adenovirus core (Corden et al., 1976).

In contrast to adenovirus, the core of SV 40 virus and polyoma

virus contains cellular histones. In SV40 and polyoma histones serve not only to package DNA in virions (Lake et al., 1973; Frearson and Crawford, 1972) but remain bound to the DNA throughout the virus life cycle (Louie, 1974; Griffith, 1975). Since the adenovirus core proteins serve the same role as histones in packaging the adenovirus DNA in virons, we have asked whether they also play a role in the infectious process. Using a combination of biophysical techniques and nuclease digestion we show in this section that adenovirus nucleoprotein isolated from purified virions shares structural properties with cellular chromatin.

Isolation of Virus Cores

The core of adenovirus has been separated from capsid components by several techniques, including exposure to acetone (Laver et al., 1967, 1968), 5M urea (Maizel et al., 1968), 10% pyridine or freezing and thawing (Prage et al., 1968, 1970), 0.5% deoxycholate at 56°C (Russell et al., 1971; Nermut et al., 1975; Harpst et al., 1977), and 0.5% sarkosyl (Brown et al., 1975). To study the structure of isolated virus cores I sought to use the mildest procedures available for their production. As a criterion for "mildness" I considered the effects of the various reagents used to prepare cores on the structure of cellular chromatin or nuclei. The detergent techniques were avoided as 0.5% deoxycholate has been shown to remove

histones from chromatin (Hadler et al., 1971) as has 0.5% sarkosyl (Gariglio, 1976). Acetone has been shown to modify primary amino groups (Means and Feeney, 1968) and was not tested on chromatin. I tested the effects of 5M urea and 10% pyridine by digesting HeLa cell nuclei with staphylococcal nuclease (nucleate 3'-oligonucleotido hydrolase, EC 3.1.4.7, referred to as staph nuclease) in their presence. Jackson and Chalkley (1975) showed that, while the activity of staph nuclease was reduced in the presence of urea, DNA could be digested to completion and chromatin to 50% acid solubility. My analysis of the early staph digestion products produced in the presence of 5M urea showed that the nucleosome DNA repeat was abolished. In contrast I found that nuclei digested with staph nuclease in the presence of 10% pyridine still showed a DNA repeat characteristic of nucleosomal chromatin. These results led me to concentrate my efforts on virus cores isolated by lysing virions with 10% pyridine.

Virus cores isolated with pyridine contain only virus proteins V and VII (Prage et al., 1970). Figure 1A shows an electron micrograph of our pyridine core preparation. The sedimentation coefficient of such adenovirus core preparations was 150S, in good agreement with values previously obtained by Harpst et al. (1977). The rosette structures seen in the electron micrographs are also similar to those previously reported for cores produced by different procedures (Nermut et al., 1975; Brown et al., 1975) but sedimentation analysis

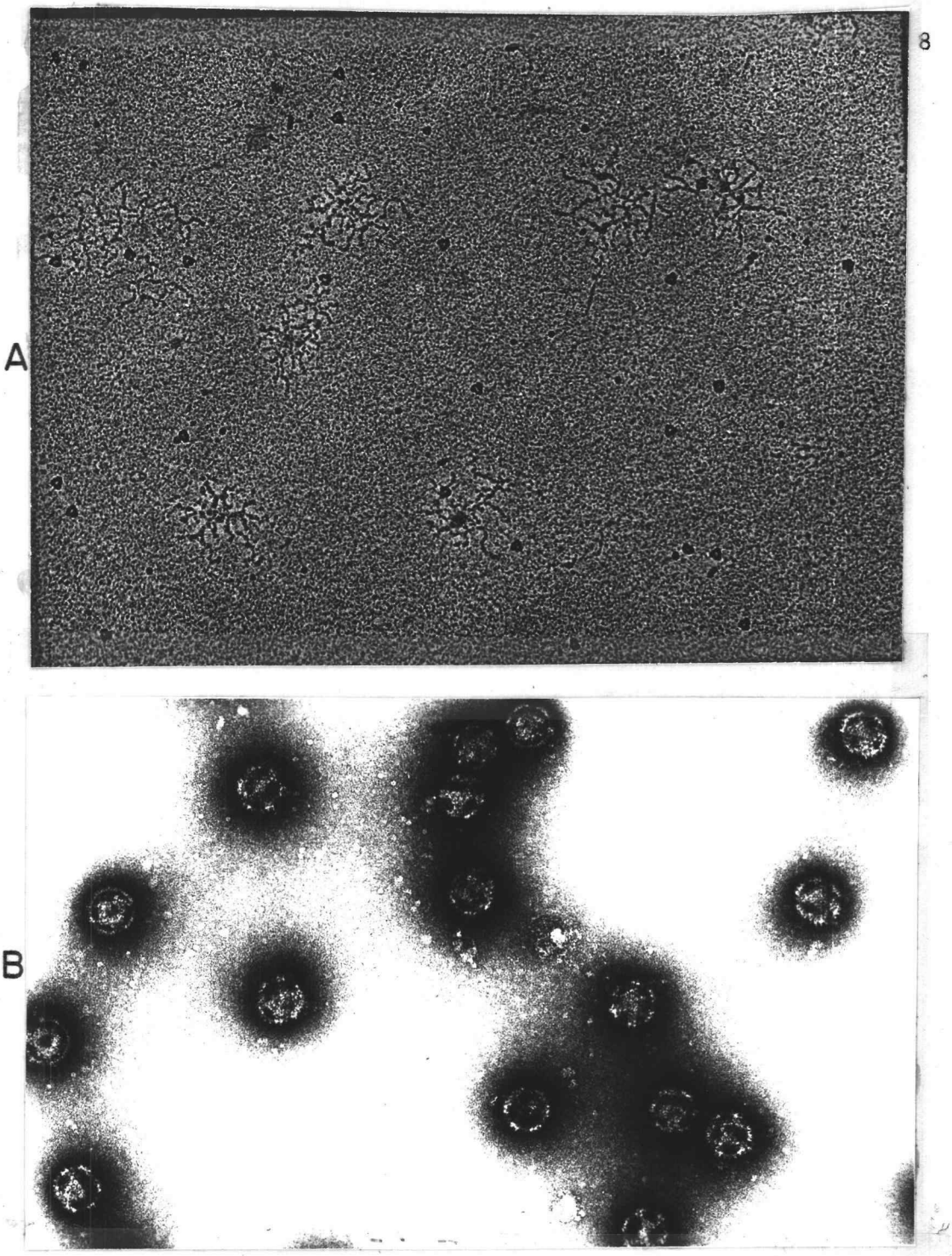


Figure 1. Electron microscopy of sub-viral particles. A) Pyridine cores 5,000 X. B) Cornerless virions 10,000 X. Both sub-viral structures were produced according to Appendix I, section (b).

showed that the pyridine cores did not progressively unfold with time as did cores produced by detergent treatment. Preparations of virus cores which had been negatively stained showed fibers of about 80 Å thickness. This is in agreement with Nermut et al. (1975) who reported 80-100 Å fibers in deoxycholate produced cores.

Figure 1B shows an electron micrograph of adenovirus which has been dialyzed at low ionic strength for several days. This procedure has previously been shown to result in the loss of the penton base and fiber proteins as well as the peripentonal hexon proteins from the vertices of the icosahedral capsid (Prage et al., 1970). For nuclease digestion studies I was able to verify the results obtained on pyridine cores by digesting virions which had been rendered cornerless by dialysis at low ionic strength. The DNA in these virus particles is completely accessible to nuclease (Corden et al., 1976) and presumably the core structure has not been altered.

Isolated pyridine cores were first analyzed for the effects of ionic strength and divalent cations. One necessity in studying macromolecular structures is having soluble, non-aggregated components. In studying the effects of salt solutions I first tested for aggregation. The assay I developed consisted of centrifuging samples of pyridine cores in various salt concentrations. Conditions of centrifugation were selected (see Appendix I, section (d)) that would result in molecules with a sedimentation coefficient greater than about 1000 S

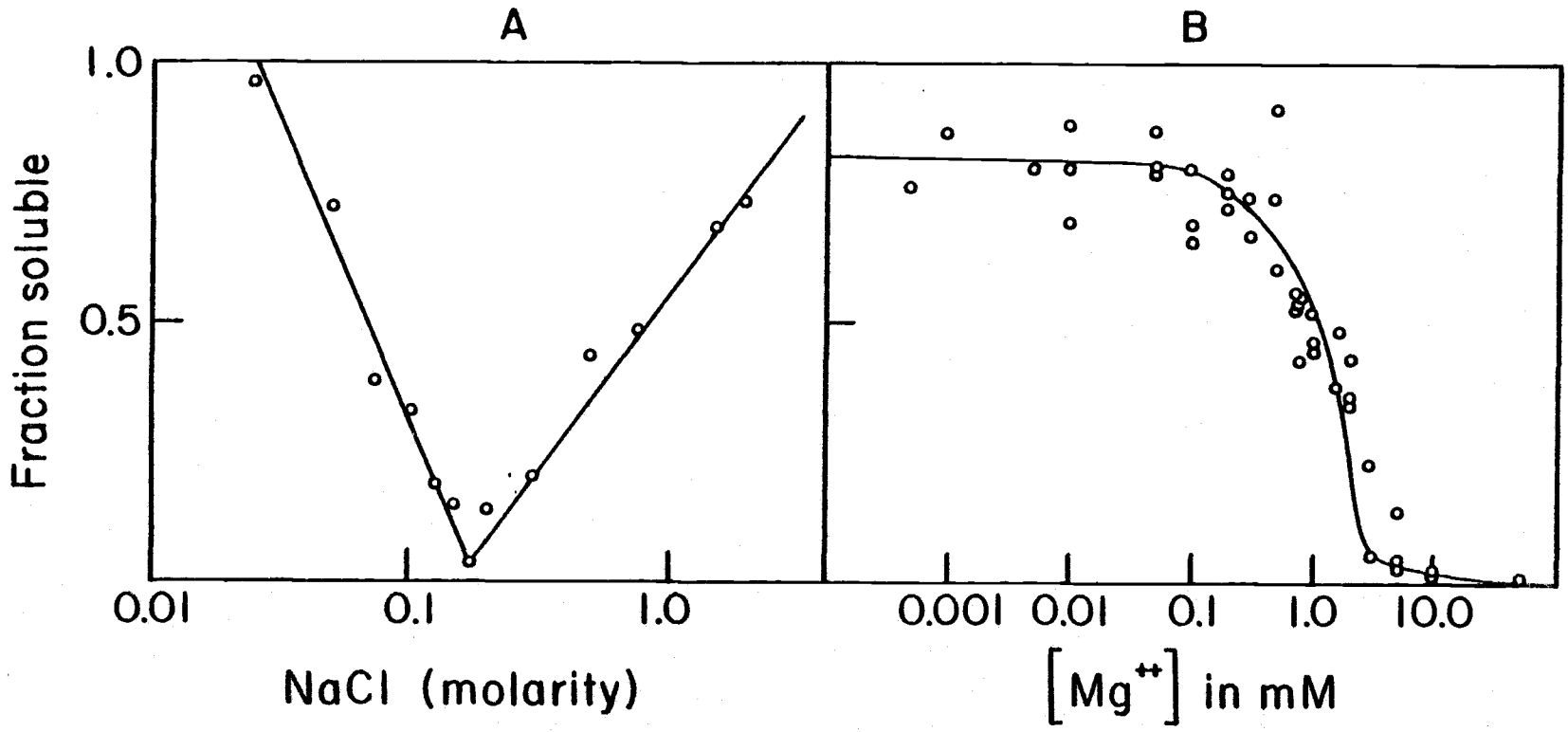


Figure 2. Salt precipitation of pyridine cores. A) NaCl B) $MgCl_2$ Assay is described in Appendix I, section (d).

sedimenting to the bottom of the tube. Aliquots were removed from the top of the liquid column, counted, and compared to a control solution which lacked salt. The results in Figure 2A show that pyridine cores have a minimum solubility in 0.175 M NaCl. This behavior in salt has been demonstrated for eucaryotic chromatin (Fredericq, 1971).

Increasing the level of Mg^{++} (Figure 2B) results in the precipitation of greater than 90% of the core solution by 2mM Mg^{++}. This concentration of Mg^{++} has previously been shown to precipitate chromatin (Marushige and Bonner, 1971). Identical results were obtained if pyridine cores were precipitated with Ca^{++} (results not shown).

The results of salt precipitation experiments show that the nucleoprotein cores of adenovirus and cellular chromatin respond similarly to increasing salt. These results also restrict the conditions under which further analysis can be undertaken.

As cellular chromatin is subjected to increasing concentrations of NaCl, histones are progressively removed (Ohlenbusch et al., 1967). Pyridine cores subjected to increasing NaCl lose protein V above 0.5M (results not shown). Protein V is also preferentially removed from adenovirus cores in urograffin (Nermut et al., 1975) or 0.5% sarkosyl (Brown et al., 1975). The protein VII-DNA complex remaining after removal of protein V is very resistant to salt. I have exposed pyridine cores to 2M NaCl and find no evidence for

removal of protein VII. Whereas salt precipitation data show that at high salt (> 1M NaCl) the core complex is soluble, ultracentrifuge analysis shows that the sedimentation coefficient of these complexes has increased from about 150S to greater than 200S. Harpst et al. (1977) have also described this phenomenon. Cores produced by the sarkosyl technique of Brown et al. (1975) can be banded to equilibrium in CsCl. In this case the cores are exposed to greater than 3M CsCl yet they band at a density characteristic of nucleoprotein. The resistance of protein VII-DNA complexes to dissociation by salt is in striking contrast to cellular chromatin.

The unique behavior of pyridine cores in salt has led us to look for other differences in the structure of pyridine cores. Circular dichroism has been used to characterize the secondary and tertiary structure of DNA (Fasman, 1977). The positive CD band between 260 and 300 nm has essentially no contribution from CD of protein peptide bonds and permits monitoring the CD of DNA in nucleoprotein complexes. The CD spectrum of pyridine cores in this region is intermediate between free DNA and cellular chromatin (Figure 3A-a). The reduced ellipticity of this band is similar to complexes of DNA and arginine rich histones (Fasman, 1977). While solutions of pyridine cores in low salt showed little evidence of light scattering, my attempt to study the effect of salt on the CD spectrum of pyridine cores was unsuccessful because of large, salt induced, scattering

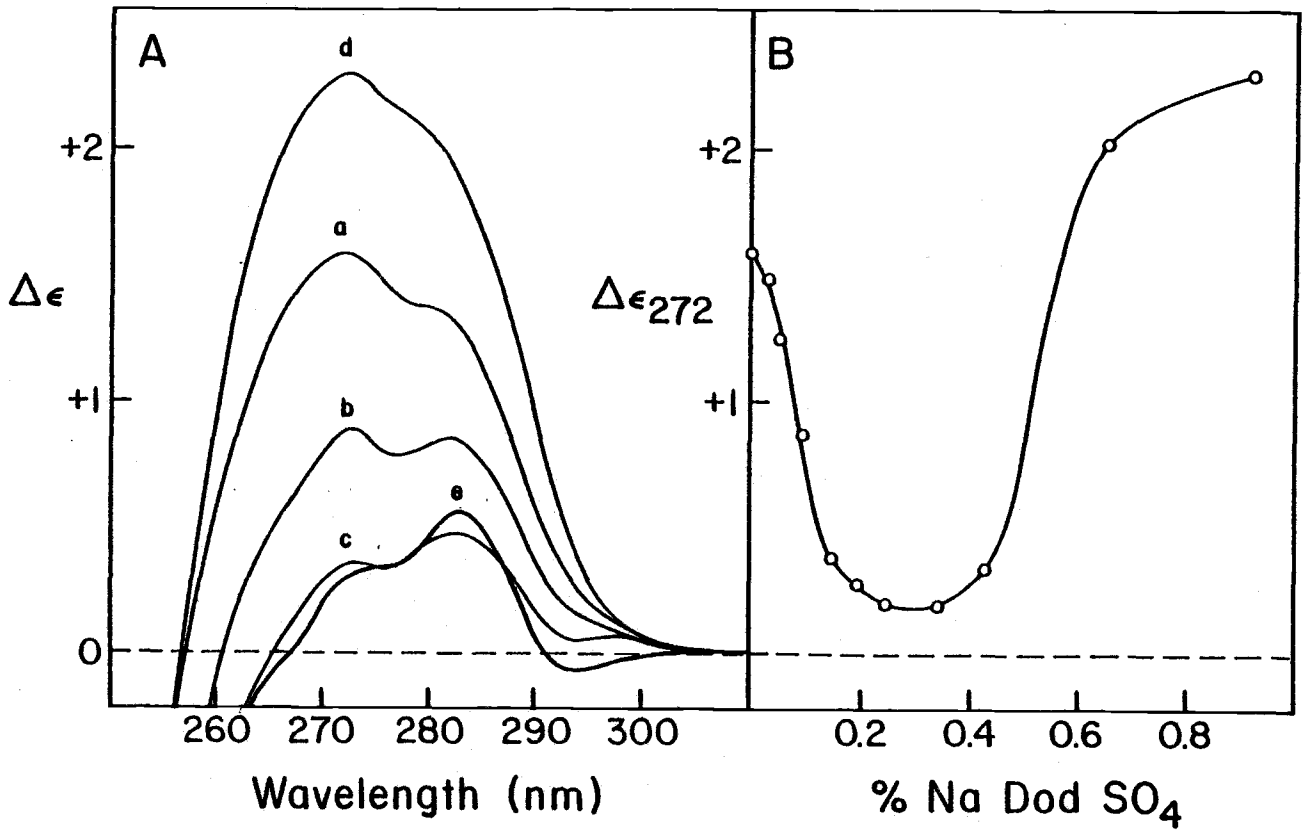


Figure 3. Circular dichroism of pyridine cores: titrations with SDS. A) CD spectrum of pyridine cores in no SDS, (a); 0.1% SDS, (b); 0.2% SDS, (c); 1.0% SDS, (d). Spectrum (e) is of chicken erythrocyte nucleosome core particles. B) CD absorbance at 272 nm plotted against SDS concentration.

artifacts.

The observation that sarkosyl cores do not contain protein V (Brown et al., 1975) led me to look at the effects of detergent on our pyridine core preparations. When I titrated a solution of pyridine cores with SDS and followed changes in DNA structure by circular dichroism an unusual result was obtained. Figure 3A shows the CD spectra of pyridine cores at various concentrations of SDS. The peak of positive ellipticity at 272 nm decreased until the SDS concentration reached about 0.3%. Figure 3B shows the ellipticity at 272 nm at various concentrations of SDS. At levels of SDS above about 0.4% the CD spectrum approaches the spectrum of free DNA. Figure 3A also shows the CD spectrum of isolated chicken erythrocyte nucleosome core particles. When nucleosomes or cellular chromatin, at the same concentration as pyridine cores, are made 0.5% SDS the CD spectrum shifts to that of free DNA (results not shown). Apparently the detergent is acting on pyridine cores to remove protein V and the resulting complex is resistant to salt and, to a limited extent, detergent. The CD spectrum of pyridine cores in 0.3-0.4% SDS is very close to that of isolated nucleosomes.

Nuclease Digestion

In the last five years the eucaryotic genetic apparatus has been intensely studied and many aspects of its structure are now well

understood (see Botchan and Watson (1978) for a compendium of recent references). The analysis of nuclease digestion of chromatin (Clark and Felsenfeld, 1971; Rill and Van Holde, 1973; Hewish and Burgoyne, 1973; Sahasrabuddhe and Van Holde, 1974) has provided the firmest evidence for the existence of a fundamental chromatin subunit (nucleosome).

In a previous report it was shown that the DNA in cornerless virions could be digested to 50% acid solubility with staph nuclease (Corden et al., 1976). Figure 4 shows that pyridine cores are also digested to this limit. This result shows that the protection from staph nuclease digestion is afforded by the internal virus proteins V and VII. To determine whether the adenovirus core proteins organize DNA in a repeating subunit, the staph nuclease digestion intermediates were analyzed on a 1.8% agarose gel (Figure 5). Clearly, there is no evidence for a series of protected DNA fragments indicative of a repeating subunit. Figure 6 shows scans of similar digestion intermediates from staph nuclease digested pyridine cores. The intermediates fall into a broad band which decreases in size with increasing digestion. This pattern of digestion intermediates is in striking contrast to the integral multiple size classes characteristic of cellular chromatin digestion (Figure 6, scan f).

Analysis of the limit digestion products on high percent gels, Figure 7, shows a broad peak at about 175 bp. This band is much

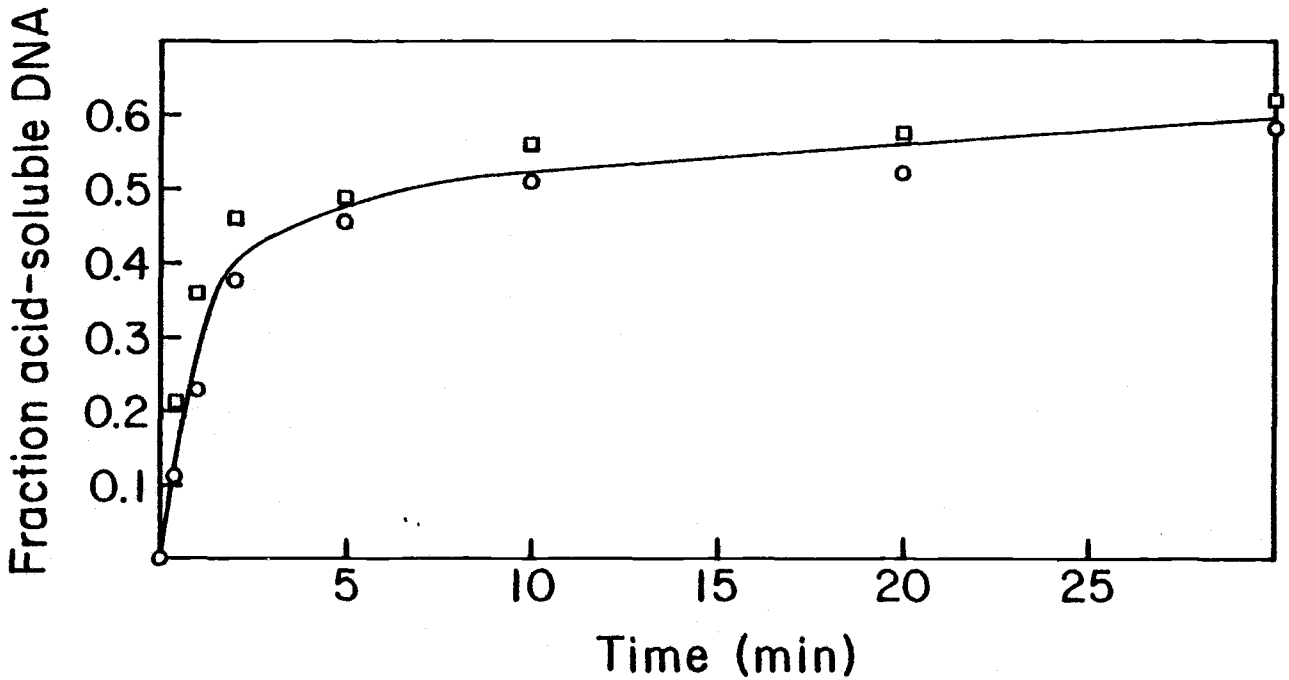


Figure 4. Time course of acid solubilization of DNA in cornerless virions (o) and pyridine cores (▫) digested with staph nuclease as described in Appendix I, section (f).

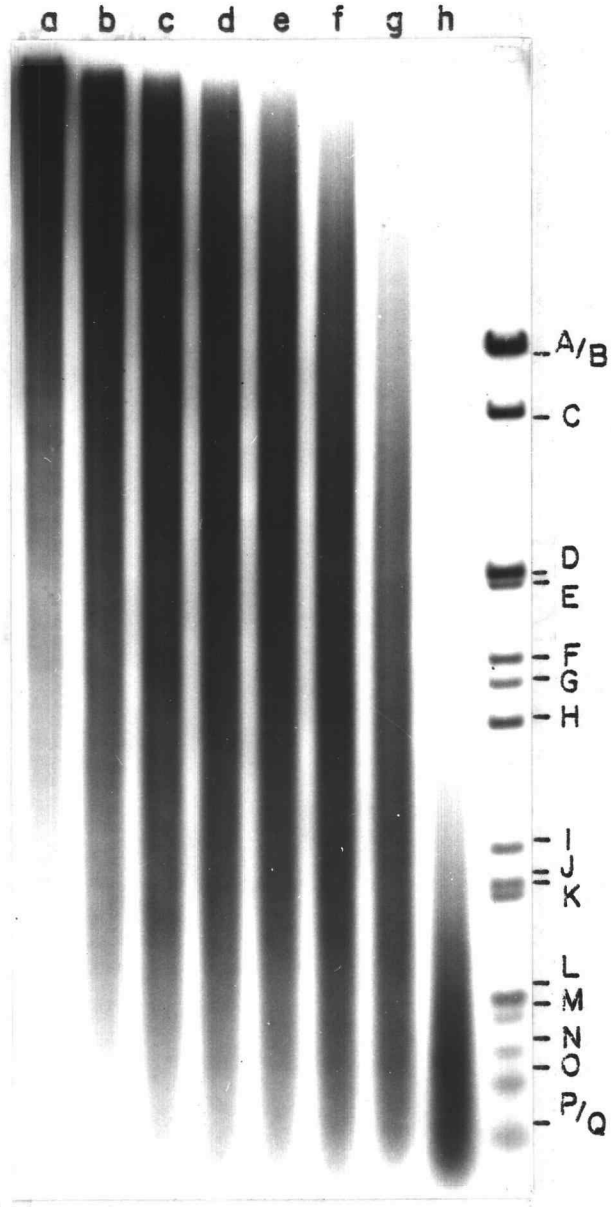
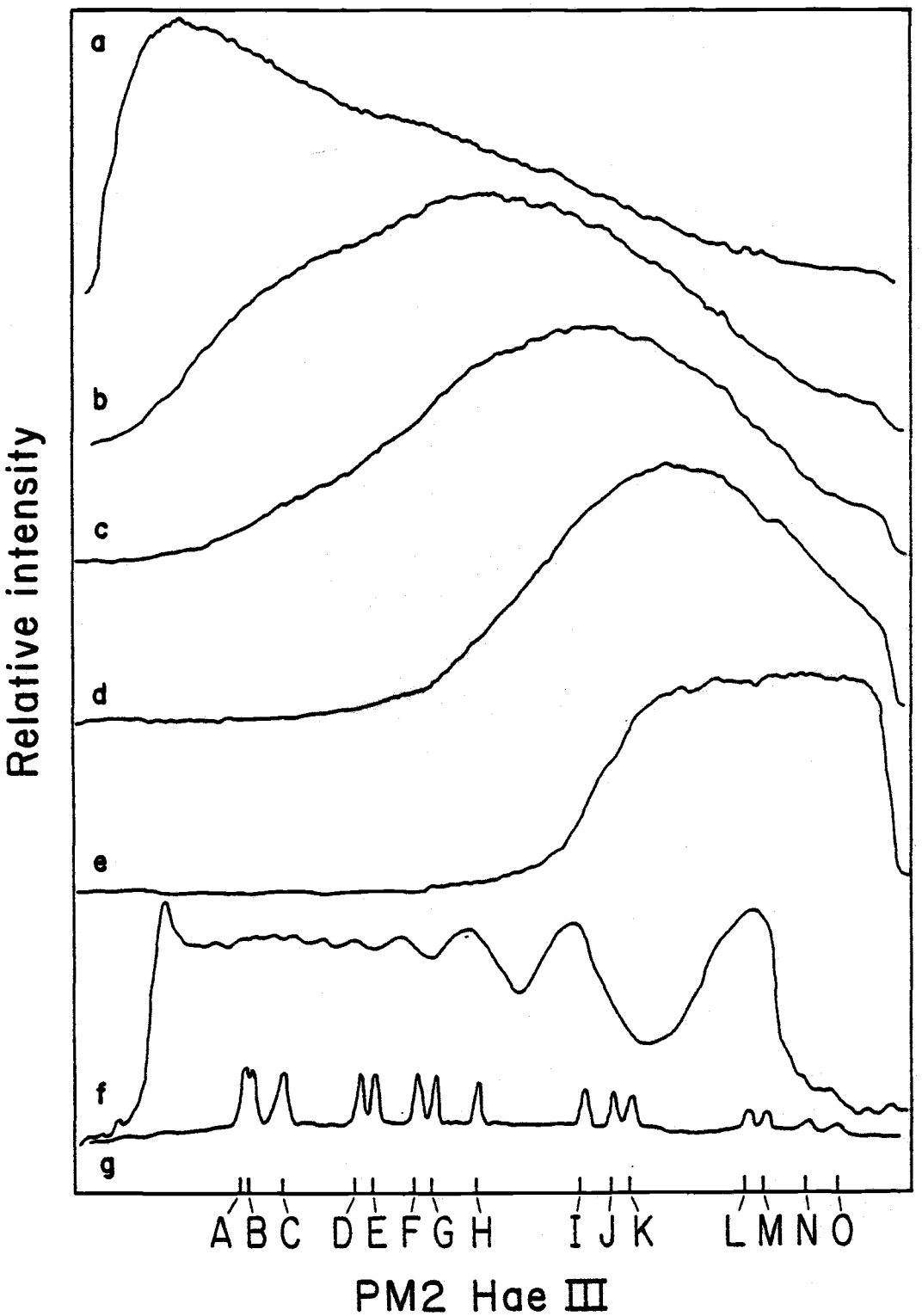


Figure 5. Electrophoretic analysis of digestion intermediates from staph nuclease digested cornerless virions. Cornerless virions were digested according to Appendix I, section (f), and separated on a 1.8% agarose slab gel. The gel was run for 10 h at 80 v at 10°C. The ^{32}P-labeled digestion intermediates were imaged by fixing the gel in 10% TCA for 15 min at 5°C followed by drying and autoradiography. 50,000-100,000 cpm (Cherenkov) were applied to each track. Tracks (a)-(h) represent digestion to 1.1, 1.4, 1.8, 2.3, 3.5, 5.0, and 25% acid soluble DNA respectively. Track (j) contains PM2 Hae III fragments the sizes of which are shown in Appendix I, section (m).

Figure 6. Electrophoretic analysis of digestion intermediates from staph nuclease digested pyridine cores. Pyridine cores were digested according to Appendix I, section (f) and separated on a non-denaturing acrylamide gel. The gel was run for 10 h at 100 v at 10°C. The ^{32}P-labeled digestion intermediates were imaged by fixing the gel in 10% TCA, drying and autoradiography. Tracks (a)-(e) represent 1.4, 2.6, 4.0, 10, and 28% acid solubility respectively. Track (f) shows a nucleosome repeat pattern from staph nuclease digested HeLa cell nuclei run on the same gel. These nucleosome oligomers are approximately integral multiples of 185 base pairs. Track (g) shows PM2 Hae III marker fragments.


a
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Relative intensity
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PM2 Hae III

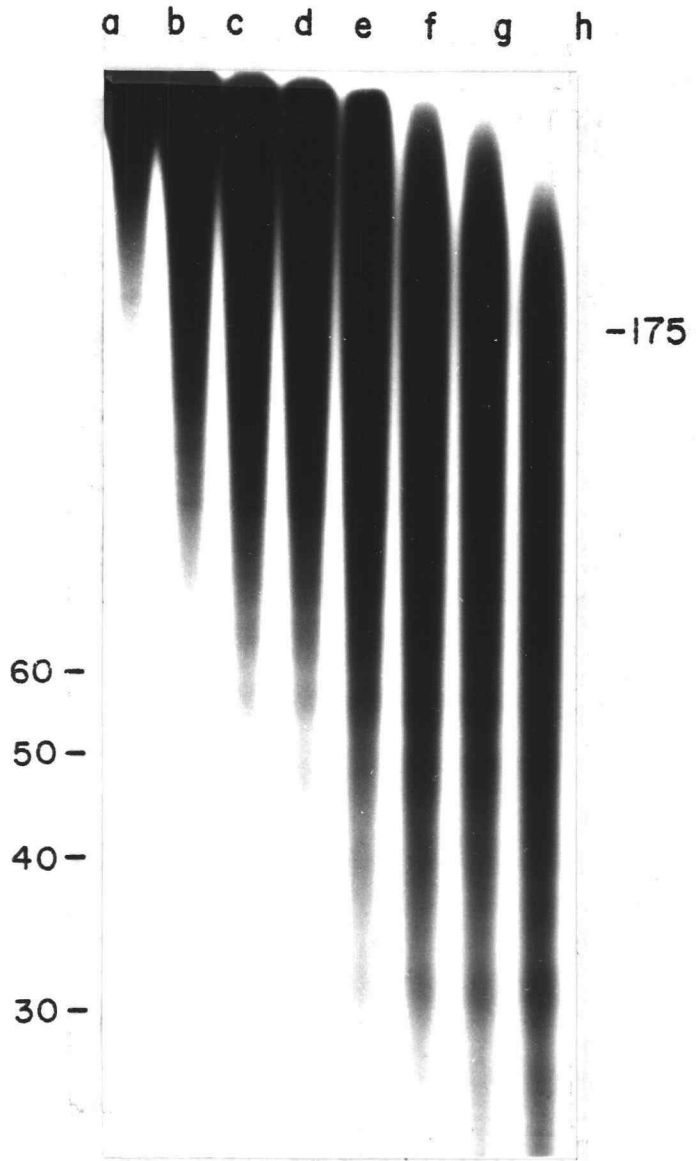


Figure 7. Electrophoretic analysis of low molecular weight digestion intermediates from staph nuclease digested pyridine cores. These are the same samples described in Figure 6 but here separated on a non-denaturing 10% acrylamide gel. Tracks (a)-(h) represent 0.5, 1.4, 4.0, 10, 22, 28, and 41% acid solubility respectively. Sizes are calibrated with respect to PM2 Hae III markers.

broader than a comparable digest of cellular chromatin. As in the digestion of cellular chromatin there are digestion products smaller than the peak size. The smaller products fall into size classes which are roughly multiples of ten base pairs. Camarini-Otero et al. (1976) have observed that the submonomer fragments from staph nuclease-digested chromatin fall into a ten base pair repeat. The coincidence of this repeat with the helical repeat of DNA has been taken to mean that DNA which is digested through these intermediates is wrapped around a protein core (Noll, 1974; Sollner-Webb et al., 1978).

Deoxyribonuclease I (EC 3.1.4.5, referred to here as DNase I) digests cellular chromatin through digestion intermediates which are roughly multiples of ten bases (Noll, 1974). Figure 8 shows the results of digestion of pyridine cores with DNase I. As for cellular chromatin, the DNA is almost totally accessible to DNase I (Figure 8A). Figure 8B shows that the digestion intermediates are multiples of ten bases. As in the staph digestion pattern, the ten base DNase I produced bands are rather broad and cannot be distinguished above about 60-80 bases. This is in contrast to the DNase I digestion intermediates of cellular chromatin which, in some cases, can be observed beyond 300 bases (Lohr et al., 1977c). The pattern of pyridine core digestion intermediates further differs from that of cellular chromatin in that the relative intensities of the bands increases with

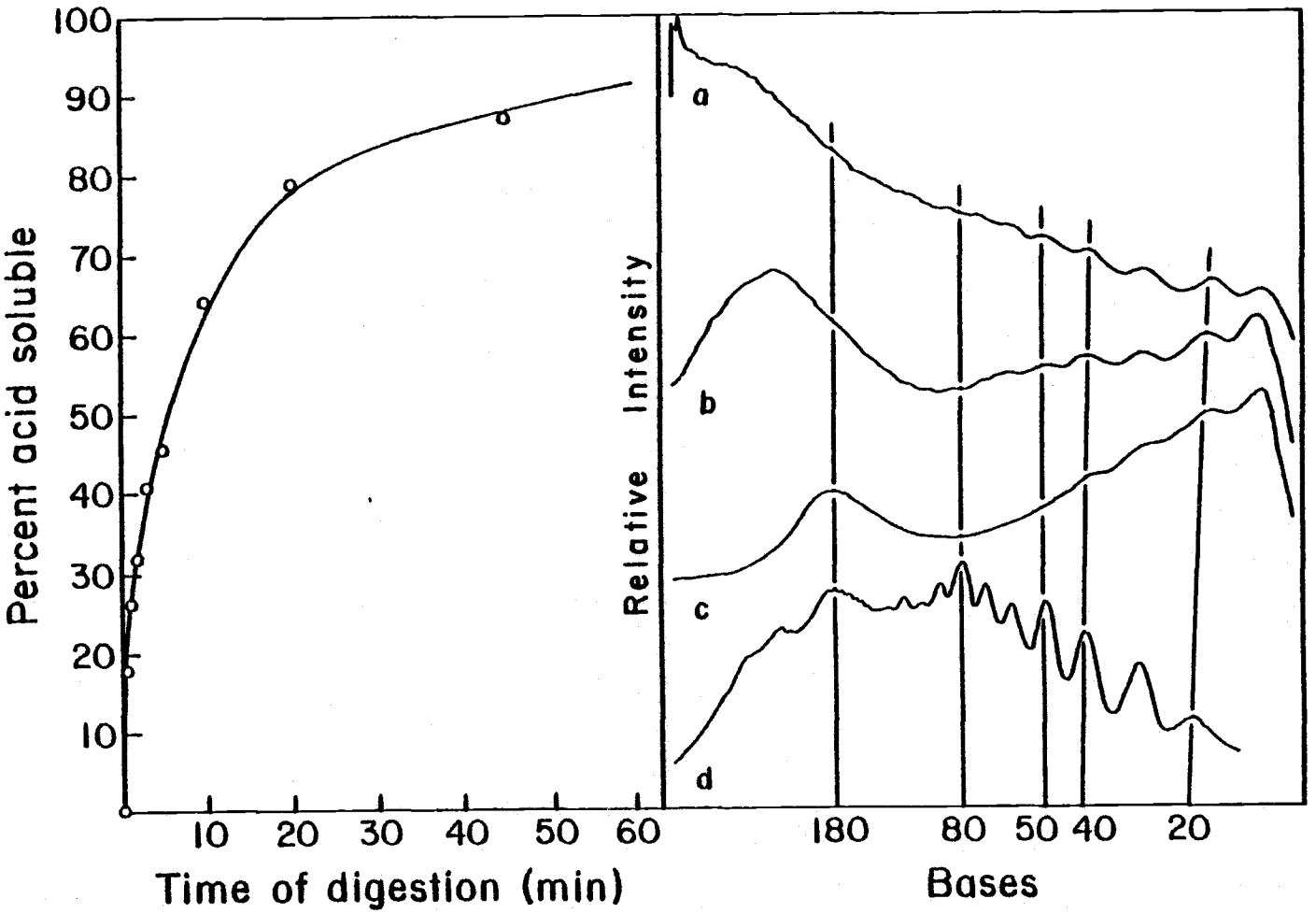


Figure 8. DNase I digestion of pyridine cores. Pyridine cores were digested according to Appendix I, section (f), and the digestion products were separated on a denaturing 8% acrylamide - 98% formamide gel. The gel was run for 8 h at 120 ma, fixed, dried, and autoradiographed. A) Percent acid soluble DNA. B) Tracks (a)-(c) represent 18, 45, and 90% acid soluble DNA. Track (d) is a DNase I digest of HeLa cell nuclei.

decreasing size as digestion proceeds. The digestion intermediates of cellular chromatin display the characteristic pattern of relative intensities seen in Figure 8B(d). The limit digest of pyridine cores with DNase I also shows a broad band at about 180 bases. It is not clear whether this band represents a structural element which exists throughout the genome or is due to protection at a few specific sites (ends?) in the genome.

The results of nuclease digestion experiments on adenovirus nucleoprotein give us the following information: (1) half of the DNA is protected from digestion by staph nuclease (2) The protected DNA falls into a broad peak centered at about 175 base pairs (3) Both staph nuclease and DNase I digest the DNA of adenovirus nucleoprotein into multiples of ten base pairs or bases.

Attempts to recover nucleoprotein complexes after nuclease digestion have not been successful due to the precipitation of the digestion products. Salt washes have also been unsuccessful in solubilizing nucleoprotein from the precipitate. Because of this failure to obtain nucleoprotein complexes from digested cores I attempted to reconstitute virus nucleoprotein from purified protein VII and DNA.

Reconstitution of Protein VII - DNA Complexes

Before the nuclease digestion data can be used to deduce a

structure for adenovirus chromatin we must ask how the virus core protein, protein VII, is spaced along the DNA strand. For example we would like to know if protein VII associates with itself in discrete complexes or long chains.

The first step in this approach was to isolate protein VII in pure form. Existing procedures (Sung et al., 1977; Everett et al., 1973) were found to be unacceptable for reasons of purity attained or difficulty of preparation. We developed two procedures for purification of protein VII. Purified virus was used as starting material in order to alleviate the problems of cellular histone contamination. Briefly, CsCl banded virus was lysed in 4m guainidine-HCl and acid soluble proteins were extracted in 0.25m HCl. Acid soluble proteins were then fractionated either electrophoretically or on the basis of their solubility in 0.25M HCl-70% ethanol. Figure 9A shows electrophoretic separation of protein VII purified according to Appendix I, section (i). Since the procedures used in these preparation schemes involved acid extraction it was necessary to show that the protein could be refolded following purification. Using fluorescence anisotropy and circular dichroism Isenberg and colleagues (Li et al., 1972; Smerdon and Isenberg, 1974; D'Anna and Isenberg, 1974) have shown that acid extracted histones can undergo conformational changes which result in folded proteins which are capable of forming complexes characteristic of cellular chromatin. Further proof that

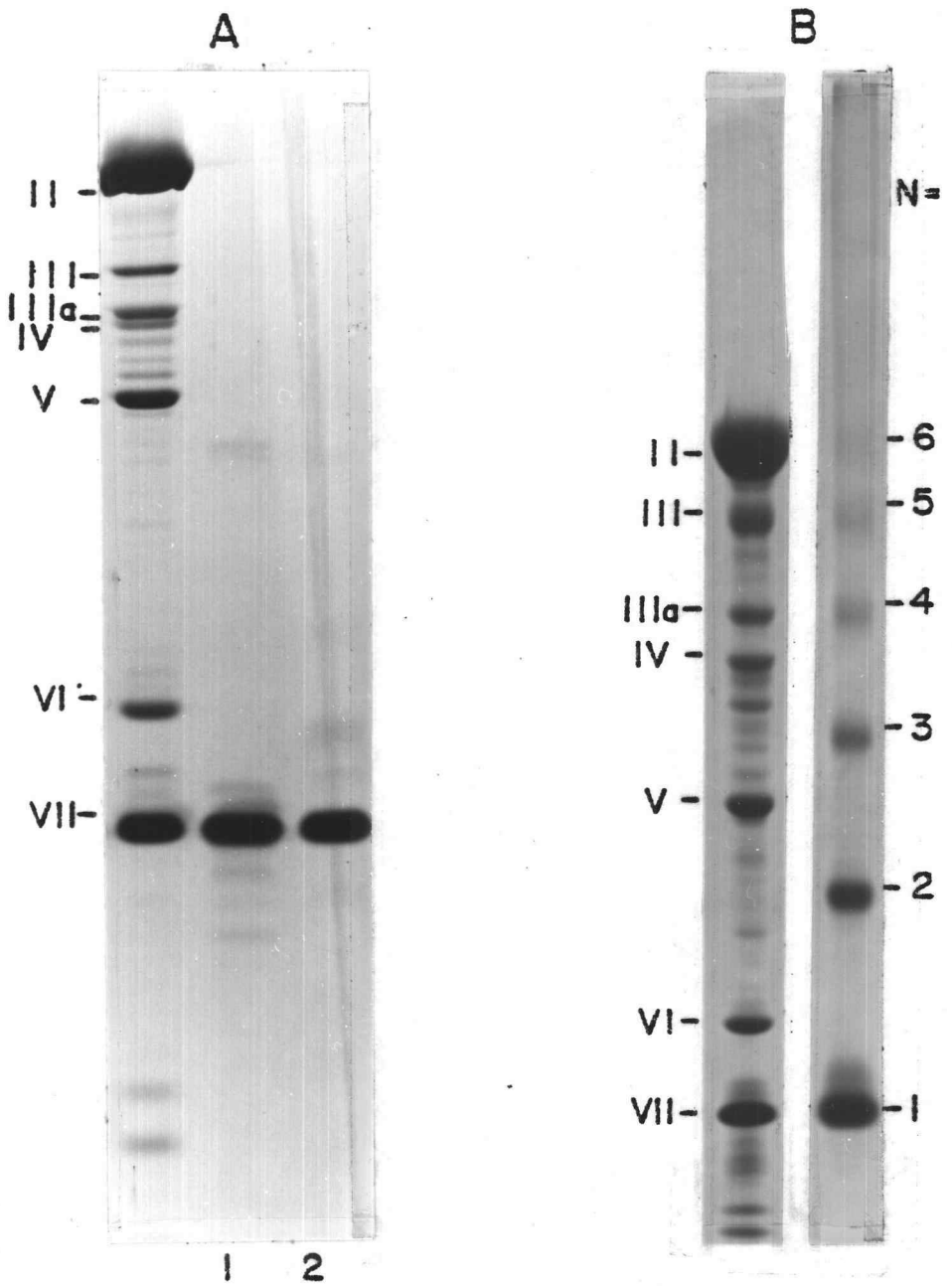


Figure 9. Electrophoretic analysis of purified protein VII and its glutaraldehyde-crosslinked products. A) Separation of protein VII purified by the method of Mardian & Isenberg (1978) (1), or by the modified Johns procedure described in Appendix I, section (i) (2). Proteins were separated on a 15% acrylamide SDS gel (Laemlii, 1970). Molecular weights of the adenovirus proteins (shown in the left-hand track) are given in Appendix I, section (m). B) Protein VII at a concentration of 10^{-3} M in 0.04 M sodium phosphate, 1.0 M NaCl was crosslinked for 1 min with 2.3% glutaraldehyde. Crosslinked protein was TCA-precipitated, acetone-washed, suspended in SDS gel electrophoresis buffer and separated on a linear 5-15% acrylamide gradient SDS gel. Adenovirus proteins are shown in the left-hand track.

can be successfully renatured comes from experiments of Laskey et al. (1977) who reconstituted nucleosomes from acid-extracted histones using a nucleosome assembly factor from Xenopus laevis.

Figure 10A shows the CD spectrum of protein VII in 0.01 N HCl and in 0.01 M $NaPO_4$ pH 7.0. The changes in CD spectrum in going from no salt (the spectrum in 0.01 N HCl) to 0.01 M sodium phosphate are instantaneous and there is no further change in the spectrum after 24h. The CD spectrum of protein VII was analyzed by the method of Baker and Isenberg (1976). This technique compares the unknown spectrum to a set of reference spectra and not only allows the calculation of the percent α-helix, β-sheet, and random-coil but provides a set of criteria for judging the trustworthiness of the data. Using as reference spectra either the poly (L-lysine) CD spectra for α-helix and β-sheet or the α-helix and β-sheet spectra of Chen et al. (1974) together with the random coil spectra of protein VII in 0.01 N HCl sums of 0.99 to 1.05 were achieved. Sums approaching unity indicate a very good fit of the calculated to the experimental spectrum. The calculated α-helix content was 7.0% and that of β-sheet was -0.2%. The negative value for β-sheet content is meaningless; within experimental error the β-sheet content is zero. The secondary structure composition of protein VII (7% α-helix, 93% random coil) is independent of the wavelength range analyzed between 200 and 250 nm. The absence of β-sheet in refolded protein VII is reminiscent of the

refolded conformation of the inner histones as analyzed by Baker and Isenberg (1976).

As a second test of protein VII refolding I analyzed the increase in tryptophan rigidity with increasing sodium phosphate concentration. Figure 10B shows a plot of fluorescence anisotropy against sodium phosphate concentration. At each concentration of phosphate the anisotropy value was stable for more than 1 h, indicating an absence of a slow aggregation step as seen with histones (Li et al., 1972).

When protein VII in 40 mM sodium phosphate was made 0.5M NaCl no further changes in anisotropy were noted. The results of the CD and fluorescence anisotropy experiments together show that by dissolving acid extracted protein VII in distilled water and adding sodium phosphate to 0.01 to 0.04 M we are able to refold the protein. Whether the refolded protein adopts the native conformation is unknown. Cellular histones, extracted with acid, refold to form complexes characteristic of native histones (D'Anna and Isenberg, 1974) and can be utilized by the nucleosome assembly factor isolated from Xenopus laevis by Laskey et al. (1977). It might be argued that, since protein VII is synthesized as a precursor protein (protein pVII contains 21 amino-terminal amino acids not found in protein VII (Sung et al., 1977)), correct refolding is not attainable. It should be pointed out, however, that histones undergo extensive post synthetic modification yet can be refolded from acid to a seemingly

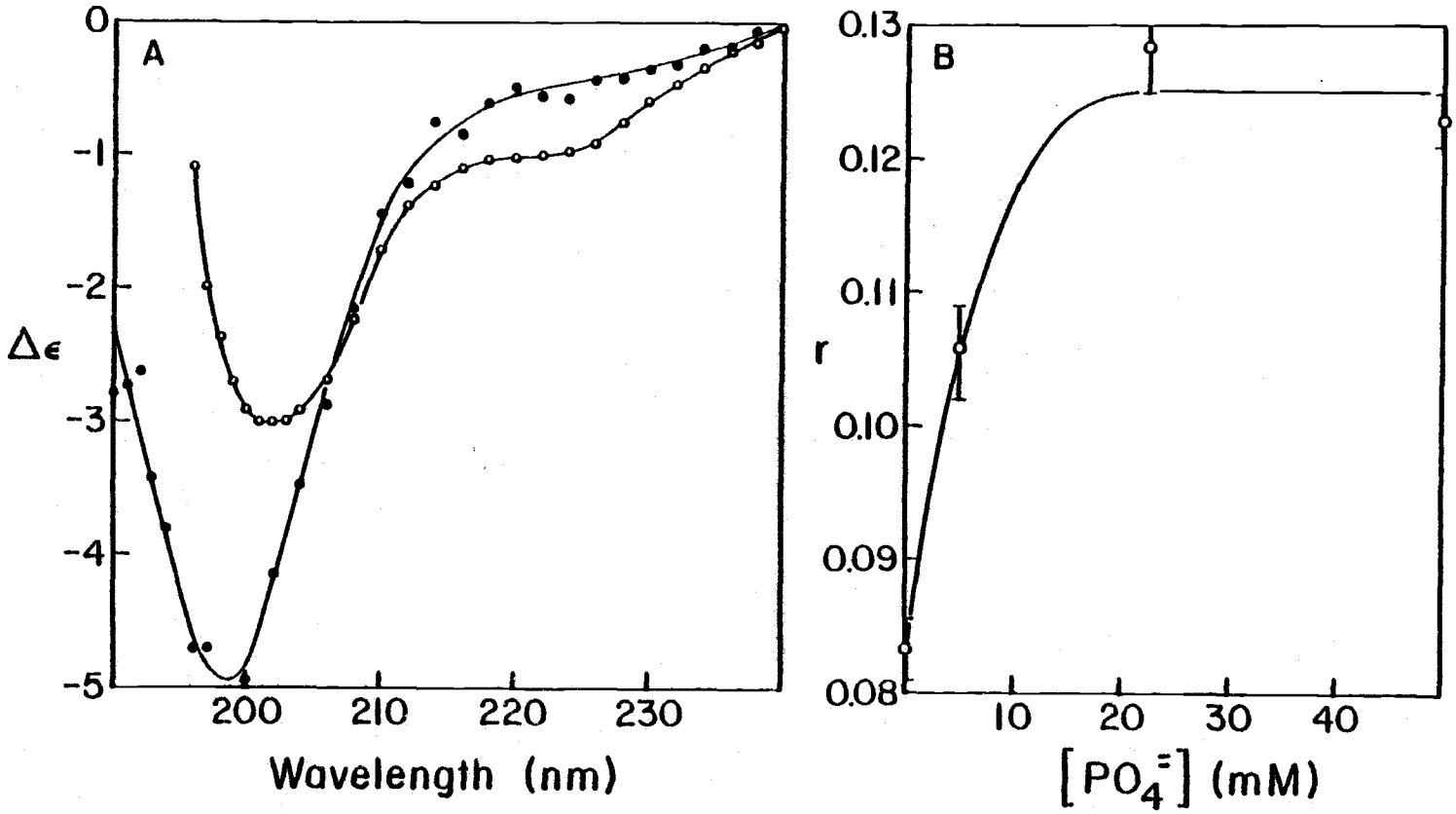


Figure 10. Demonstration of protein VII refolding. A) CD spectrum of protein VII in 0.01 N HCl (●) or 0.01 M sodium phosphate (o). B) Fluorescence Anisotropy.

native conformation.

Does protein VII form a complex with itself? We observe no indication of such a complex from the CD or anisotropy data. If, however, a 10^{-5}M solution of protein VII in 0.04 M sodium phosphate is made 1M in NaCl the solution becomes visibly turbid in a matter of minutes indicating protein aggregation. Figure 9B shows that when the aggregated protein is briefly cross-linked with glutaraldehyde a series of protein VII multimers is observed. This series of multimers extends at least up to hexamer but probably extends further as judged by the presence of material in the cross-linking solution which remains insoluble when boiled in SDS electrophoresis buffer. More extensive cross-linking results in more insoluble material.

Cross-linking of aggregated protein VII with dimethyl suberimidate results in dimer formation but few higher oligomers (not shown). The cross-linking of virus particles with tartaryl diazide also demonstrates a protein VII dimer (Everitt et al., 1975). Since these reagents link only amino groups, the absence of higher oligomers linked by them indicates that interaction between dimers may take place through different binding surfaces. Thus, the aggregation of protein VII may occur through the polymerization of dimers much as described for the aggregation of individual histones (Sperling and Bustin, 1975).

Because of the aggregation of protein VII in high salt we chose

to form protein VII - DNA complexes by salt gradient dialysis in the presence of 5M urea (Bekhor et al., 1969). Camarini-Otero and Felsenfeld (1977) showed that nucleosomes can be successfully reconstituted by salt gradient dialysis in the presence or absence of 5M urea. When I formed complexes of protein VII with adenovirus DNA according to the procedures described in Appendix I, section (j), the resulting complex precipitated during the final dialysis step. The precipitated complex was accessible to staph nuclease and showed the same limit of acid solubility (50%) as pyridine cores (results not shown). Electrophoretic analysis of staph nuclease digestion intermediates showed no evidence of a repeating DNA unit (results not shown). When complexes of protein VII were formed with 145 bp DNA derived from chicken erythrocyte core particles (Tatchell and Van Holde, 1978), by the same gradient dialysis procedure, the resulting complex again precipitated. Figure 11 shows the result of sucrose gradient analysis of protein VII - 145 bp DNA complexes formed with different protein to DNA ratios. As the ratio of protein to DNA increases, more DNA sediments to the bottom of the tube with the protein. The DNA remaining in solution has the same sedimentation coefficient as the input DNA and no protein VII can be detected in these fractions. These results might be taken to indicate a cooperative binding process. We interpret the results, however, as a consequence of protein VII first aggregating and then binding DNA.

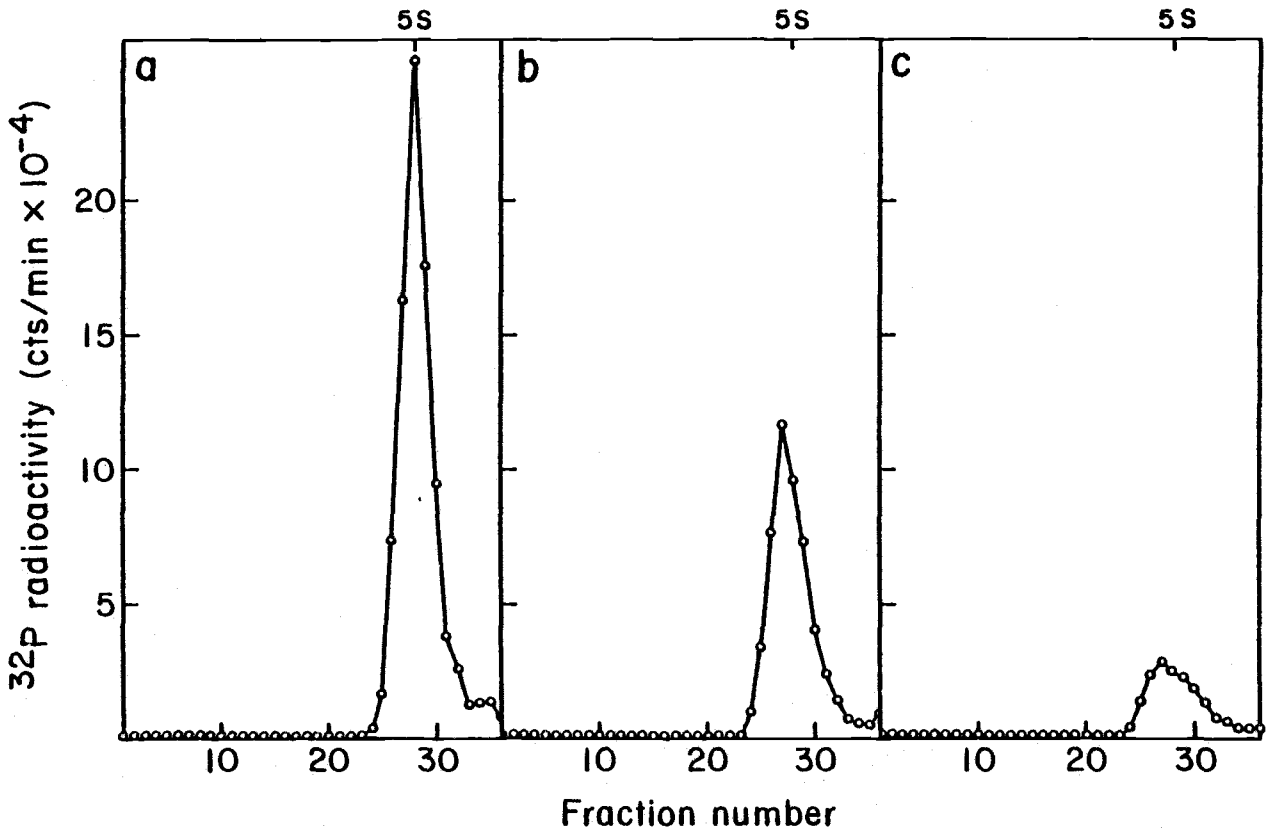


Figure 11. Sucrose gradient analysis of reconstituted Protein VII - DNA complexes. Reconstitution procedures are described in Appendix I, section (j). Starting protein - DNA ratios are 0.5, (a); 1.0, (b); and 1.5, (c).

The reconstituted complexes are not equivalent to the core structures isolated from virions. The failure to reconstitute soluble adenovirus nucleoprotein is not surprising considering the in vivo assembly of the core complex. Protein VII is a cleavage product of a 20,000 dalton precursor protein, pVII (Anderson et al., 1973). The precursor is found complexed with DNA in immature virions (Ishibashi and Maizel, 1974). Thus, for successful in vitro reconstitution it may be necessary to start with formation of pVII - DNA complexes.

Discussion

In characterizing the nucleoprotein structure of the adenovirus core I have used a variety of experimental approaches previously successfully applied to the structure determination of eucaryotic chromatin. For this reason discussion of our results will rely on comparison with the results of similar experiments on eucaryotic chromatin or histone-DNA complexes.

Electron microscope data on adenovirus core preparations (Figure 1A; Nermut et al., 1975; Brown et al., 1975) show that adenovirus DNA can be released from virions in a rosette structure with emanating 100 Å figers. These fibers are smooth, showing no evidence of a beaded, nucleosome-like structure. The core complexes contain, as a major protein component, the highly basic virus protein VII. This protein is present in approximately equal weight as

DNA in the virus core.

The salt precipitability of virus cores is similar to that of eucaryotic chromatin; however, the response of cores to high salt (> 1M) or low levels of detergent is unusual. At greater than 0.5M NaCl or between 0.1 and 0.4% detergent protein V is removed from pyridine cores and they sediment more rapidly. Raising the detergent concentration above 0.4% causes removal of all of the proteins, but raising the salt as high as 2M does not remove protein VII from adenovirus DNA. A possible cause of this observation is that protein VII forms a matrix at high salt which traps the DNA even when primary electrostatic interactions have been abolished. We have observed aggregation of protein VII alone in high salt.

The circular dichroism spectrum of pyridine cores is reduced, compared to free DNA, in the region of 260-300 nm. This property is also seen in chromatin and individual histone-DNA complexes (Fasman, 1977). Under conditions similar to those which remove protein V from adenovirus cores, the CD spectrum is further reduced in the region of 260-300 nm. Both secondary and tertiary structure of DNA can contribute to its CD spectrum. Changing the linking number of DNA does not necessarily change the CD spectrum unless accompanied by a change in the base tilt with respect to the helix axis (Johnson and Tinoco, 1968). Based on the supercoil structure of the nucleosome model of Finch et al. (1977), Cowman and Fasman (1978)

have proposed that the reduced ellipticity of nucleosomes at 260-300 nm is caused by an increased contribution of a ψ-like CD spectrum produced by interaction between adjacent superhelical turns. This interpretation is supported by their data on the CD spectrum of nucleosomes with various linker lengths and the observed CD spectrum of spacerless dimer nucleosomes (Klevan and Crothers, 1977; Tatchell and Van Holde, 1978). A possible explanation for our CD results on pyridine cores is that SDS removes protein V, thus allowing virus DNA to wrap around protein VII, in turn forming regions of ordered tertiary structure in the viral nucleoprotein. Our results do not rule out the possible explanation that the CD changes are brought about by changes in the DNA secondary structure.

Nuclease digestion studies of pyridine cores have yielded two kinds of information about the organization of the viral nucleoprotein. Separation of staph nuclease digestion products on high-percentage non-denaturing gels (Figure 7) or DNase I digestion products on denaturing gels (Figure 8B) reveals fragments which are multiples of ten base-pairs (or bases) upt to about 60 base pairs (bases). If nuclease-susceptible sites are spaced roughly ten base pairs apart, then the failure to see higher order repeats is understandable. Why the ten base repeat upon DNase I or staph nuclease digestion is not resolved beyond 60 bases is not clear. The origin of the ten base repeat in eucaryotic chromatin is thought to be the result of DNA

wound around a histone core thus exposing a nuclease digestion site only at those points, approximately every ten base pairs, where the backbone is in the preferred conformation. This explanation is bolstered by the recent results of Liu and Wang (1978), who observed a ten base DNase I repeat upon digestion of DNA complexed with DNA gyrase.

The reason for the lack of a higher order repeat analogous to the nucleosome might also be attributed to the structure of complexes formed by protein VII. Rather than forming discrete complexes like the histone core complex, protein VII forms large aggregates when exposed to high salt or when complexed with DNA. This behavior is similar to that of individual histones under similar conditions (Sperling and Bustin, 1975).

While our results do not allow us to unequivocally deduce the nucleoprotein structure of the adenovirus core, they do allow the rejection of certain kinds of possible structures. Eikbush and Moudrianakis (1977) have recently proposed that DNA, in response to changes in its hydration shell, adopts one of two fundamentally different structures. These are (1) a supercoil formed by dehydration in low salt and (2) a folded-fiber formed when charge-shielded DNA is dehydrated. The folded-fiber arrangement seems to be the method of choice for packaging virus DNA in, for example, λ phage

(Cummings et al., 1965) T4 phage (Earnshaw et al., 1978) and Herpes virus (Furlong et al., 1977). Supercoiled DNA, on the other hand, has been adopted as the packaging conformation of DNA in chromatin (Finch et al., 1977), and thus in SV40 virus as well.

The rosette structue of isolated adenovirus cores is not consistent with the torroidal or folded-fiber packaging of DNA. In this connection it is interesting to note that Olins and Olins (1971) observed rosette structures in T7 DNA-histone H4 complexes. In contrast T7DNA - H1 complexes adopted a torroidal structure. The CD spectrum of DNA complexed with histone H1 is very different from that of DNA-H4 complexes. The very non-conservative spectrum of the H1-DNA complex has been interpreted as being due to the DNA adopting a ψ conformation (Fasman, 1977). The CD spectrum of pyridine cores is much closer to the DNA-like spectrum of individual inner histones (H2A, H2B, H3, and H4) complexed with DNA (Fasman, 1977). This result is then also consistent with a supercoil DNA structure rather than the folded-fiber. When protein V is removed from cores by SDS (Figure 3) the CD spectrum approaches more closely the CD spectrum of nucleosomal DNA.

While this spectrum can be obtained by increasing ψ-like structure (Cowman and Fasman, 1978), the spectrum shape is more consistent with a super-coil conformation. In this case the ψ-like spectrum is contributed by interactions between adjacent superhelix turns. The

increased sedimentation coefficient of pyridine cores in high salt or 0.3% SDS is consistent with more highly compacted DNA structure and a ψ-like contribution to the CD spectrum.

The nuclease digestion patterns of pyridine cores are consistent with a supercoiled DNA structure. The lack of higher order staph nuclease generated repeats is likely due to the lack of discrete complexes formed by the underlying core protein, and/or the irregular spacing of nuclease sensitive sites. Laemmli (1975) has demonstrated digestion products of 200-400 bases in collapsed DNA structures in the ψ conformation, using single strand specific nuclease. Preferential accumulation of digestion intermediates in this size range are not seen when pyridine cores are digested with staph nuclease, further supporting the supercoil structure. DNase I digestion intermediates of cellular chromatin have been taken to reflect the periodic exposure of nuclease binding sites on DNA wrapped around a protein core (Noll, 1974; Liu and Wang, 1978). Our results are certainly consistent with this interpretation; however, the ten base repeat seems less sharp and extends only up to about 60-80 bases. A possible explanation for this difference is that the precise cutting site for DNase I is not as restricted as in eucaryotic chromatin. If the enzyme could hydrolyze phosphodiester bonds several bases either side of a periodic enzyme binding site, then the ten base repeat pattern would blur at larger DNA sizes.

It is interesting that dinoflagellate chromatin, which contains a single H4-like histone, has a staph nuclease digestion pattern similar to adenovirus cores (J. R. Allen, unpublished observation). Carter (1978) has argued that several different histones are necessary to build a closed or beaded structure. Sperling and Bustin (1975) have shown that, contrary to complexes containing all four histones, interaction between individual or pairs of histones results in extended rod-like structures. Chromatin constructed from a single type of histone does not display a repeating subunit structure (adenovirus cores, dinoflagellates). It thus seems likely that the digestion pattern observed for adenovirus core chromatin is due to the extended, nondiscrete nature of the underlying protein complex.

In this section I have described a partial characterization of the nucleoprotein structure of the adenovirus core. Is the general similarity in structure between the virus core and cellular chromatin reflected in a similar functional role? In the following section I make use of the differences in staph nuclease digestion patterns of virus cores and cellular chromatin to follow the fate of virus cores during productive infection.

PART II: ADENOVIRUS DNA IS ASSEMBLED INTO NUCLEOSOMES EARLY DURING PRODUCTIVE INFECTION

Introduction

The core of adenovirus contains no histones but rather a virus-coded arginine-rich histone-like protein (Russell et al., 1968; Laver, 1970; Lischwe and Sung, 1977; Sung et al., 1977). Based on core protein stoichiometry and protection of virus DNA from nuclease digestion we earlier proposed a model for the chromatin-like organization of the adenovirus core (Corden et al., 1976). More recent work has shown that the major and minor adenovirus core proteins, namely, protein VII, and protein V, respectively, organize virus DNA into a structure which resembles chromatin but differs most notably in the lack of a repeating DNA unit upon digestion with staph nuclease (Part I).

Because of the ubiquity of nucleosomal DNA in eucaryotes as a template for transcription and replication I sought to define the role of the virus core proteins in the infectious cycle. Two interesting possibilities were imagined. First, adenovirus core proteins might act in a fashion similar to histones during the SV40 infectious cycle and serve both to package the virus DNA in virions and fold the intracellular DNA into the proper template structure. If this were true then common structural features between adenovirus and nucleosomal chromatin might be related to common function. Alternatively, adenovirus core proteins might act only to package DNA in the virion. In

this case we might expect naked intracellular adenovirus DNA or replacement of core proteins with cellular histones early in infection.

To differentiate between these possibilities I have made use of the different patterns of DNA sizes protected from staph nuclease digestion by adenovirus core proteins and cellular histones. Cells were infected with ^{32}P-labeled virus and the fate of the labeled virus DNA was followed during the course of infection. Early during infection (before the onset of viral DNA synthesis) staph nuclease digestion of nuclei reveals size classes of protected input viral DNA which are integral multiples of a unit size. This protection pattern differs drastically from the digestion of virus cores (Part I) and resembles closely the digestion pattern of the host chromatin.

This report will describe experiments which show that adenovirus DNA is assembled into nucleosomes containing cellular histones early during productive infection. I will further show that newly assembled adenovirus chromatin differs from the bulk of the host chromatin in at least three respects: (1) the nucleosome repeat length of adenovirus is 160 bp compared to 185 bp for the host; (2) adenovirus nucleosome linker regions are more susceptible to staph nuclease digestion; and (3) nucleosome length adenovirus DNA does not accumulate during staph nuclease digestion.

By 9 h post infection I have observed a class of adenovirus nucleosomes which appear to resemble more closely the host

nucleosomes and may represent the final stage of a nucleosome assembly process. I have also studied the effects of inhibitors of protein synthesis and DNA synthesis on the assembly of adenovirus chromatin. Finally, I have observed the persistence of adenovirus nucleosomes late in infection (18 h) at a time when newly synthesized adenovirus DNA is complexed in quite different structures.

^{32}P Remains in Unit Length Adenovirus DNA

The prime concern in studying the fate of radioisotopically labeled virus is that the isotope remains in the molecule under study. To demonstrate that the ^{32}P from labeled virus remains in adenovirus DNA, total DNA from infected cell nuclei at 1, 5, 9, and 18 hours post infection was isolated and cleaved with Eco RI restriction endonuclease. The resulting DNA fragments were separated on a 1% agarose gel, as shown in Figure 12. The autoradiograph of the gel shows that ^{32}P is predominantly in the six bands unique to adenovirus type 2 (Petterson et al., 1973). Samples which were not treated with RNase showed no additional bands on autoradiographs. These results show that adenovirus DNA is not degraded and the products recycled into host polynucleotides. Lawrence and Ginsberg (1967) have shown that only 3% of the cell associated parental virus DNA is acid soluble after five hours of infection.

Our results show further that very little of the nuclear adenovirus

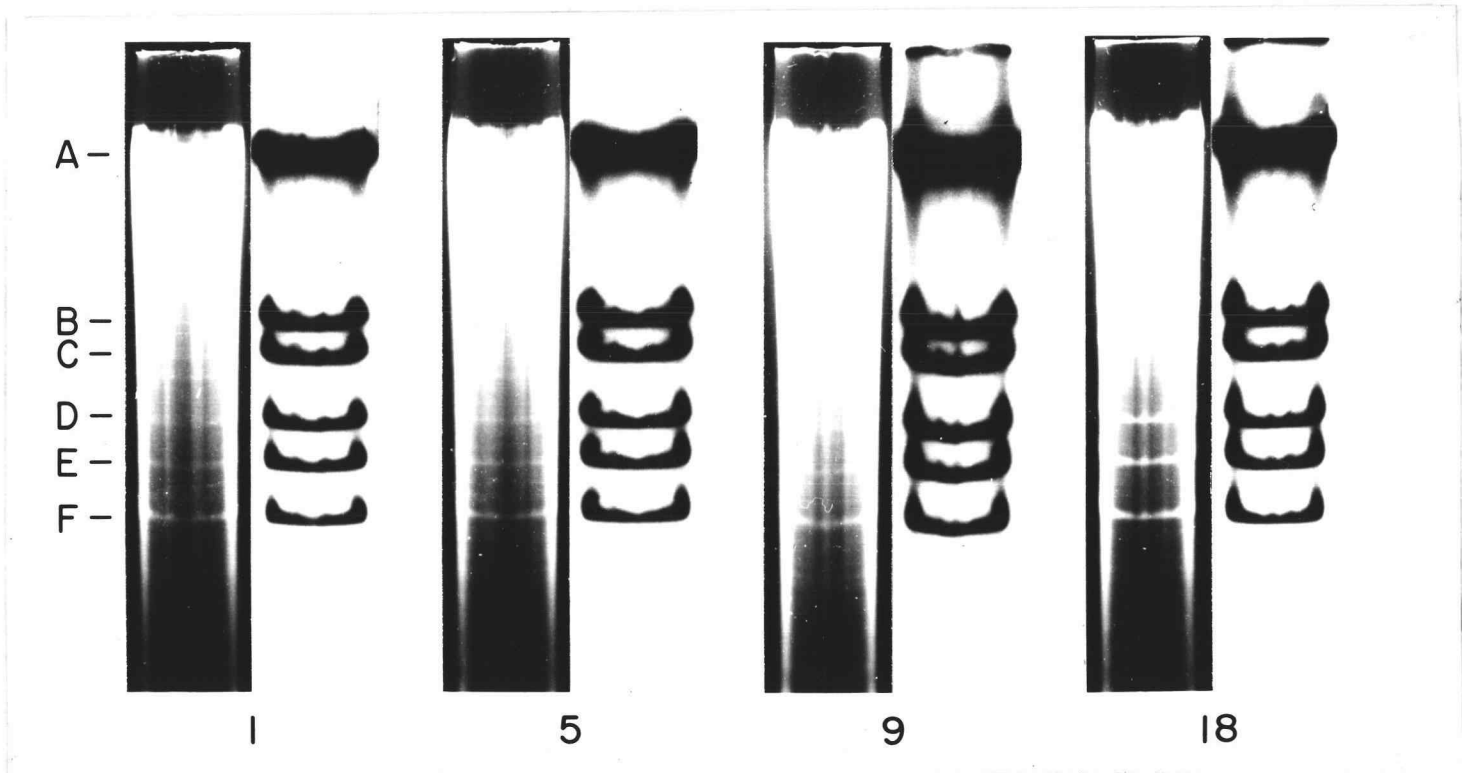


Figure 12. EcoR1 digested infected cell DNA. Total nuclear DNA from cells infected with ^{32}P-labeled adenovirus was isolated 1, 5, 9, and 18 h after infection and cleaved with restriction endonuclease EcoR1. Digested DNA (50 μg) was analyzed by electrophoresis on a non-denaturing 1.4% agarose gel. The gel tracks shown to the left at each time are photographs of the ethidium bromide stained gel (total DNA). Tracks to the right are the autoradiographs of the same gel (adenovirus DNA). The position of the six EcoR1 fragments of purified adenovirus DNA are shown to the left.

DNA is integrated into cellular DNA. Two observations support this claim: (1) The autoradiographs show very little background intensity and (2) bands EcoRI A and EcoRI C, which contain the DNA termini, have the relative intensities expected for free unit length adenovirus DNA. The results shown for 1, 5, and 9 hrs post infection are unchanged at 18 hrs post infection. At this time after infection Shick et al. (1976) have shown that 5-20% of the total intracellular viral DNA is integrated into cellular DNA. Tyndall et al. (1978) have further shown that about 15% of input virus DNA is integrated into cellular DNA during infection of HEK cells with adenovirus type 5 mutants ts36 and ts125 at the non-permissive temperature. My failure to observe this level of integrated DNA is probably due to differences in virus strain, cell line, or growth conditions used in our experiments.

Nuclear Adenovirus DNA is Accessible to Nuclease

DNase I has been shown to cleave cellular chromatin (Mirsky, 1971) and adenovirus cores (PART I) to greater than 95% acid solubility. Under similar conditions purified adenovirus is not digested (Lawrence and Ginsberg, 1967). This property makes DNase I an excellent probe to monitor the process of virus uncoating regardless of the fate of the uncoated DNA. Adenovirus uncoating begins in the cytoplasm after the virus passes through the cell membrane (Lawrence and Ginsberg, 1967; Lonberg-Holm and Philipson, 1969). In my

experiments half of the cell-associated virus is associated with the nucleus by 1 hr post infection. Nuclei were isolated and digested with DNase I as detailed in Appendix I, section (b). Acid solubility was measured by precipitation with 10% TCA. Figure 13 shows the time course of digestion with DNase I at 1, 5, and 9 hours post infection. A plot of the limit of solubility against time of infection shows a steady increase in the amount of virus uncoated (Figure 13B). My values for the plateau or limit of digestion are lower than those previously reported. I believe that the reason for this discrepancy is that my use of lower levels of DNase I (Lonberg-Holm and Philipson, 1969) and the absence of detergent from the reaction buffer (Lawrence and Ginsberg, 1967) makes my assay sensitive only to completely uncoated virions and not to virions with capsid discontinuities. Through the use of inhibitors of protein and RNA synthesis, Lawrence and Ginsberg (1967) and Philipson (1967) have shown that uncoating is carried out by pre-existing enzymes, either viral or cellular. Evidence implicating a cellular enzyme, nuclear membrane ATPase, has come from the studies of Chardonnet and Dales (1972). Together these results suggest an uncoating process where virus arrives at the nuclear membrane and is enzymatically uncoated. Before 1 hr post infection, the uncoating enzyme is not saturated (Fig. 2B) but after this time the uncoating sites are saturated and uncoating takes place at a steady rate.

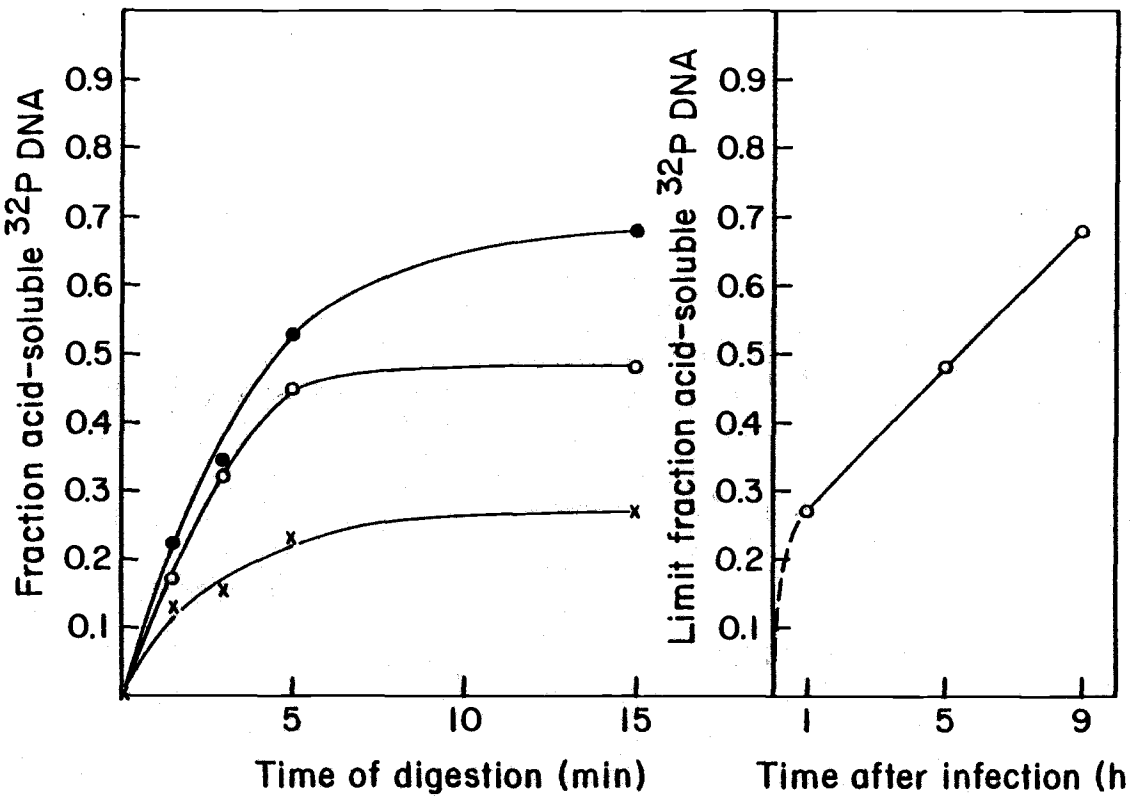


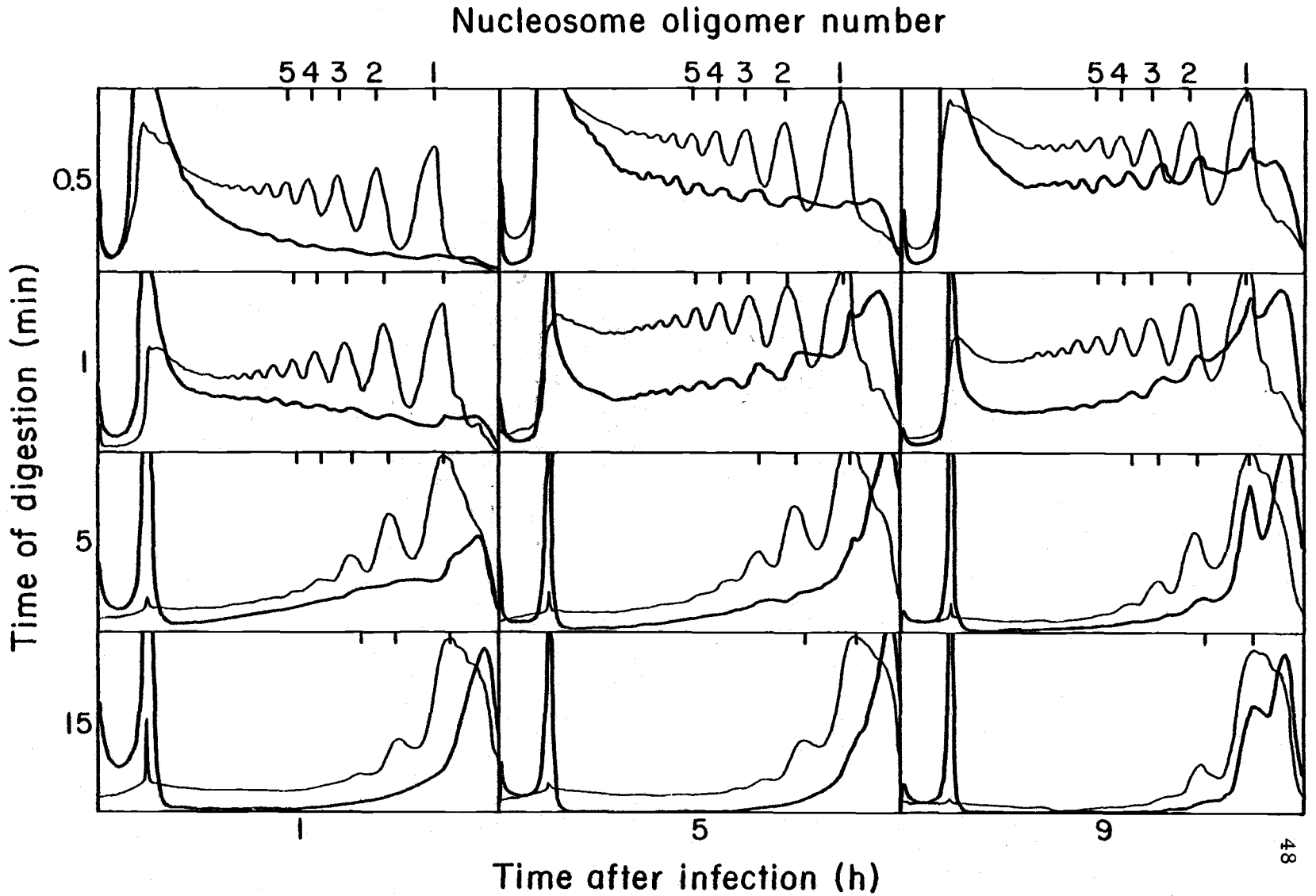
Figure 13. DNase I digestion time courses of infected cell nuclei. Nuclei from cells infected with ^{32}P-labeled adenovirus were isolated 1 h (x), 5 h (o), or 9 h (o) after infection and digested with DNase I according to Appendix I, section (f). Acid solubility was determined by counting ^{32}P released to the supernatant when digested nuclei were precipitated with cold TCA and centrifuged at 5000 x g for 10 min.

Adenovirus DNA is Repackaged in a Nucleosome Repeat Structure

To analyze the nature of the uncoated adenovirus DNA we chose as probe staph nuclease. This choice is based on the difference in size distribution of digestion intermediates of virus cores and host cell nuclei. Digestion of virus cores with staph nuclease produces a single broad band which decreases in size with increasing time of digestion. In contrast digestion of HeLa cell nuclei produces a series of bands which are integral multiples of a unit size. Figure 3 shows the results of agarose gel electrophoresis of staph nuclease generated DNA fragments from infected cell nuclei. It is apparent from the densitometer scans that even as early as 1 h after infection some adenovirus DNA is protected in size classes which are integral multiples of a unit size. This pattern is very different from the staph nuclease digestion pattern of virus cores and suggests that core proteins have been replaced by cellular histones.

At each time of digestion, each member of the series of adenovirus DNA size classes is smaller than the corresponding member of the host series. As previously observed (Sollner-Webb and Felsenfeld, 1975; Shaw et al., 1976) the size of each nucleosome oligomer band decreases with increasing digestion. In my experiment the host nucleosome oligomers late in digestion are smaller than the comparable adenovirus band early in digestion. To show that the size

Figure 14. Electrophoretic analysis of intermediates from staph nuclease digested infected cell nuclei. Cells were infected with ^{32}P-labeled adenovirus and nuclei were isolated and digested with staph nuclease as described in Appendix I. Purified digestion intermediates were separated on a 20 x 20 x 0.2 cm non-denaturing 1.8% agarose gel. The gel was run for 10 h at 80 v at 10°C. Total DNA was imaged by staining in 0.5 μg/ml ethidium bromide and photographing under uv illumination. ^{32}P-labeled adenovirus DNA was imaged by autoradiographing the fixed and dried gel. Unlabeled and labeled PM2 Hae III fragments were run in tracks adjacent to the staph digested samples. The negative of the ethidium bromide picture and the autoradiograph were scanned on a Joyce-Lobel microdensitometer and the scans were digitized and replotted to scale using PM2 Hae III fragments to align the two sets of scans. The thick line represents adenovirus DNA and the thin line represents total cellular DNA.


Nucleosome oligomer number
5 4 3 2 1
5 4 3 2 1
5 4 3 2 1
Time of digestion (min)
0.5
1
5
15
1
5
9
Time after infection (h)

difference at each time of digestion is not due to an increased rate of digestion of the adenovirus chromatin we carried out the following control. Cells were pre-labeled with ^{3}H thymidine, the thymidine was then removed and the cells infected with ^{32}P adenovirus. Infected cell nuclei were isolated 5 h post infection. Production of acid soluble ^{3}H and ^{32}P during staph nuclease digestion was followed and nuclease resistant DNA was isolated, run on gels and sized. After correction for ^{32}P in intact virus, the percent acid soluble ^{3}H and ^{32}P were found to be the same at each time of digestion (results not shown). Analysis of the DNA showed, as before, that each adenovirus band was smaller than the corresponding host band (results not shown). This result says that the smaller size of the adenovirus repeat is not due to a higher rate of digestion and shows unequivocally that adenovirus DNA is repackaged, early during infection, into a nucleosome repeat structure shorter than that of its host.

Sizes of dimer and trimer DNA are shown in Figure 15. To obtain the average repeat size the zero time (extrapolated) values for oligomer nucleosome DNA were divided by the oligomer number (bp/n). The values obtained were 185 bp for the host HeLa cell chromatin and 160 bp for adenovirus chromatin. The values for oligomer bands at the earliest time of digestion were not used for the extrapolation because, on the gel system used, their sizes, against markers in adjacent tracks, were consistently overestimated by about 4%. The

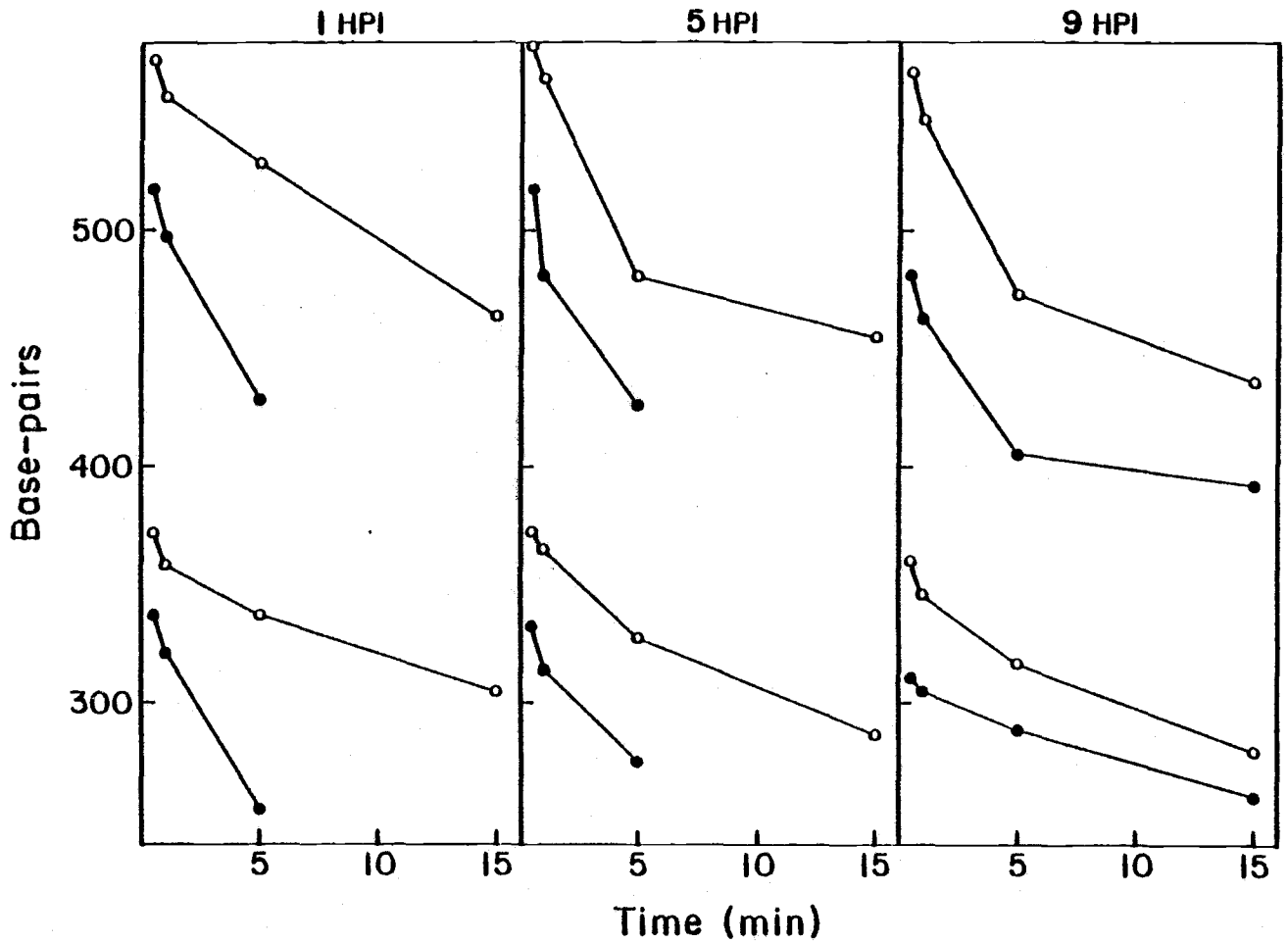


Figure 15. Sizes of dimer and trimer nucleosome DNA from adenovirus-infected cells. DNA sizes from the gel shown in Figure 14 are plotted against time of digestion. Sizes of adenovirus ( ) and total cell ( ) nucleosome oligomers were determined relative to adjacent PM2 Hae III markers.

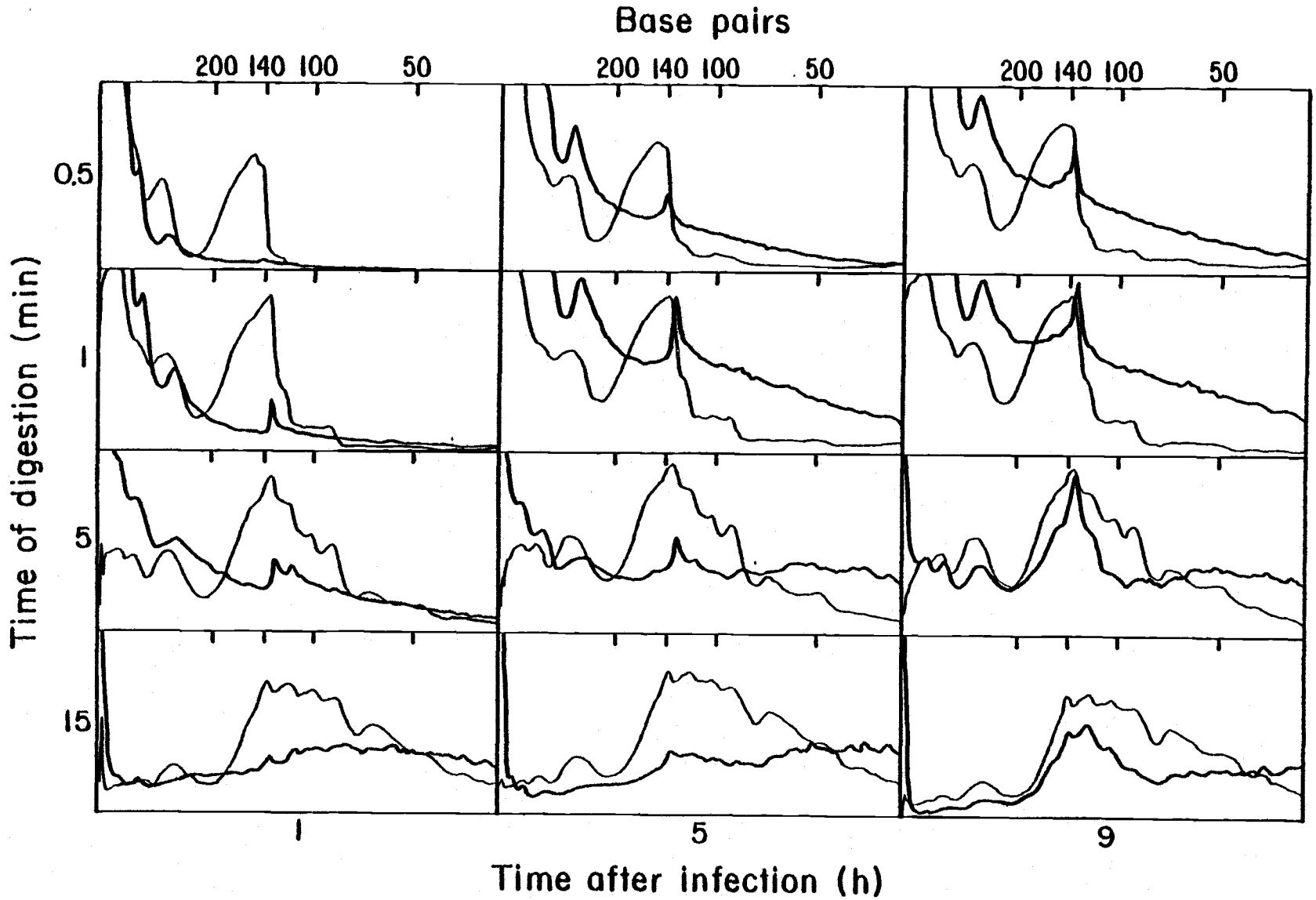
precise cause of this anomaly is unknown; however, the presence of very large DNA in the early samples indicates that perhaps the high viscosity of the sample retards the series of smaller bands. The magnitude of the retardation was calculated by mixing PM2 HaeIII fragments with early time digestion samples and running the mixture next to markers alone. The retardation effect was not evident on acrylamide or composite gels.

Adenovirus Chromatin is Digested into Two Classes of Nucleosomes

Individual nucleosomes from various sources have been shown to contain 140-200 bp of DNA. Analysis of nucleosomal DNA from staph nuclease digested nuclei has revealed a characteristic series of monomer and sub-monomer DNA fragments down to about 40 bp (Axel et al., 1974; Weintraub and Van Lente, 1974). Analysis of staph nuclease digestion products from adenovirus-infected cell nuclei on a 6% acrylamide gel (Figure 16) shows that adenovirus chromatin not only differs from cellular chromatin in repeat length but that differences exist in the accessibility of nucleosome linker and core regions.

The homogeneity and size of adenovirus monomer nucleosome DNA shows that it does not proceed through the same digestion intermediates as the bulk of cellular chromatin. The DNA between 140

Figure 16. Electrophoretic analysis of low molecular weight digestion intermediates from staph nuclease-digested infected cell nuclei. The same samples shown in Figure 14 were separated on a non-denaturing 6% acrylamide gel and imaged as described in the legend to that figure. The thick line represents adenovirus DNA and the thin line represents total cell DNA.


Base pairs
200 140 100 50
200 140 100 50
200 140 100 50
0.5
1
5
15
Time of digestion (min)
1
5
9
Time after infection (h)

and 200 bp has been shown to be due to nucleosomes containing histone H1 (Shaw et al., 1976). Early in our digestions (0.5 min, 1 min) the adenovirus monomer peak contains little DNA larger than about 130-140 bp. Whether the absence of 200-140 bp DNA means that histone H1 is not present in adenovirus chromatin cannot be determined from our data. Later in infection (5 and 9 h post infection) I observe more DNA in the size range 200-140 bp. The implication of these results is that a maturation process is occurring which results in increasingly stable linker regions, possibly due to the addition or modification of histone H1.

A second observation from Figure 16 is that, at each time of infection, the size of the predominant monomer species gradually decreases with increasing digestion. Starting at 0.5 min the predominant peak is about 140 bp. This size decrease parallels the decrease in the size of the leading edge of the cellular monomer peak and probably represents digestion at or near the ends of core particle DNA. Since the size decrease of the adenovirus monomer peak does not seem to be more rapid than the comparable decrease in the host we conclude that the end digestion of nucleosome core particles takes place at the same rate for both types of particles.

The staph nuclease digestion pattern also yields information about the stability of the nucleosome core particle. As predicted by random polymer degradation theory (Montroll and Simha, 1940)

and observed experimentally by Lohr et al. (1977b) for yeast chromatin, the ratio of monomer to dimer DNA increases during the period of digestion after one half of the linker regions have been cut. The digestion of HeLa chromatin seems to follow this relationship. In Figure 16 (0.5 min and 1.0 min) it can be seen that, even when the cellular monomer peak is several times larger than dimer, the adenovirus monomer is smaller than its dimer. This result could be obtained in two ways: 1) some spacers are refractory to staph nuclease digestion, thus giving rise to an unusually large proportion of dimers or 2) monomer nucleosome DNA does not accumulate during digestion. All my evidence points to the second case. At no time of digestion do we observe a larger than expected dimer peak (Figure 14). Furthermore, the spacers of adenovirus chromatin seem to be more accessible to staph nuclease. Finally, analysis of limit digests finds a larger proportion of adenovirus DNA in the submonomer region (120-40 bp). Thus, the failure of adenovirus monomer to accumulate during digestion is due to an increased rate of digestion, probably due to cuts within the nucleosome core. The conclusions reached from analysis of the monomer region DNA are that the newly assembled adenovirus nucleosomes are more sensitive to staph nuclease digestion both in the linker region between nucleosomes and within nucleosome cores.

Later in infection (9 h post infection) analysis of the staph

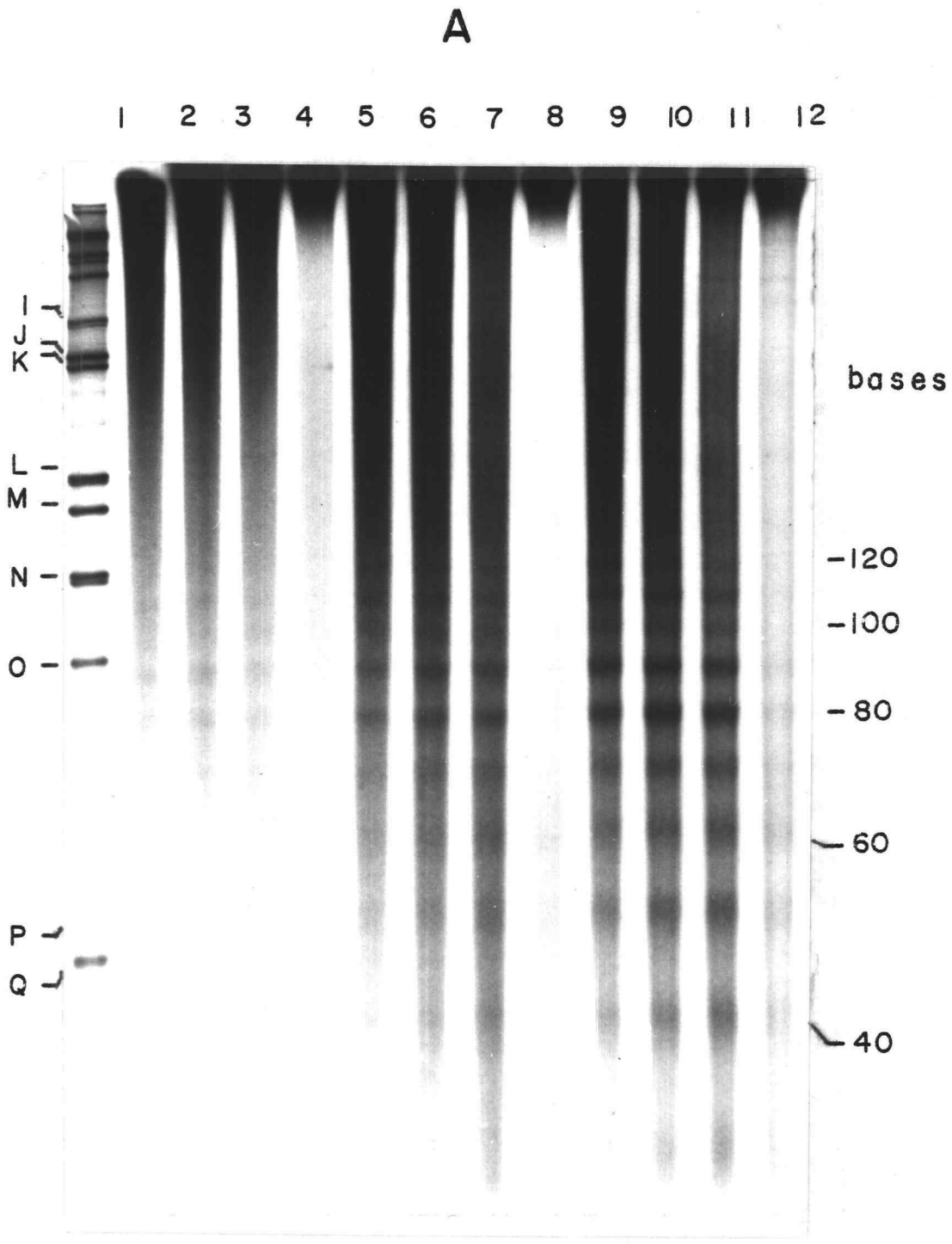
nuclease limit digest pattern (15 min digestion) reveals the presence of a cellular-like monomer. At 1 and 5 h post infection the peak representing submonomer DNA is greater in intensity than the monomer length DNA. Apparently the sharp 130-140 bp monomer is degraded to submonomer fragments and does not accumulate as monomer-length DNA. The picture at 9 h is very different. By this time of infection the pattern of limit digest fragments parallels closely the host pattern. Early in digestion at 9 h post infection a sharp, homogeneous adenovirus monomer peak exists. As digestion proceeds this peak is digested away. The pattern of the remaining DNA, late in digestion, reveals a staph nuclease resistant monomer. The temporal appearance of a class of resistant adenovirus nucleosomes suggests an assembly process in which the smaller, homogeneous, labile nucleosomes assembled early in infection mature into nucleosomes with a resistance to staph nuclease closer to that of host nucleosomes. While the labile, newly assembled adenovirus nucleosomes persist at 9 h post infection, a fraction of these have been stabilized to internal cuts.

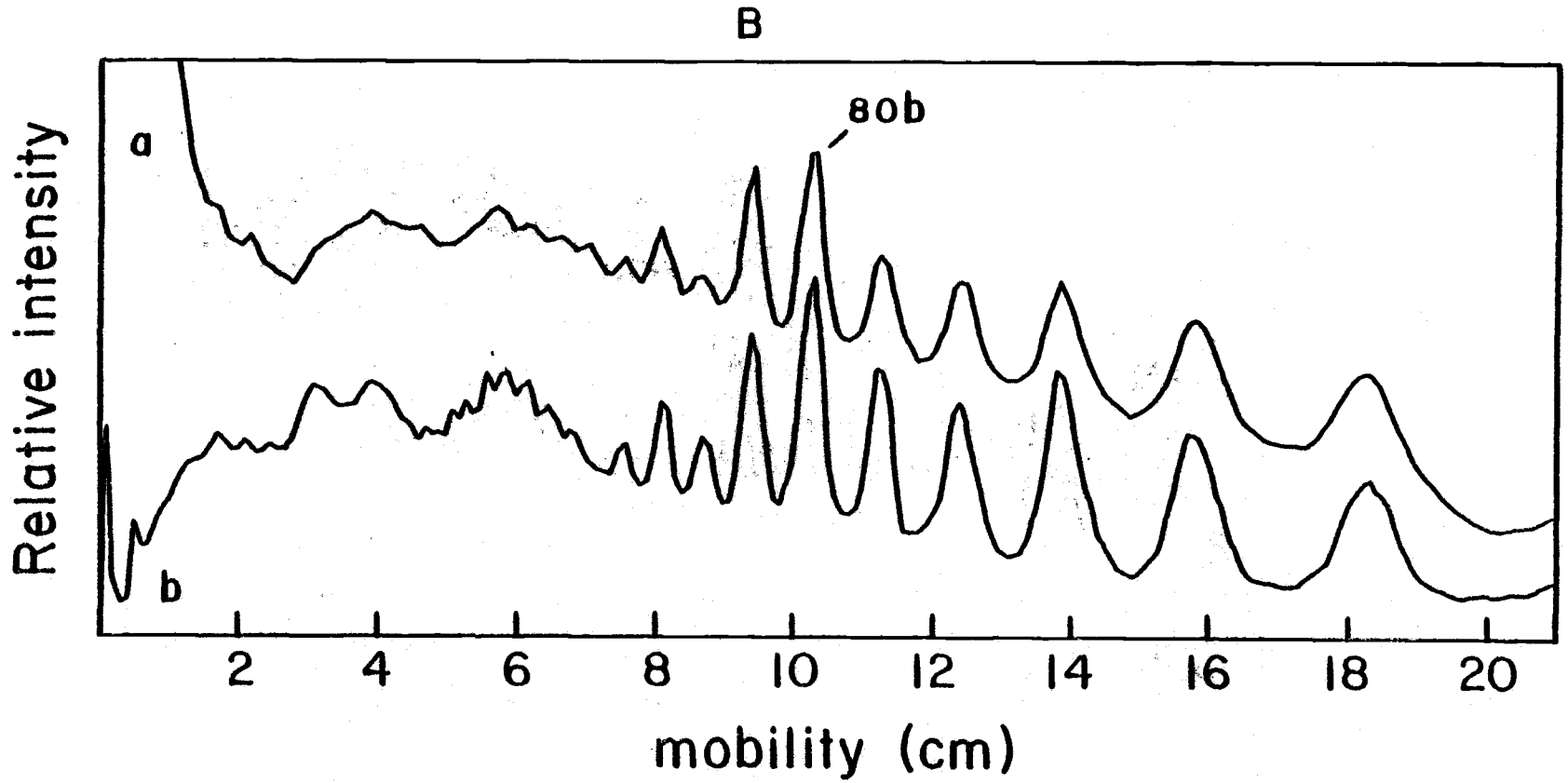
DNase I has been shown to cleave within nucleosomes at characteristic ten base intervals. To compare the internal structure of host and adenovirus nucleosomes, infected cell nuclei were digested with DNase I and the products analyzed on denaturing gels. Figure 17A shows the autoradiograph of an 8% acrylamide, 7M urea gel. It is

Figure 17. DNase I digested infected cell nuclei. Nuclei were isolated 1, 5, and 9 h after infection with ^{32}P-labeled adenovirus and digested with DNase I as described in Appendix I. Purified DNA digestion intermediates were separated on a 20 x 20 x 0.1 cm denaturing gel (8% acrylamide, in 98% formamide). The gel was run for 7 h at 120 ma at 20°C. Total DNA was imaged by staining the gel in 0.5 μg/ml ethidium bromide in 0.4 mM EDTA pH 7.0. ^{32}P-labeled adenovirus DNA was imaged by autoradiographing the fixed and dried gel.

A) Adenovirus DNA digestion intermediates from nuclei digested for 0.5, 1, 5, and 15 min are shown for 1 h post infection (1-4), 5 h post infection (5-8), and 9 h post infection (9-12). PM2 Hae III fragments are shown to the left while calculated sizes of digestion intermediates are shown to the right. (Page 58)

B) Adenovirus DNA from track 10 (a) is compared to the scan of the same track from the ethidium bromide negative (b). (Page 59)


A
1 2 3 4 5 6 7 8 9 10 11 12
I
J
K
L
M
N
O
P
Q
bases
120
100
80
60
40


B
Relative intensity
a
b
80b
2
4
6
8
10
12
14
16
18
20
mobility (cm)

apparent from this gel that with increasing time after infection more of the adenovirus DNase I cleavage products are in the ten base repeat pattern characteristic of cellular chromatin. Figure 17B shows scans of track 9-2 of Figure 17A and the same track from the ethidium bromide stained gel (not shown). Clearly, the sizes as well as the relative concentration of the adenovirus and HeLa digestion products are the same. The temporal appearance of the ten base repeat coincides with the appearance of staph nuclease-resistant adenovirus monomers. These results are consistent with the notion that stability is generated by organizing the internal structure of the nucleosome. The DNase I digestion pattern of the labile, newly assembled adenovirus nucleosome cannot be determined from gels because of the high background intensity at the time (1 h post infection) when only this nucleosome is apparent. To analyze the internal structure of the newly assembled, labile nucleosome it will be necessary to isolate these particles from infected cell nuclei.

Isolation of Adenovirus Nucleosomes

The demonstration of a DNA repeat with nuclease digestion does not rigorously show that the DNA is packaged into compact nucleoprotein particles. Johnson et al. (1978) have shown, for example, in Phsarum, that the ribosomal genes are digested into a nucleoprotein fraction containing nucleosome length DNA and histones. This

complex sediments at 5S, much lower than the 11S of compact nucleosomes, and indicates that unfolded particles can be protected from staph nuclease digestion. Ribosomal cistrons have also been demonstrated in the DNA repeat in Xenopus laevis (Reeves, 1978) and Tetrahymena (Mathis and Gorovsky, 1976). Ribosomal chromatin in Oncopeltus faciatus has been shown by electron microscopy to have a smooth, non-beaded, morphology (Foe, 1978). Thus, at least for ribosomal chromatin, the possibility exists for an altered, extended, chromatin which retains the property of protecting DNA from staph nuclease in multiples of a unit size.

To determine whether adenovirus nucleosomes are in a compact or extended form I separated nucleoprotein particles released from staph nuclease-digested infected cell nuclei on sucrose gradients. The result of such a separation of nucleoprotein particles isolated 9 h post infection is shown in Figure 18A. Adjacent fractions were pooled for gel electrophoresis. The sedimentation coefficient of material in pooled fractions 10 and 11 was determined by analytical ultracentrifugation to be 11S. Gradient separation of nucleoprotein particles isolated 1 h and 5 h post infection revealed no peak of radioactivity co-sedimenting with cellular monomer. Figure 18B shows the DNA of selected fractions from the 9 h post infection gradient. Clearly, the peaks representing dimer and monomer nucleosomes contain both cellular and adenovirus DNA. This indicates that adenovirus DNA

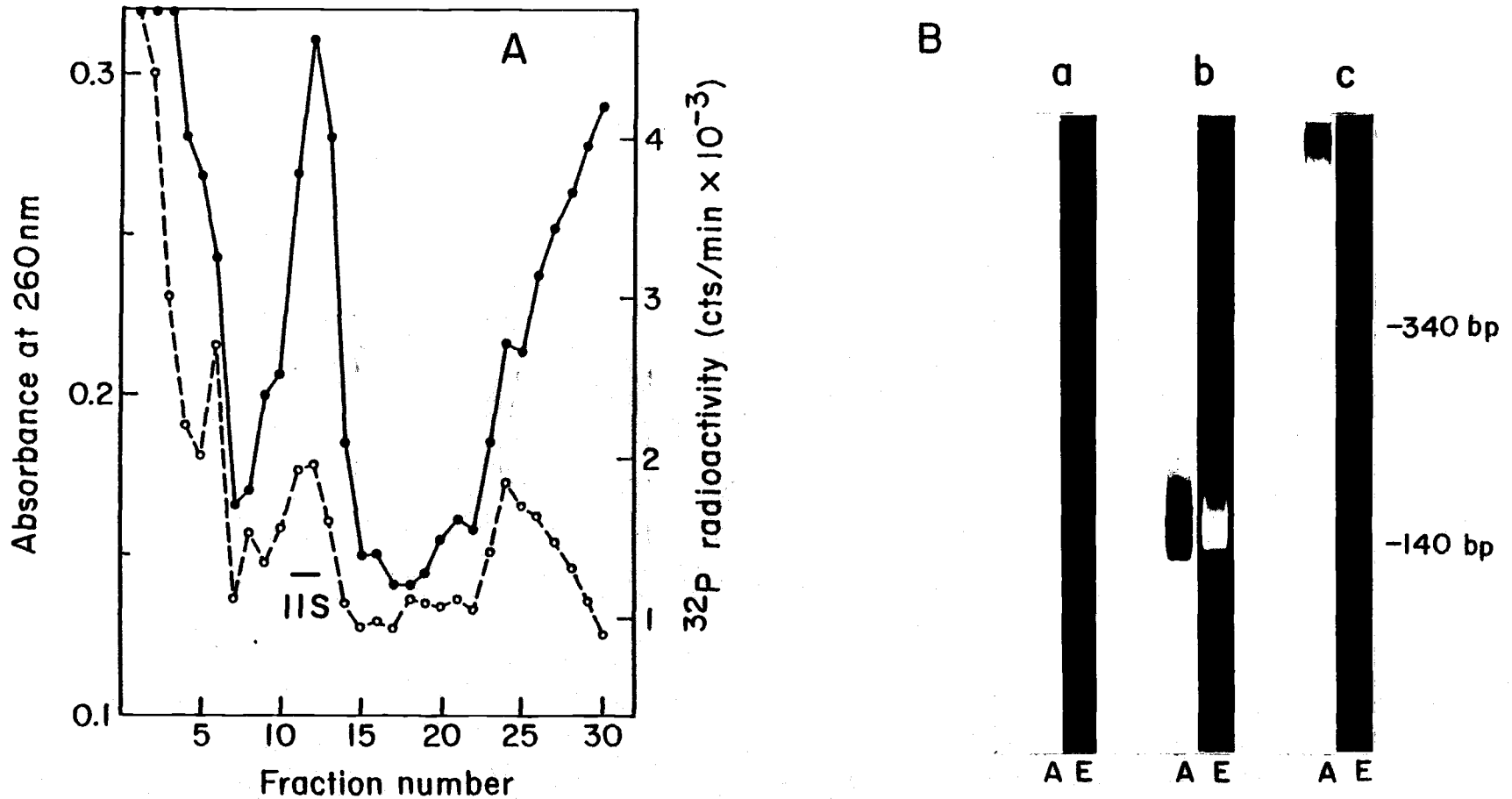


Figure 18. Isolation of adenovirus nucleosomes. A) Sucrose gradient separation of chromatin particles released from infected cell nuclei. Nuclei were isolated 9 h post infection, digested with staph nuclease for 3 min, and the chromatin particles released from EDTA lysed nuclei (see Appendix I, section (k)) were analyzed on a linear 5-20% sucrose gradient. After centrifugation for 24 h at 24,000 rpm in a SW27 rotor the gradient was fractionated from the bottom and samples read for absorbance at 260 nm (•) or and ^{32}P radioactivity by Cerenkov counting (o). Centrifugation was from left to right. The sedimentation coefficient of pooled fractions 11 and 12 was determined to be 11 S by analytical ultracentrifugation. B) Fractions 5 and 6 (a), 11 and 12 (b), and 24 and 25 (c) were pooled, the DNA extracted and separated on a non-denaturing 6% acrylamide gel. E (ethidium bromide) A (autoradiograph).

is present in compact nucleoprotein particles. Gel analysis of material from the peak between the monomer peak and the top of the gradient revealed no monomer DNA, indicating that extended nucleosomes (or free nucleosome length DNA) are not released from nuclei under these conditions. From the size of the released adenovirus nucleosomal DNA I judge these particles to be the fully assembled cellular-like nucleosomes seen in Figure 16 (9 h post infection, 5, 15 min. digestion). I would like to know the sedimentation coefficient of the monomer with very homogeneous 140-130 bp DNA. In the infection used for sucrose gradient analysis, assembly of adenovirus nucleosomes was judged to be almost complete by 9 h post infection. This judgment is based on the increased abundance of 200-140 bp DNA in the total staph nuclease-resistant fraction. As discussed earlier, the appearance of longer monomer DNA later in infection is most likely due to a maturation event which increases the nuclease resistance of the linker region of newly assembled nucleosomes. Since very little radioactivity was evident in the cellular monomer peak at 1 and 5 h post infection, when the homogeneous immature monomer is predominant, we conclude that this DNA does not sediment with the cellular nucleosome fraction.

A possible explanation for our failure to observe the newly assembled monomer particle on sucrose gradients is that it precipitates upon digestion with staph nuclease. Clark and Felsenfeld (1971)

first observed the precipitation of chromatin during staph nuclease digestion. More recently Noll and Kornberg (1977) have studied the release of chromatin particles from rat liver nuclei after staph nuclease digestion. The monomer released from nuclei under their conditions contained DNA from about 200 down to 140 bp. They also noted insoluble material containing a series of DNA fragments 140, 120, 100, 80 bp and smaller. Whether this insoluble material is created by the act of digestion or exists prior to digestion in an insoluble form in the nucleus is not known. Until methods are developed for the release of insoluble particles from the nucleus, the study of newly assembled adenovirus nucleosomes cannot be advanced.

Mode of Adenovirus Nucleosome Assembly

The characteristic nucleosome repeat of greater than 140 bp is unique to biologically assembled chromatin. Two systems have been described which will carry out this task: (1) cellular replication machinery not only replicates DNA but assembles DNA into a nucleosome repeat with a spacing characteristic of the organism (Seale, 1978b) (2) a protein from unfertilized Xenopus laevis eggs has been isolated and shown to assemble nucleosomes in vitro with a spacing of about 200 bp (Laskey et al., 1977, 1978). The main difference between these processes is that the Xenopus factor will assemble nucleosomes in the complete absence of DNA replication. To

determine which process is involved in the assembly of adenovirus nucleosomes the effect of inhibitors of DNA synthesis on the structure of intranuclear adenovirus chromatin was studied.

Both cytosine arabinoside and cycloheximide have been shown to block DNA replication (Kornberg, 1974). When these drugs are introduced at the time of infection and the pattern of DNA protected from staph nuclease digestion is determined 6 h later, the following results are obtained (Figure 19). Neither araC nor cycloheximide has stopped the assembly of adenovirus chromatin. In both cases digestion intermediates up to octamer are seen. Since the modes of action of the inhibitors used are different, this result tells us something about the mechanism of adenovirus nucleosome assembly.

Cytosine arabinoside has been shown to block DNA synthesis at the level of DNA chain elongation. Assembly of adenovirus chromatin in the presence of ara C indicates that the assembly process is independent of chain elongation. This implies the existence of a nucleosome assembly factor similar to that isolated by Laskey et al. (1977, 1978).

The results of experiments with cycloheximide are more difficult to interpret. In the current model of chromatin replication nucleosomes assembled in the presence of cycloheximide contain histones which were previously bound to the parental DNA strand. Binding of the parental histone octamers to adenovirus DNA would

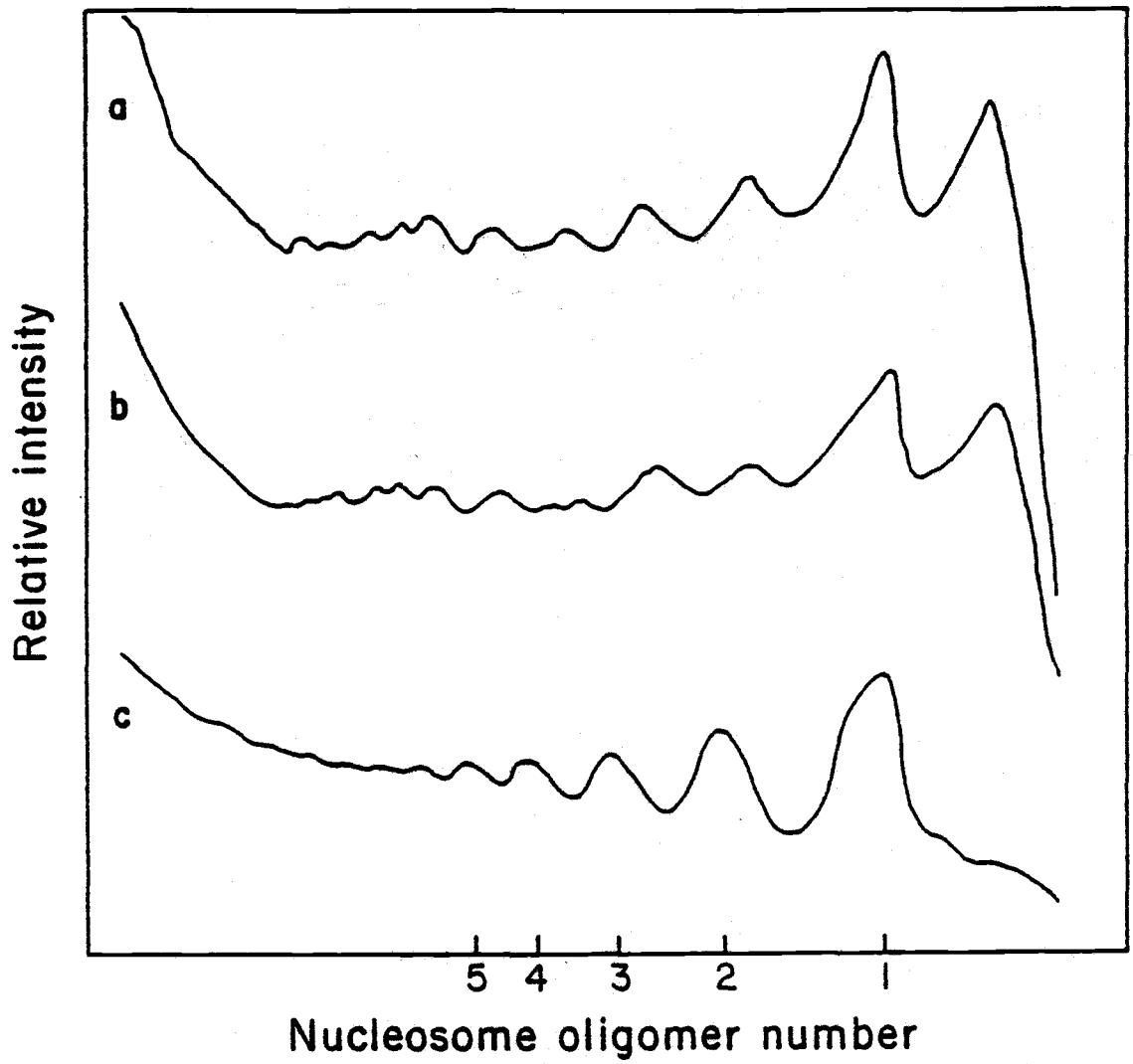


Figure 19. Effect of inhibitors of DNA and protein synthesis on adenovirus nucleosome assembly. Cells infected with ^{32}P-labeled adenovirus were made 20 μg/ml cytosine arabinoside (a), or 25 μg/ml cycloheximide (b) at the time of infection. After 6 h nuclei were isolated and digested with staph nuclease as described in Appendix I. Digestion intermediates were separated on a non-denaturing 2% agarose gel. Scans of the autoradiograpn are shown in (a) and (b). Scan (c) is the scan of the negative of the ethidium bromide stained gel track shown in (a). Inhibition of DNA synthesis was verified by the failure of the infected cells to incorporate ^{3}H-thymidine into acid precipitable material 6 h after infection (data not shown).

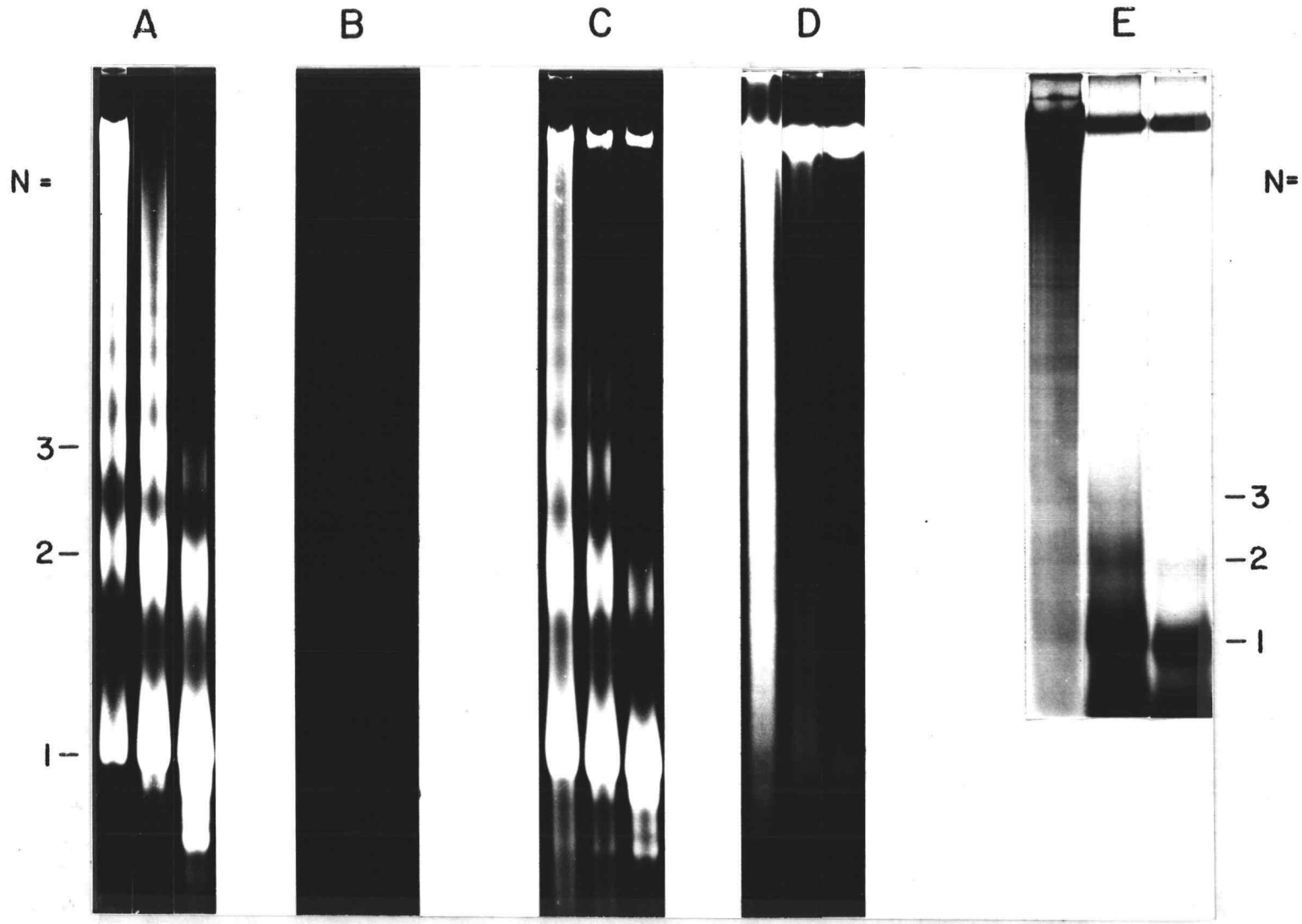
entail their removal and reassembly on new DNA. A more likely explanation is that the assembly of adenovirus chromatin in the presence of cycloheximide utilizes the existing histone pools and/or nascent histones. If methods can be developed for separating adenovirus chromatin or nucleosomes from their cellular counterparts, then labeling studies could determine the origin of the histones involved in adenovirus nucleosome assembly.

Adenovirus Nucleosome Repeat Structure Persists Late in Infection

The onset of DNA replication at nine hours post infection signals a switch from the early to the late phase of infection. Early transcription and translation result in the formation of products necessary for the initiation and maintenance of virus DNA replication. Concomitant with the initiation of viral DNA replication is an alteration in the pattern of viral transcription resulting in the expression of genes coding for virus structural proteins. The results of the preceding sections showed that parental genomes are packaged in nucleosomal chromatin which would be available to serve as templates for early transcription. The experiments described in this section were designed to look at the nuclease digestion pattern of intranuclear adenovirus DNA after the onset of viral DNA replication. The staph nuclease pattern of intranuclear adenovirus DNA was

visualized by the blotting technique of Southern (1975). Direct labeling of viral DNA (Corden et al., 1976) was not used because of ambiguities arising from residual host DNA synthesis or repair. Unlabeled adenovirus infected HeLa cell nuclei were isolated late in infection, cleaved with staph nuclease, and the resulting DNA fragments purified, and separated by gel electrophoresis. After staining with ethidium bromide the DNA contained in the gel was transferred to a nitrocellulose sheet (Southern, 1975), and the adenovirus DNA sequences were imaged by hybridization with ^{32}P-labeled adenovirus DNA. Figure 20A-D shows the digestion patterns of total cellular and adenovirus DNA from infected cell nuclei isolated 8 and 36 h post infection. The 8 h experiment serves as a control. Since adenovirus DNA replication has not started by this time, failure of adenovirus DNA to hybridize to this filter means that probe DNA is binding specifically to adenovirus DNA. Secondly, it shows that, with the sensitivity employed, the digestion products of input virus DNA are not imaged. The resulting pattern of adenovirus digestion products 36 h post infection (Figure 20C and D) looks very different from the host digestion pattern. The nascent adenovirus DNA is not protected in a regular repeat. This result is consistent with the observations of Kedinger et al. (1978) on isolated adenovirus replication complexes. By electron microscopy they identified two types of structure, uncomplexed (20 Å diameter) DNA and single stranded DNA bound by the

Figure 20. Persistence of adenovirus nucleosomes late in infection. Total nuclear and adenovirus digestion intermediates from staph nuclease digested nuclei (unlabeled) isolated 8 h post infection (A and B) or 36 h post infection (C and D) were separated on a 3% acrylamide gel as described in Appendix I, section (1). A and C are pictures of ethidium stained gels. B and D are autoradiographs of filters containing the DNA from the gels shown in A and C transferred by the Southern procedure and imaged by hybridization with ^{32}P-labeled adenovirus DNA. Input adenovirus DNA 18 h post infection (E) was imaged by autoradiographing separated staph nuclease digestion products on a 2% agarose gel. The three gel tracks shown in each figure are 0.5, 5, and 15 min of digestion. Nucleosome oligomer numbers shown at the left were determined from the position of PM2 Hae III markers.


A
B
C
D
E
N =
3
2
1
N=
3
2
1

adenovirus-coded 72,000 dal protein in a smooth 200 Å dia. fiber. Some nucleosomes were seen in their preparations; however, these could apparently be explained as artifacts of their isolation procedure. By our technique some monomer length DNA seems to be present late in digestion of 36 hpi nuclei but due to the decreased hybridization efficiency of small DNA fragments bound to nitrocellulose (Melli and Bishop, 1970) it is unclear whether this small DNA is truly nucleosomal or represents a cutoff of a random size distribution. Experiments looking at total nuclear adenovirus DNA by this technique at 12, 18 and 24 hours post infection show essentially the same pattern.

In contrast to this result, input virus DNA at 18 h post infection is still present in a nucleosome repeat pattern (Figure 20E). The persistence of the nucleosome repeat means that most of the input genomes are not rearranged into replicative intermediates. Vlak et al. (1976) have shown, through density shift experiments, that most of the input genomes undergo at least one round of replication. At first look their result seems to contradict my own. Consideration of the template for initiation of virus replication provides a possible explanation for this contradiction. By the time virus replication starts, cellular protein synthesis has largely been shut off (Ginsberg et al., 1967). Thus, as the first round of viral replication occurs, the histones on the parental virus DNA segregate non-randomly to one of the daughter strands (as in cellular replication). The other daughter strand would

then bind virus-coded 72K protein and carry out the remainder of the adenovirus replication cycle.

The persistence of the nucleosome repeat pattern further shows that a nucleosomal adenovirus is available as a template for transcription late during virus infection.

Discussion

The results of Part II show that adenovirus DNA is reorganized, early during productive infection, into a structure which differs from the structure of the virus core. The most dramatic difference is the presence of a chromatin-like nucleosome repeat upon staph nuclease digestion of infected cell nuclei. Digestion of pyridine cores or cornerless virions with staph nuclease results in the appearance of a single broad band of digestion intermediates. The peak of this size distribution decreases with increasing time of digestion. In contrast, the digestion products of adenovirus chromatin early during productive infection (digestion of infected cell nuclei) fall into DNA size classes which are approximately integral multiples of a unit size. The presence of integral multiple size classes of protected DNA is common to eucaryotic chromatin and is thought to reflect the nucleosomal organization of histones and DNA.

The limit staph nuclease digestion pattern of pyridine cores consists of a broad band with a peak at about 175 bp but stretching

from greater than 300 bp down to 20-30 bp. A series of low molecular weight bands that are integral multiples of 10 bp can be seen below 60 bp. Analysis of the nucleosome monomer region on gels containing protected DNA from staph nuclease-digested infected cell nuclei reveals a very homogeneous size class of adenovirus DNA at about 140 bp, the size of cellular monomer nucleosome core particle DNA. While a series of adenovirus DNA bands that are multiples of approximately 10 bp can be seen in the digestion products from infected cell nuclei, the relative intensities of these bands are different from the bands seen in pyridine core digests and more closely resemble the limit pattern from cellular chromatin.

Digestion of chromatin or nucleosomes with DNase I results in a series of DNA bands which are multiples of approximately ten bases (b) when the adenovirus DNase I digestion intermediates from infected cell nuclei are analyzed (Figure 17B) the pattern is nearly indistinguishable from that of the host. Comparable digestions of pyridine cores with DNase I reveals a series of 10 b repeating size classes; however, this pattern extends only to 60-80 b (rather than beyond 180 b in infected cell digests) and the relative intensities are not the same as for chromatin (Figure 8B).

The isolation of 11S nucleoprotein particles from staph nuclease-digested infected cell nuclei is also in contrast to the insoluble nature of the nuclease digestion products of pyridine cores.

Taken together, the results of nuclease digestion experiments on infected cell nuclei strongly suggest that adenovirus DNA is repackaged with cellular histones early during productive infection. Our experiments cannot, however, rule out the possibility that the adenovirus core proteins remain bound to adenovirus DNA and undergo structural changes resulting in a more chromatin-like protection from nuclease digestion. Several observations argue against this possibility. Chardonnet and Dales (1970) have shown, early during infection that the amount of virus protein bound to the nucleus at 37°C is not significantly greater than the amount bound at 5°C. The amount of virus DNA they found bound to the nucleus at 37°C is severalfold higher than at 5°C, indicating that virus protein is not following virus DNA into the nucleus. Since these experiments were performed with protein labeled with ^{3}H-arginine, and virus protein VII is 23 mole percent arginine, their experiment should have been able to detect the presence of protein VII bound to adenovirus DNA early during productive infection. A second indication that protein VII is not involved in the infectious process comes from the infectivity of deproteinized virus DNA (Nicolson and McAllister, 1972; Graham and VanDer Eb, 1973).

Although the nuclease digestion patterns of newly assembled adenovirus chromatin clearly indicate a nucleosomal organization, subtle differences from the digestion patterns of total cellular

chromatin have been observed. The repeat distance, or spacing of nucleosomes, in the newly assembled adenovirus chromatin is 160 bp compared to 185 bp for the host HeLa cell chromatin. Repeat distances are known to be different from species to species (Compton et al., 1976; Lohr et al., 1977a) and to vary within individual cell types (Martin et al., 1977). While shorter repeat distances have been correlated with transcriptional activity (Lohr et al., 1977a; Thomas and Thompson, 1977) exceptions do exist (Lipps and Morris, 1978; Gorovsky et al., 1978). Gottesfeld and Melton (1978) have shown that the nucleosome repeat distance of DNA which is expressed as polysomal mRNA in rat liver cells is the same as the repeat distance of total rat liver chromatin. Thus, while the adenovirus genome is certainly transcriptionally active, the shorter nucleosome repeat distance it displays may not be related to this activity.

The linker and core regions of newly assembled adenovirus nucleosomes are more accessible to nuclease than the average cellular nucleosome. This can be seen in the lack of protected DNA fragments in the size range 200-140 bp and in the lack of accumulation of monomer nucleosome length DNA. That a change in the internal structure of the newly assembled adenovirus nucleosomes is responsible for the increased lability is suggested by the DNase I digestion patterns. As seen in Figure 17A the amount of adenovirus DNA in the ten base "ladder" characteristic of eucaryotic nucleosomes increases with

increasing time of digestion. I have interpreted this result as due to an assembly process where newly assembled, labile adenovirus nucleosomes are precursors to the more stable, cellular-like nucleosomes seen in the limit staph nuclease digestion patterns (Figure 16) and the DNase I digestion patterns (Figure 17) at 9 h after infection. This idea is consistent with recent results of Worcel et al. (1978), who showed that histones H3 and H4 are added first to newly replicated DNA, followed by H2A and H2B, and later by histone H1. The properties of newly assembled adenovirus chromatin are those expected for chromatin lacking H1 and possibly H2A and H2B. The nucleosome monomer in the size range 200-140 bp has been shown to be due to histone H1 bound to DNA 'tails" on nucleosome core particles (Shaw et al., 1976). Absence of adenovirus DNA in this size range at 1 and 5 h post infection indicates that histone H1 is not present in newly assembled adenovirus chromatin. By 9 h after infection we see some DNA in the 200-140 bp size range. Whether histones H2A and H2B are associated with the newly assembled chromatin is not clear. H3 and H4 alone can protect DNA from nuclease digestion in much the same way as all four histones (Camarini-Otero et al., 1976). Seale (1978c) has shown that the repeat distance of newly replicated HeLa chromatin is shorter than that for total HeLa chromatin. This result suggests that the shorter repeat of newly assembled adenovirus chromatin is due not to

transcriptional activity but rather results from the fact that it is newly assembled.

Experiments in which cellular DNA or protein synthesis are inhibited at the time of infection have shown that the assembly of adenovirus chromatin is independent of cellular DNA and protein synthesis. These results suggest that adenovirus is assembled by a nucleosome assembly factor similar to that described by Lasky et al. (1977, 1978). It is not known whether a Xenopus-like nucleosome assembly factor is involved in nucleosome assembly during replication. A possible role for nucleosome assembly factor would be to assemble the daughter fork which does not receive parental histones. This assembly is not linked to elongation as shown by continued replication in the presence of cycloheximide (Weintraub, 1976; Seale, 1976). During adenovirus infection the uncoated virus genomes could mimic the free daughter arm and be assembled in much the same way as the restoration of nuclease resistant DNA after removal of cells from a cycloheximide block (Seale and Simpson, 1975). Infection of growing cells with adenovirus results in the eventual shut-down of cellular DNA synthesis (Ginsberg et al., 1967). Infection and resting human primary cells, however, results in a transient stimulation of cellular DNA synthesis (Ledinko, 1967), indicating a need for cellular DNA replication factors early during infection.

Is the assembly of adenovirus chromatin a virus-specific event or is it the consequence of a generalized cellular mechanism? My experiments do not allow us to answer this question. By transfecting cells with labeled DNA we should be able to tell if virus proteins are necessary for the assembly of adenovirus chromatin.

Do the newly assembled nucleosomal adenovirus genomes serve as templates for early transcription? My results do not provide an unequivocal answer to this question. Figure 14 shows that, while much of the adenovirus DNA is in the nucleosome repeat pattern, there is a high background of DNA which does not fall into the pattern. The amount of DNA in the repeat compared to background DNA which does not fall into the pattern increases with increasing time of digestion (Figure 14). This increase suggests that some of the background DNA seen early might be due to cuts within newly assembled nucleosomes. These internal cuts would be expected to blur the nucleosome repeat pattern. Flint and Sharp (1976) and Berk and Sharp (1977) have shown that the concentration of early mRNA in HeLa cells is the same at multiplicities of infection from 3-100. The ratio of infectious virus particles to physical virus particles is 1:10 (Green et al., 1967); therefore, there will be the same amount of early mRNA synthesized with 30 or 1000 templates. Two mechanisms have been considered to explain these observations: (1) cytoplasmic early mRNA or its translation products exert feedback control on their own synthesis

or (2) there are a limited number of transcription initiations per cell per unit time. The uv irradiation and mixing experiments of Berk and Sharp (1977) point strongly to the second alternative. This explanation would mean that perhaps as little as 10% of our input genomes may be acting as templates for early transcription. It will clearly be necessary to isolate early transcription complexes to verify their nucleosomal nature.

PART III. GENERAL DISCUSSION

The results presented in my thesis demonstrate differences between the nucleoprotein structure of adenovirus DNA in virus cores and in infected cell nuclei. In mature virions the virus DNA is complexed with virus-coded core proteins. The virion nucleoprotein complex has some properties in common with cellular chromatin but several differences exist. The lack of a nucleosome repeat in adenovirus core nucleoprotein has been demonstrated through the analysis of staph nuclease digestion intermediates. When the staph nuclease digestion pattern of intranuclear adenovirus nucleoprotein is analyzed early during productive infection a pattern similar to cellular chromatin is observed. This change in the pattern of staph nuclease digestion intermediates to a nucleosome repeat pattern is probably mediated by the replacement of virus core proteins with cellular histones early during productive infection. DNase I digestion experiments support this contention. Experiments using inhibitors of protein and DNA synthesis show that the assembly of adenovirus DNA into nucleosomes, early during infection, is independent of cellular DNA replication. This result implicates a nucleosome assembly factor similar to that isolated by Laskey et al. (1977, 1978). The observation of a structural reorganization of adenovirus nucleoprotein during the infection process raises several questions.

If virus core proteins are removed early during infection what is the role of protein VII in the virus life cycle? An answer to this question has come from work in our laboratory (Corden et al., Appendix II) on an adenovirus mutant which does not process the precursor to protein VII. Adenovirus type 2 mutant tsl (Weber, 1976) does not process protein pVII to VII at the nonpermissive temperature yet forms virions containing the precursor protein. These virions are thought to be assembly intermediates. The presence of protein pVII in closed capsid virus particles means the protein VII-DNA complexes do not exist as cores late in infection. This is consistent with the results of Everitt et al. (1977) and D'Halluin et al. (1978a, 1978b) who failed to detect virus core structures as assembly intermediates.

Our experiments with tsl virions were analoguos to those described in Part II. ^{32}P-labeled tsl virions, synthesized at the nonpermissive temperature, were used to infect cells. Nuclei were isolated and digested with nucleases. The protected DNA was isolated and run on gels (Appendix II). The results of these experiments show that tsl mutant virions are defective in uncoating. The implication is that protein VII is necessary for proper virus uncoating. The presence of protein VII only in mature virions suggests a mechanism for uncoating where protein VII plays a crucial role. Cleavage of the internal core protein, pVII→ VII, the final virus maturation event, can be imagined as "cocking" the virus into readiness for infection.

Since the cleavage takes place internally there must be a conformational change manifested externally. In ts1 mutant virions this change has not occurred and thus, early during infection the "trigger" is not recognized by the cell and uncoating does not occur.

The role of protein VII thus appears to be restricted to packaging the virus DNA in virions and facilitating the uncoating of virions early during infection. In many ways this role is similar to the role of protamine in packaging DNA in the male gamete of many organisms.

What role do histones play in the adenovirus infection process? There is now good evidence that most of the DNA in the eucaryotic nucleus is complexed with histones to form nucleosomes. Both transcriptionally active (Lacy and Axel, 1975; Axel et al., 1975; Kuo et al., 1976; Mathis and Gorovsky, 1976; Bellard et al., 1978) and replicating (McKnight et al., 1978; Cremisi et al., 1978; Seale, 1978a) chromatin are packaged in nucleosomes. It seems likely therefore, that histones are involved, actively or passively, in the processes of transcription and replication. The adenovirus genome is transcribed (Price and Penman, 1972) and replicated (de Jong et al., 1977; van der Vliet and Kwant, 1978) by the host polymerases. Considering these observations, and my own results, I would like to propose that the assembly of adenovirus DNA into nucleosomes, early during infection, is necessary for the formation of the template for transcription and replication.

Recent experimental results suggest that nucleosomal chromatin structure is a necessary, if not sufficient, template for transcription. Experiments in which protein free DNA is injected into Xenopus laevis oocytes have shown a striking correlation between conditions where the injected DNA is assembled into nucleosomes and conditions where transcription occurs (Mertz and Gurdon, 1977; Wyllie et al., 1978). Parker and Roeder (1977) have shown, using a chromatin template, that purified RNA polymerase III selectively and accurately transcribes the 5S RNA genes of Xenopus laevis in vitro. Similar experiments using DNA as template result in nonselective transcription (Parker and Roeder, 1977). The results of my thesis clearly show that, during infection, adenovirus DNA meets the necessary condition for transcription; namely, it is assembled into nucleosomes. Is this assembly sufficient for transcription to occur? Although the transcribed cellular sequences are present in the nucleosome repeat pattern upon staph digestion (Mathis and Gorovsky, 1976; Bellard et al., 1978), evidence exists that the nucleosomes responsible for this repeating pattern are altered. Weintraub and Groudine (1976) have shown that transcriptionally active genes are preferentially rendered nonhybridizable by digestion with DNAase I. While the basis of increased DNase I sensitivity of active genes is not known, several possibilities have been discussed. Transcriptionally active chromatin fractions are enriched in nonhistone proteins (Gottesfeld

and Butler, 1977). Particularly interesting, in this connection, is the release from nuclei of HMG proteins during DNase I digestion (Vidali et al., 1977). The involvement of nonhistone proteins as necessary template factors is further suggested by the high protein to DNA ratio (2.7). (Parker and Roeder (1977) observed for their in vitro transcription system (nucleosomal chromatin would be expected to have a ratio of about 1). A second possible factor implicated in the generation of DNase I sensitive transcriptionally active chromatin is histone modification, especially acetylation (Allfrey, 1977). Thus, the assembly of adenovirus DNA into nucleosomes, early during infection, may be necessary but not sufficient to form the correct template for virus transcription. The results of my thesis show that the newly assembled adenovirus nucleosomes are more susceptible to staph nuclease than those of cellular chromatin. Whether this susceptibility holds for DNase I needs to be tested before an altered conformation similar to active cellular chromatin can be claimed. Because of the presence of partially uncoated but not yet assembled virus genomes, nuclei are not suitable for the tests of adenovirus nucleosome DNase I susceptibility. The isolation of adenovirus transcription complexes early during infection would not only allow the test for DNase I susceptibility, but also the characterization of the histone and nonhistone components of such complexes.

The involvement of histones in eucaryotic DNA replication has

been intensely studied yet is not well understood (Seale, 1978b; Chambon, 1978). It seems certain that nucleosomes are rapidly a ssembled on newly replicated DNA (McKnight et al., 1978; Cremisi et al., 1978a). Kedinger et al. (1978) have shown that histones are absent from adenovirus replication complexes. This result suggests that, contrary to cellular replication, histones are not involved in adenovirus DNA replication. The mechanism of adenovirus DNA replication is decidedly different from the host mechanism (Levine et al., 1977), taking place by a displacement mechanism similar to mitochondrial DNA replication. In fact, van der Vliet and Kwant (1978) have shown that the DNA polymerase activity responsible for adenovirus DNA replication is DNA polymerase γ. This polymerase has many properties in common with mitochondrial DNA polymerase (Robberson et al., 1972). Although these results strongly suggest that histones are not involved in adenovirus replication, they do not rule out the possibility that the initial round of adenovirus replication takes place on a nucleosomal template. Vlak et al. (1976) have shown that most of the input viral genomes are replicated at least once. Coupled with my results showing that input virus genomes are maintained in a nucleosome structure late in infection, the indication is that the initial round of adenovirus replication takes place on a nucleosomal template. Whether the initial round of adenovirus DNA replication is catalyzed by DNA polymerase γ is

not known.

How do other animal viruses satisfy the apparent requirement for a nucleosomal template? Clearly the papova viruses have solved the problem by utilizing histones to package the viral DNA in virions as well as during the growth cycle. Other viruses, such as Herpes, which do not package their DNA with histones in the virion, but replicate in the nucleus, would be expected to utilize histones in a manner similar to adenovirus.

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APPENDIX

APPENDIX I

a) Cells and Virus

HeLa S3 cells were grown in suspension culture in Joklik modified minimal essential medium (Gibco F13) supplemented with 7% fetal calf serum (Sterile Systems). Adenovirus type 2 was obtained from Dr. Joseph Weber, Department of Microbiology, University of Sherbrooke, Quebec, Canada. Virus stocks were kept as crude lysates which were prepared by sonicating infected cells and clearing the lysate by low speed centrifugation. Virus was labeled in phosphate-free Joklik modified MEM (Gibco) supplemented with 2% dialyzed calf serum (Gibco). Infected cells were suspended in phosphate-free medium two hours post infection and ^{32}P orthophosphate (NEN) was added to 10 μCi/ml at six hours post infection. Infected cells were harvested at 48 h post infection and virus was purified by twice CsCl banding according to Doerfler (1969).

Studies on the fate of infecting virus were carried out by infecting cells with ^{32}P-labeled virus. Cells were concentrated to 10^7 cells/ml in serum-free medium and stirred for 30 min at 37°C with 10^3-10^4 virions per cell. The concentration of the inoculum was determined by reading the absorbance at 260 nm of an aliquot of the purified virus lysed in 0.5% SDS. After a 30 min adsorption period the cells were diluted with Joklik MEM, 7% fetal calf serum to 5×10^5 cells per ml.

b) Virus Degradation Products

Cornerless virions and pyridine cores were prepared as described by Prage et al. (1970). Cornerless virus was produced by dialyzing purified virus for 18 h at 5°C against three changes of 1000 volumes of 5 mM Tris-maleate, 0.1 mM EDTA, pH 6.4. Pyridine cores were produced by first dialyzing CsCl purified virus for 18 h at 5°C against three changes of 1000 volumes of 5 mM Tris-HCl, 0.1 mM EDTA, pH 8.1. Dialyzed virus was made 10% pyridine and incubated at room temperature for 1 h. The pyridine-virus suspension was then layered on a 15-40% sucrose gradient and centrifuged for 3 h at 24,000 rpm in a SW27 rotor at 10°C. Gradients were fractionated through a hole punctured in the bottom of the tube and fractions were read for ^{32}P by Cherenkov radiation or for A_{260} in a

Cary 14 spectrophotometer. The peak of pyridine-released virus cores sedimented to the bottom third of the gradient. Peak fractions were pooled and dialyzed against 5 mM Tris-HCl, 0.1 mM EDTA, pH 8.1.

c) Electron Microscopy

Cornerless virions and pyridine cores were applied directly to parlodian, carbon-coated grids and blotted dry. A drop of uranyl acetate was then placed on the grid for 1 min and washed off with several drops of water and blotted dry. Pyridine cores were further rotary shadowed at 30° with platinum. Samples were observed in a Philips 300 electron microscope operated at 60 kv.

d) Salt Precipitation Assay

Aliquots (0.1 ml) of ^{32}P-labeled pyridine cores in 5 mM Tris-HCl, 0.1 mM EDTA, pH 8.1, containing about 10 μg of DNA per ml were mixed with an equal volume of salt solution to give the final desired concentration. Samples were rapidly mixed and held on ice for 30 min, centrifuged for 30 min at 5000 rpm in a Sorvall HB-4 rotor, and 0.05 ml of the supernatant was removed and counted. The percent soluble was calculated with respect to a solution of cores to which only buffer was added. The centrifugation force applied to the solution was sufficient to clear molecules with a sedimentation coefficient greater than 1000 S.

e) Circular Dichroism and Flourescence Anisotropy

Circular dichroism was measured on a Jasco Model J-40 CD recorder at 20°C. Spectra of pyridine cores are reported as $\Delta\varepsilon$ in units of $1.\,cm^{-1}\ mol^{-1}$ of DNA phosphate assuming a molecular extinction coefficient of 6800 (Felsenfeld and Hirschman, 1965). Spectra of protein VII are reported as $\Delta\varepsilon$ in units of $1.\,cm^{-1}\ mol^{-1}$ of residue. Concentrations of protein VII were estimated spectrophotometrically using the amino acid compositions of Sung et al. (1977) and the molecular extinction coefficients of Edelhoch (1967) for tyrosine (4) and tryptophan (2) to calculate a molar extinction coefficient for protein VII of $275.5 = 17 \times 10^3\ 1.\,cm^{-1}\ mol^{-1}$. CD spectra of protein VII were analyzed for α-helix, β-sheet, and random coil by the method of Baker and Isenberg (1976) using the spectrum of protein VII in 0.01 N HCl as the random coil reference spectrum.

Fluorescence anisotropy $r = (I_{\parallel}-I_{\perp})/(I_{\parallel}+2I_{\perp})$ was measured on a computer controlled polarization spectrometer (Ayres et al., 1974). Measurements were made at 20°C. Samples were excited at 295 nm and emission was measured at 340 nm. At these wavelengths only tryptophan is excited, eliminating the complication of depolarization by tyrosine to tryptophan energy transfer.

f) Nuclease Digestion of Virus Cores

Cornerless virions or pyridine cores at about 10 μg DNA/ml were adjusted to 0.5 mM Ca^{++}, 0.5 mM Mg^{++} and digested with 5 units/ml staph nuclease (Worthington) or 5 units/ml DNase I (Worthington). DNA concentration was measured spectrophotometrically on a sample of virus or cores lysed in 0.5% SDS. Acid solubility was measured by spotting aliquots of the staph nuclease digestion mixture on polyethyleneimine (PEI) cellulose (Merck) strips which were pre-treated with a drop of 0.1 M EDTA to stop the digestion. Thin-layer PEI cellulose sheets containing a complete time course of digestion were developed by ascending chromatography in 3.5% perchloric acid. Under these conditions acid soluble nucleotides run with the solvent front. After the front reached the top of the plate it was dried and the strips were cut into top and bottom halves, counted in PPO, POPOP, toluene fluor, and the ratio of counts in the top half (acid soluble) to the total or top and bottom was used to calculate the percent acid soluble. This technique was found to give the same results as centrifugation of TCA precipitated digests but was quicker and more reproducible for the low concentrations and small volumes employed in the digestion of cornerless virus or pyridine cores. Since DNase I limit digests of free DNA contain polynucleotides up to n=10, the PEI cellulose assay underestimates the percent acid soluble and thus centrifugation of digest aliquots made 10% TCA was used to follow DNase I digests.

Digestion samples for electrophoretic analysis were stopped with 0.1 vol. of M EDTA, 1.6 M NaCl, 10 mg/ml pronase, 22% sarkosyl. These samples were incubated at 37 C for 2 h, extracted once with phenol, isoamyl alcohol, chloroform (25:1:24), twice with isoamyl alcohol, chloroform (1:24), ethanol precipitated with three volumes of 95% ethanol, washed with 70% ethanol, and air dried. Samples for electrophoretic analysis were resuspended in 0.1 x E buffer (Loening, 1967) for non-denaturing gels or in buffered 98% formamide for denaturing gels (Maniatis et al., 1975). Non-denaturing acrylamide gels were prepared as previously described (Lohr et al., 1977a).

Agarose gels were prepared by melting agarose (BioRad) in E buffer. Denaturing gels were prepared according to Staynov et al. as modified by Lohr et al. (1977). ^{32}P-labeled digestion intermediates were imaged by autoradiography of wet or dry gels by using Kodak R X-ray film and Kodak intensifying screens. Films were scanned on an Ortec 4310 densitometer. Sizes of nuclease digestion intermediates were determined by coelectrophoresis of Hae III restriction endonuclease fragments of bacteriophage PM2. The sizes of these fragments are given in the figure legends as well as in Appendix I, section m).

g) Isolation and Digestion of Nuclei

Cells were pelleted at 500 x g for 5 min at 37°C. The cell pellet was resuspended at 5×10^7 cells/ml in 5°C nuclear isolation buffer containing 0.25 M sucrose, 1 mM Tris-HCl (pH 7.5), 3 mM $CaCl_2$, 0.5% NP-40. The lysis buffer was adjusted immediately prior to use to 1 mM PMSF with a stock dissolved in isopropanol. Cells in nuclear isolation buffer were held on ice for 10 min and were then pelleted at 500 x g for 5 min. After one further wash in nuclear isolation buffer the nuclei were washed in once in digestion buffer containing 0.25 M sucrose, 1 mM Tris-HCl (pH 7.5), 0.3 mM $CaCl_2$, 0.1 mM PMSF. Nuclei were suspended in 37°C digestion buffer at 30 μg/ml DNA. Staph nuclease digestion was carried out directly with 100 units/ml or $MgCl_2$ was added to 0.1 mM and DNase I digestion was carried out at 125 units/ml.

h) DNA Purification and Gel Electrophoresis

Nuclease digestion was stopped by removing an aliquot from the digestion solution and placing it in ice cold 0.1 M EDTA (pH 7.1), to a final concentration of 10 mM. Samples were first treated with 0.2 volume of 5 mg/ml pancreatic RNase (Worthington), 5000 units/ml RNase T1 (Worthington) for 15 min at 37°C. The solution was then made 1.0 mg/ml pronase, 0.5% SDS, 0.3 M Na Acetate and incubated for 1 h at 37°C. DNA was extracted as described in section (f). Gels were poured as described in section (f) with voltages and running times described in figure legends. Gels were stained with 0.5 μg/ml ethidium bromide, illuminated on a black-ray light box (UVProducts) and photographed using Polaroid type 55 film. When type 55 negatives and autoradiographs of the same gel were compared

they were both scanned on a Joyce-Loebl microdensitometer. Scans were digitized and replotted to scale by using a Hewlett-Packard 9821A calculator.

i) Purification and Crosslinking of Protein VII

Two procedures were used to isolate the arginine-rich major core protein, protein VII. Both procedures started with twice CsCl banded virions. Main band virus was dialyzed against 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.1. Virus, at a concentration of 1 mg/ml DNA was lysed by addition of an equal volume of 8 M guanidine-HCl at 20°C. After the virus solution became clear all procedures were performed at 5°C. Acid soluble virus proteins were extracted by adding an equal volume of 0.5 M HCl, stirring for 1 h, and removing the precipitate by centrifugation at 2000 x g for 30 min. The supernatant was then dialyzed for 8 h against 2 changes of 100 volumes of 0.25 M HCl. The precipitate which formed during this time was removed by centrifugation at 2000 x g for 30 min. The supernatant at this point contained essentially all of virus proteins IV, V, VI, and VII as detected by SDS polyacrylamide gel electrophoresis. Two approaches were used to separate protein VII from this mixture. The first approach utilized the preparative gel electrophoresis system described by Mardian and Isenberg (1978). This technique consists of separating the proteins in an acid-urea polyacrylamide gel (Panyim and Chalkey, 1969), followed by electroelution of individual bands. The eluted protein was desalted on a Sephadex G-75 column in 0.01 N HCl. Purified protein VII was then dialzyed exhaustively against twice distilled water, lyophilized, and stored at -20°C.

The second procedure we used to isolate protein VII is based on the histone purification scheme of Johns (1976). Acid-soluble virus proteins in 0.25 M HCl were dialyzed vs. 70% ethanol, 0.25 M HCl overnight at 5°C. The precipitate which formed was removed by centrigugation at 40,000 rpm in a 50 Ti rotor for 1 h. The pellet contained proteins IV, V, and VI. Protein VII remained in the supernatant in essentially pure form as determined by SDS polyacrylamide gel electrophoresis. The supernatant was dialyzed exhaustively against twice distilled water, lyophilized, and stored at -20°C.

Crosslinking of protein VII was carried out with gluteraldehyde (Sperling & Bustin, 1975) or dimethyl suberimidate (Davies & Stark, 1970). After re-folding the protein in 0.04 M $NaPO_4$, crosslinking was performed as described in the figure legends. Crosslinked

proteins were separated by SDS gel electrophoresis according to Laemmli (1970).

j) Reconstitution Procedures

Protein VII-DNA complexes were prepared by the following procedure. Protein VII was first brought to 40 mM sodium phosphate pH 7.0. Protein and DNA were then made 50 μg/ml each in 2 M NaCl, 5 mM Tris HCl, 0.1 mM EDTA, pH 7.5 with or without 5 M urea mixed. The mixed solution was dialyzed slowly (48 h) against a NaCl gradient (2.0 M to 0.6 M) at 5°C. When the dialysate reached 0.6 M NaCl the remaining salt and urea were removed by dialysis against 5 mM Tris HCl, 0.1 mM EDTA, pH7.5 at 5°C.

k) Sucrose Gradient Analysis of Chromatin Particles

Digestion of nuclei was stopped by adding EDTA to 10 mM and samples were immediately frozen in liquid nitrogen. Samples were thawed at room temperature and dialyzed at 5°C for 4 hrs in 10 mM Tris (pH 7.5), 0.7 mM EDTA, 0.1 mM phenylmethylsulfenyl fluoride (PMSF). After dialysis the samples were cleared of nuclei by centrifugation at 10,000 x g for 20 min and applied to 5% to 20% sucrose gradients made in dialysis buffer. Gradients were spun at 27,000 rpm in a SW 27 rotor for 24 hrs at 4°C. Samples were collected through a hole in the bottom of the tube and read for ^{32}P Ad DNA (Cerenkov radiation) or cellular DNA (absorbance at 260 nm).

Protein VII-DNA complexes formed with purified HeLa nucleosome DNA were separated on linear 5-20% sucrose gradients. Sucrose solutions were made in 5 mM Tris HCl, 0.1 mM EDTA, pH 7.5. Gradients were run in a SW27 rotor at 24K for 24 h at 5°C.

l) Blotting and Hybridization

Nuclei from cells infected with unlabeled virus were digested as in section (b). The resulting purified DNA fragments were separated electrophoretically on a composite acrylamide or agarose gel as previously described (Lohr et al., 1977a), but with the following modifications: the agarose composition was raised to 1%. The

acrylamide concentration was raised to 3% and diallyltarardiimide (DATD, BioRad) was substituted for bis acrylamide and used at a final concentration of 0.3%. Following electrophoresis the gel was stained in 0.5 μg/ml ethidium bromide and photographed as in section (b). The acrylamide matrix was then broken down by soaking the gel in 2% sodium periodate at room temperature for 30 min (Righetti & Drysdale, 1974). The gel was then processed for blotting as described by Southern (1975), except that 1 M ammonium acetate was used in the blotting reservoir and to soak the nitrocellulose. Transfer in my system approached the efficiency of an agarose gel but afforded the resolution of an acrylamide gel. After transfer the filter was air dried for 4 hr and then baked in vacuo at 60°C for 1 hr. Hybridization was carried out in 50% formamide, 0.6 M NaCl, 0.2 M Tris HCl (pH 8), 0.02 M EDTA (Dawid, 1976). Purified ^{32}P-labeled adenovirus DNA with a specific activity of 10^5 cpm/μg was sonicated in 10 mM Tris (pH 7.5) at 100 μg/ml for 1 minute at full power on a Kontes sonicator. This DNA was then boiled for 3 min and quenched on ice. Denatured sonicated DNA (10 μg) was added to 10 ml of hybridization buffer containing the filter (5 x 15 cm) and was incubated in a rotating bottle for 24 hr at 48°C. After hybridization the filter was washed two times for 15 min in 50% formamide, 2 x SSC at room temperature followed by two washes for 15 min each in 2 x SSC at room temperature. The filters were then air dried and autoradiographed.

m) DNA and Protein Size Markers

DNA fragments from bacteriophage PM2 DNA cleaved with restriction enzyme Hae III were used as size markers for electrophoretically separated DNA. Unlabeled DNA was used for gels imaged by ethidium bromide staining. Markers for gels which were dried and autoradiographed were produced by end-labeling the restriction fragments with ^{32}P using γ^{32}P-ATP and polynucleotide kinase (McParland et al., 1977). The sizes for the DNA fragments reported by Kovacic & Van Holde (1977) were revised by running the PM2 Hae III fragments on slab gels in tracks adjacent to sequenced markers. The Hae III fragments of 0X175 RF DNA (New England Biolabs) were used as reference sizes. By running the PM2 Hae III fragments against the sequenced markers on 4 different porosity acrylamide gels (10%, 6%, 4.5%, and 3.9%) the following sizes were calculated: A, 1772; B, 1669; C, 1416; D, 944; E, 867; F, 698; G, 635; H, 525; I, 336; J, 301; K, 272; L, 166; M, 152; N, 121; O, 97; P, 51; Q, 48.

Molecular weights of adenovirus type 2 proteins have been compiled by Weber (1976); II, 120,000; III, 85,000; IIIa, 66,000; IV, 62,000; V, 48,500; VI, 22,000; VII, 18,500; VIII, 13,000; IX, 12,000.

APPENDIX II

Adenovirus Type 2 Uncoating Mutant

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Adenovirus 2 temperature-sensitive mutant ts1 does not process virus precursor proteins P-VI, P-VII, or Vb at the restrictive temperature although a normal yield of non-infectious virus particles is obtained (1). These virions contain the uncleaved precursor proteins but lack virus structural proteins VIII and X-XII. The mutant virions have been shown to adsorb efficiently, thus indicating that the blocked infection step is subsequent to adsorption (2). Weber has argued (1), on the basis of the infectivity of free adenovirus DNA, that the mutant ts1 virions are defective in uncoating. In this report we provide evidence to support this claim.

The early stages of adenovirus infection can be summarized as follows: i) attachment to the cell membrane, ii) penetration, iii) transport to the nucleus, iv) uncoating at the nuclear membrane. Nucleases have been used to probe the structure of virions at these stages of infection. Lawrence and Ginsberg (3) have shown that whereas purified adenovirus is not susceptible to pancreatic DNase (DNase I), intracellular virus is rendered 80% digestible within 1 h after infection. Sussenbach (4) has shown that the initial uncoating product has lost only a small fraction of its protein and Lonberg-Holm and Philipson (5) have demonstrated the cytoplasmic location of this partially uncoated product. Lonberg-Holm and Philipson further show that about half of the cytoplasmic virus is transported rapidly to the nucleus where uncoating is completed. Electron microscopy of infected cells has supported the nuclear location of uncoating (6, 7, 8).

Both mutant and WT were propagated in HeLa cells. WT virus was grown at 37°C. Infectious ts1 was grown at 33°C while non-infectious mutant virions were grown at 39°C. Analysis of virion proteins by SDS gel electrophoresis verified the phenotype of ts1. Virus was labeled with $^{32}P_i$ (NEN) in phosphate-free Joklik-modified Eagle minimal essential medium (Gibco, F13) supplemented with 2% dialyzed calf serum (Gibco). Label was added to 10 μCi/ml at 6 h after infection and virus was purified after 48 h according to Doerfler (9). For studies following the fate of infecting virus, HeLa cells were infected with twice CsCl banded ^{32}P-labeled virus at a multiplicity of 5×10^3 particles/cell. Virus concentration was determined by specific activity. Adsorption was carried out in suspension culture at 5×10^6 cells/ml in Joklik-modified MEM. After 30 min. at 37°C the cells were diluted 10-fold with 37°C Joklik-modified MEM supplemented with 7% fetal calf serum (Sterile Systems). Cells were harvested 9 h after infection and nuclei were isolated, nuclease digested, and DNA extracted as previously described (10). Agarose gels were prepared by melting agarose (BioRad) in E-Buffer (11) in a microwave oven followed by stirring at 90°C in a water bath for 1 h.

Denaturing gels were made according to Maniatis et al. (12).

Figure 1 shows an EcoR1 restriction endonuclease digest of total nuclear DNA extracted from ^{32}P-labeled WT infected cells 9 h after infection. The autoradiograph of this gel shows that the input ^{32}P is contained in the 6 fragments characteristic of adenovirus 2 DNA (13). This result shows that the labeled input virus DNA has not been degraded or integrated into host DNA.

When HeLa cells are infected with ^{32}P-labeled ts1 virions grown at the restrictive temperature we observe that the first three stages of infection occur with only a slight variation from WT. While we typically observe 40-50% of the attached virus in the nuclear fraction by 9 h after infection, ts1 infection results in only about 30% of the cell associated virus in the nuclear fraction at this time. Digestion of isolated nuclei, 9 h after infection, with either DNase I or staph nuclease, reveals a striking difference between ts1 39C and WT (figure 2). Both nucleases digest ^{32}P-labeled WT intranuclear adenovirus DNA to acid soluble material. In contrast, intranuclear ^{32}P-labeled ts1 DNA is not digested to acid soluble products. This observation is consistent with a block in infection at the level of uncoating.

The failure to observe acid soluble material does not rigorously show that DNA has not been cleaved. To see if any input ts1 DNA had been digested we separated the purified digestion products on gels. Figure 3A shows the pattern of digestion intermediates of staph nuclease digested nuclei from ts1 and WT infected cells separated on a nondenaturing agarose gel. From the autoradiographs it is clear that the input ts1 DNA has not been digested. While some WT input virus DNA has not been digested, a significant fraction appears as smaller digestion intermediates. The unit-length adenovirus DNA which remains at the top of the gel is apparently due to intact virus because it does not diminish with increasing digestion.

We used DNase I digestion to see if input ts1 virus could be nicked. Figure 3B shows the results of DNase I digestion of nuclei isolated from ts1 and WT infected cells. Purified digestion intermediate DNA was separated on a denaturing gel. The input adenovirus ts1 DNA remains at the top of the gel indicating that it has not been nicked. This result, together with the results from staph digestion experiments, shows that ts1 39C virions are not uncoated in vivo to form the nuclease susceptible structures characteristic of WT virus.

The inaccessibility of viral DNA to nuclease probes could be due to either the failure of the cell to remove the virus capsid, or

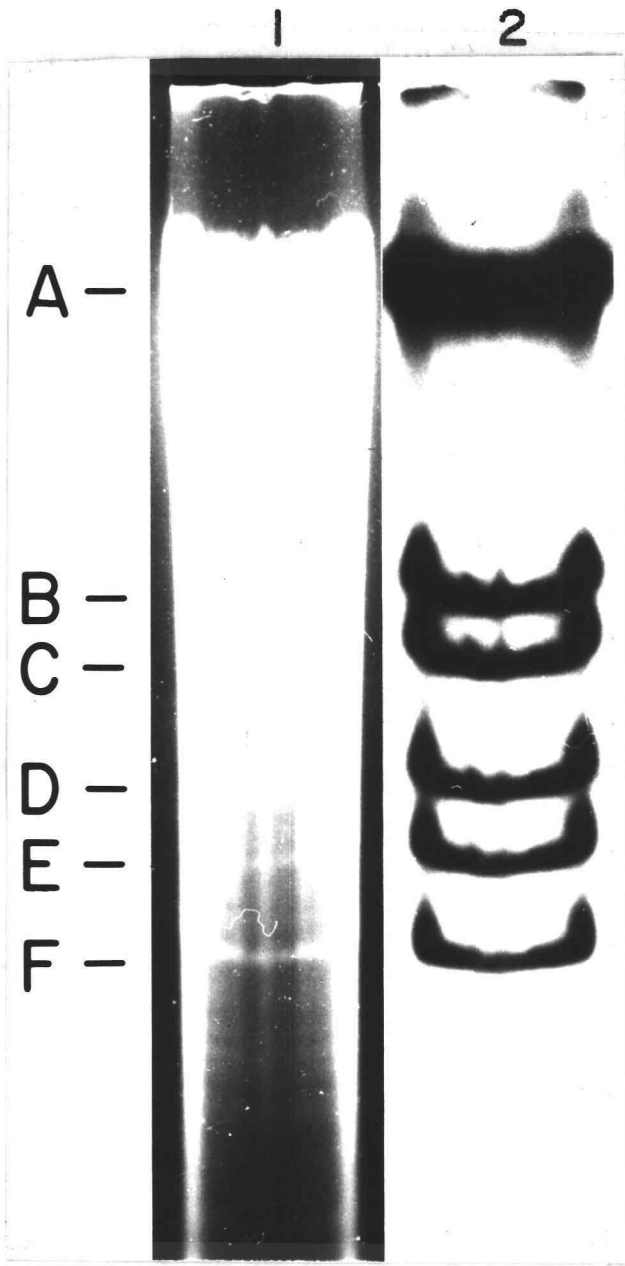


Figure 1. Electrophoretic analysis of EcoR1 digested total nuclear DNA from ^{32}P WTAd2 infected cells. DNA was extracted from nuclei isolated from cells harvested 9 h post infection, digested with EcoR1 restriction endonuclease, applied to a 1.4% agarose gel made in E buffer and electrophoresed at 2 volts/cm for 12 h. The gel was then stained in 0.5 μg/ml ethidium bromide for 1 h, photographed under long wavelength u.v. illumination, dried, and autoradiographed.
1) Ethidium bromide stained gel (total nuclear DNA), 2) autoradiograph (input adenovirus DNA), 3) EcoR1 digested purified Ad2 DNA.

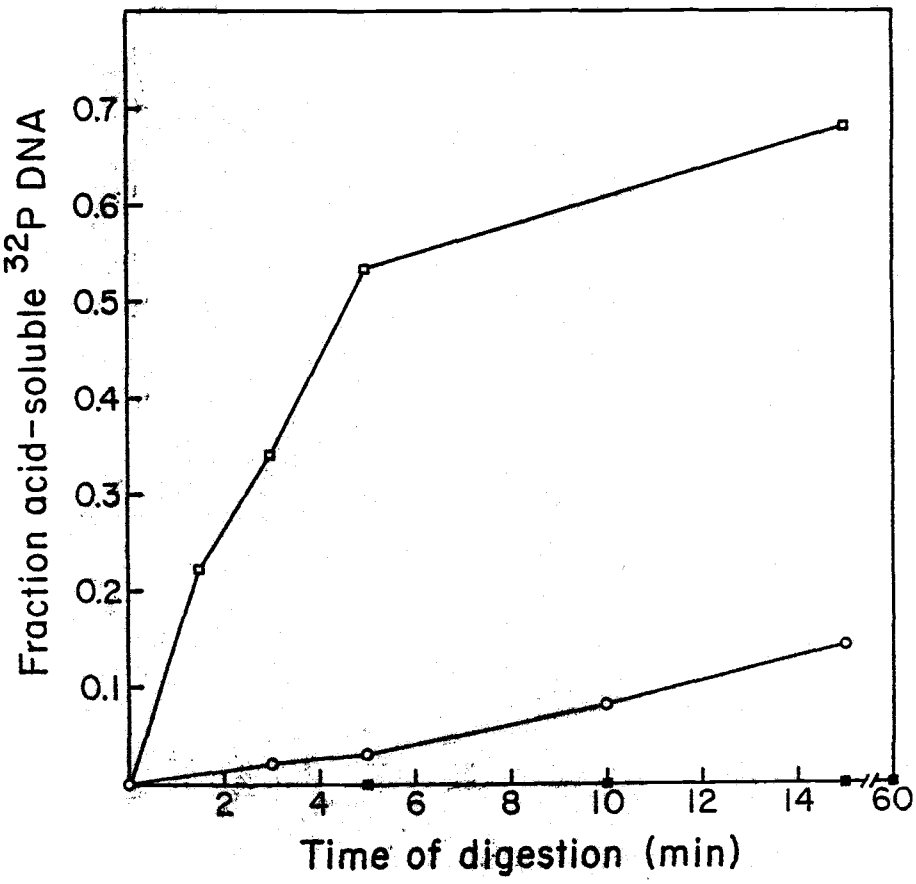
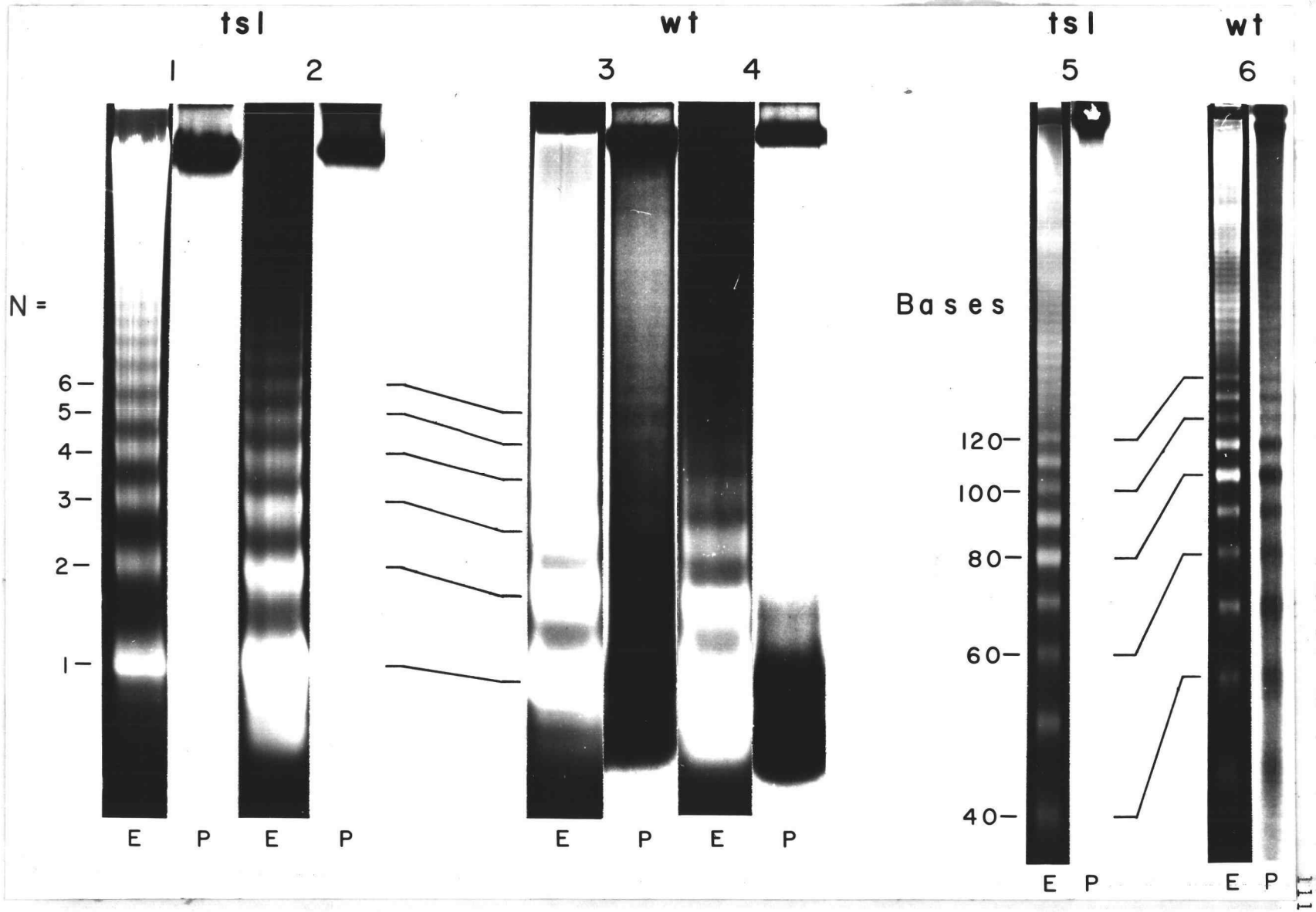


Figure 2. Nuclease digestion of infected cell nuclei. Nuclei were isolated 9 h post infection from cells infected with ^{32}P labeled WT or ts1 39C virus. Nuclei at 300 μg DNA/ml were digested at 37°C with 100 units/ml DNase I (Worthington) or 125 units/ml staph nuclease (Worthington). Samples for acid solubility measurements were removed at various times and the reaction stopped by making the sample 20 mM EDTA, 1% SDS. Acid soluble ^{32}P was measured by precipitating samples, on ice, with 1/10 volume of 100% TCA, followed by centrifugation. DNase I digestion of WT infected nuclei (□). Staph nuclease digestion of WT infected nuclei (○). DNase I digestion of ts1 infected nuclei (■). Staph nuclease digestion of ts1 infected nuclei (●).

Figure 3. Electrophoretic separation of DNA from nuclease digested infected cell nuclei. Nuclei were digested as described in Figure legend 2. Digestion samples for electrophoretic analysis were stopped with EDTA, RNase digested, pronase digested, phenol extracted and ethanol precipitated (10).
(A) Staph nuclease digested samples were resuspended in 0.1 X E buffer and electrophoresed at 2 volts/cm in a 2% agarose gel for 12 h. The gel was stained in ethidium bromide, photographed, dried, and autoradiographed. WT digested for (1) 5 min. (2) 15 min. ts1 digested for (3) 5 min, (4) 15 min. (B) DNase I digested samples were resuspended in buffered 98% formamide and electrophoresed on an 8% acrylamide, 7 M urea gel at 120 m amps for 5 h. The gel was stained for 15 min. in ethidium bromide, photographed, dried, and autoradiographed. WT 1 min digestion (1), ts1 1 min. digestion (2).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greater protection afforded the DNA by virus core components. Since the different polypeptide pattern of ts1 is confined to core components, we tested the nuclease resistance of ts1 pyridine cores. Staph nuclease digested ts1 pyridine cores to at least 80% acid solubility (results not shown). This result shows that ts1 pyridine cores are more labile than WT to sarkosyl and high salt (14). Thus, it appears that the failure of ts1 39C virions to uncoat is caused by the inability of the cell to remove the virus capsid.

Virus proteins VI, VII, VIII, X, XI, and XII, missing in ts1 39C virions, do not label when intact virus is enzymatically radioiodinated (15). This result demonstrates their internal location. Proteins VI and VIII are, however, associated with the hexon, or major capsid protein (15). How does the altered core structure of the ts1 39C mutant virion cause the capsid to become refractory to removal? The uncoating defect of the ts1 virion suggests a role for processing of the internal virus components in the adenovirus infectious cycle. Clearly, the structural assembly of adenovirus can take place without cleavage of the internal components. If the ts1 virion is considered as an assembly intermediate, then the final cleavage must be accomplished by a virion component. Indeed, Ad2 ts1 fails to complement Ad5 ts2 (16), a mutant whose lesion has been mapped in the hexon gene. Cleavage of the internal components; the final maturation event, might then be imagined as cocking the virion into readiness for infection. Since the cleavage takes place internally there must be a conformational change which is manifested externally. In ts1 39C virions this change has not occurred and thus, early during infection, the "trigger" is not recognized by the cell and uncoating does not occur. What is the trigger mechanism that leads to virus uncoating? While we have no answer to this question, several aspects of the uncoating process have been described. The process if reversibly inhibited by low temperature but is uninhibited by puromycin indicating the involvement of a pre-existing enzyme(s), host or viral (3). Inhibition of nuclear membrane ATPase blocks uncoating as monitored by electron miroscopy (17). We are currently looking for modification or cleavage of input virus proteins early during infection in order to discover the mechanism which triggers adenovirus uncoating.

We have also observed that the digestion intermediates of intranuclear ^{32}P-labeled WT adenovirus show some interesting similarities to their cellular counterparts. These similarities suggest that adenovirus DNA may be complexed with cellular histones early during infection and is the subject of a separate paper (Corden et al., in preparation).

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