

Adenovirus Chromatin Structure at Different Stages of Infection

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We investigated the structure of adenovirus deoxyribonucleic acid (DNA)-protein complexes in nuclei of infected cells by using micrococcal nuclease. Parental (infecting) DNA was digested into multimers which had a unit fragment size that was indistinguishable from the size of the nucleosomal repeat of cellular chromatin. This pattern was maintained in parental DNA throughout infection. Similar repeating units were detected in hamster cells that were nonpermissive for human adenovirus and in cells pretreated with *n*-butyrate. Late in infection, the pattern of digestion of viral DNA was determined by two different experimental approaches. Nuclear DNA was electrophoresed, blotted, and hybridized with labeled viral sequences; in this procedure all virus-specific DNA was detected. This technique revealed a diffuse protected band of viral DNA that was smaller than 160 base pairs, but no discrete multimers. All regions of the genome were represented in the protected DNA. To examine the nuclease protection of newly replicated viral DNA, infected cells were labeled with [³H]thymidine after blocking of cellular DNA synthesis but not viral DNA synthesis. With this procedure we identified a repeating unit which was distinctly different from the cellular nucleosomal repeat. We found broad bands with midpoints at 200, 400, and 600 base pairs, as well as the limit digest material revealed by blotting. High-resolution acrylamide gel electrophoresis revealed that the viral species comprised a series of closely spaced bands ranging in size from less than 30 to 250 base pairs.

Histones, the ubiquitous structural proteins of eucaryotic chromatin, are complexed with deoxyribonucleic acid (DNA) in repeating units called nucleosomes. The repeating element of chromatin structure has been characterized by electron microscopy and by digestion with micrococcal nuclease, which cuts between nucleosomes, yielding a multimeric array of DNA fragments (19, 29).

A variety of mammalian adenoviruses have been isolated and studied as simple model systems for eucaryotic gene expression. Upon adsorption and penetration of adenovirus into a cell, the viral DNA becomes uncoated in the nucleus, where viral transcription and replication are carried out by cellular polymerases (1, 6, 46). In contrast to other DNA viruses which replicate in the nucleus, such as simian virus 40 (18), adenoviruses do not use cellular histones to package DNA into virus particles; instead, virally coded proteins are complexed with DNA in the virus core (17). Polypeptide VII, the major

core protein, is a small arginine-rich protein that has an amino-terminal basic domain similar to that of histones (34, 43). Two minor core proteins (polypeptides V and X) are also basic. The mass of the core proteins in the virion is equal to the mass of the viral DNA, which is a linear molecular with a molecular weight of 25×10^6 (48).

Viral cores have been isolated by a variety of procedures (7, 37), and their physical properties have been investigated (24). Although there is considerable disagreement in the literature about solubility and resistance to degradation of cores, it has been generally established that protein VII is tightly bound to the DNA (5). It has been proposed that protein VII is distributed evenly along the DNA (7) or, alternatively, that clusters of six molecules of protein VII and one molecule of protein V are closely complexed with about 200 base pairs of DNA (10) in the core. The intracellular forms of adenoviral chromatin have been characterized even less well. Sedimentation studies have indicated that when infecting viral DNA-protein complexes penetrate a nucleus, the final product of uncoating

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resembles naked DNA (32). However, micrococcal nuclease digestion of this parental chromatin has revealed a nucleosome-like repeating pattern, which is thought to reflect an association between histone and input DNA (38, 44).

Protein VII is synthesized in the form of a 20,000-dalton precursor (pVII), which binds to viral DNA and then enters the viral capsid before proteolytic processing of the 20 N-terminal amino acids to form mature protein VII (3, 17, 31). Late in infection, pVII is synthesized at high levels concomitantly with viral DNA synthesis. Cellular DNA synthesis and histone synthesis have ceased at this stage of infection (28). Electron microscopy of infected cell nuclei has revealed smooth fibers which are the length of adenovirus DNA and have no evidence of a nucleosomal repeat (27; O. L. Miller and L. D. Hodge, *J. Cell Biol.* 67:284a, 1975). It seems likely that pVII bound to DNA is the major structural protein in late transcription complexes.

In this report we describe the digestion of adenoviral chromatin by micrococcal nuclease in isolated nuclei at different stages of infection. We examined nonpermissive and permissive infections and the effect of *n*-butyrate on early viral chromatin structure. Quantitation and comparison of different experimental protocols were stressed in this study. We also describe a novel repeating unit of viral DNA labeled late in infection.

MATERIALS AND METHODS

Cells and viruses. HeLa cells and BSC-1 cells (13, 14) were cultured in monolayers in Dulbecco modified Eagle medium supplemented with 5% calf serum. Baby hamster kidney cells (BHK-21) were obtained from Edward Penhoet. Temperature-sensitive mutants of adenovirus 5 (Ad5) (H5ts1, H5ts18, and H5ts19) were generous gifts from Jim Williams.

Butyrate-treated cells. Sodium butyrate (7.5 mM; J. T. Baker) was added to tissue culture medium 20 to 24 h before virus infection. A 0.5 M stock solution of sodium butyrate prepared in Dulbecco buffer was stable for several months at 4°C. In each experiment, [³H]thymidine incorporation was measured on a test plate as described previously (12) to confirm that cellular DNA synthesis was shut off before infection.

Labeled virus particles: preparation and infection. HeLa cells were infected with 10 to 50 plaque-forming units (PFU) of Ad3 or Ad5 per cell. [³H]-thymidine (20 µCi/ml) was added 20 h after infection. Cells were harvested 48 h after infection, and virus was purified by Freon extraction and centrifugation in cesium chloride, as described previously (11). Virus bands were dialyzed into phosphate-buffered saline, and the concentration of virus particles was estimated by measuring the optical density at 280 nm (11). The number of PFU was calculated from the data of Green et al. (22). Plaque titration of the labeled particles in selected preparations confirmed these estimates

within a factor of 3; the number of PFU per milliliter was sometimes two- to threefold lower than the estimates, but it was never higher.

Virus prepared in this way had a specific activity of about 5×10^{-5} cpm per virus particle (or 1.2×10^6 cpm/µg of DNA). A total of 10^7 cells were infected in a suspension or on plates at a multiplicity of infection of 500 to 2,000 particles per cell; this resulted in 5×10^5 input counts in each preparation. A multiplicity of infection of 1,000 particles represented 100 and 50 PFU/cell for Ad3 and Ad5, respectively. For baby hamster kidney cells, 10- to 20-fold higher multiplicities of infection were used in order to obtain enough labeled DNA in nuclei to detect bands on gels.

Labeling of DNA late in infection. Infected cell monolayers were labeled at different times by adding 10 µCi of [³H]thymidine (60 Ci/mmol; Schwarz/Mann) per ml to the medium. When an unlabeled chase was performed, cells were washed twice with 5 ml of unlabeled medium and then incubated in unlabeled medium.

Cell harvest, nucleus preparation, and micrococcal nuclease digestion. At different times after infection, cells that were infected with [³H]thymidine-labeled virions or were labeled late in infection were harvested by scraping plates after they were washed with phosphate-buffered saline. Nuclei were prepared by the method of Honda et al. (25), as described previously (14). A sample was taken before nuclease digestion to measure the total counts and to confirm that all counts were in viral sequences.

Nuclei were suspended in sucrose buffer [0.05 M tris(hydroxymethyl)aminomethane, pH 7.4, 0.025 M KCl, 0.001 M MgCl₂, 0.25 M sucrose, 0.015 M β-mercaptoethanol] at a concentration of approximately 10^7 nuclei per ml; 1 mM CaCl₂ was added, the suspensions were prewarmed at 37°C for 2 min, and micrococcal nuclease (Sigma Chemical Co.) was added to a final concentration of 0.6 U/ml. (One Sigma unit was equal to 100 Worthington units of micrococcal nuclease; the values given in the text and figures are Worthington units, as this system is used more widely in chromatin literature.) Digestion was stopped by pipetting samples into 0.2 volume of 10 mM ethylenediaminetetraacetate-2% sodium dodecyl sulfate and blending this preparation with a Vortex mixer. After twofold dilution with 10 mM tris(hydroxymethyl)aminomethane (pH 7.9), pronase was added (final concentration, 1 mg/ml) for 1 h at 37°C. The pronase-treated sample was extracted twice with phenol and once with chloroform and then precipitated with 2.5 volumes of cold 90% ethanol.

In each experiment, DNA was extracted from a sample of nuclei taken before nuclease was added. Gel electrophoresis and restriction analysis confirmed that the label was in viral sequences which were the size of full-length Ad5 DNA.

Gel electrophoresis, blotting, and fluorography. Micrococcal nuclease-digested DNA was electrophoresed on 10-cm 1.4% agarose gels at 50 V for 4 h at 4°C or for 3 h at 25°C. Buffers, staining, and photography were as described previously (14). DNA was blotted onto either nitrocellulose paper (42) or diazotized paper (2) by the method of Wahl et al. (45). To detect [³H]thymidine counts by autoradiography, gels

were dehydrated by soaking in methanol (twice the gel volume three times for 45 min each) and then soaked in 2 volumes of 5% PPO (2,5-diphenylazole; Packard) in methanol for 1 to 1.5 h. The gel was then rinsed in water and soaked in water containing 6% glycerol to precipitate PPO and prevent cracking when the gels were dried. Gels were dried under a vacuum and then autoradiographed with preflashed Kodak X-R5 film at -70°C ; a DuPont Cronex Intensifying Screen was used for enhancement when necessary.

For quantitation and size determinations, autoradiographs were scanned with a Joyce-Loebl densitometer. Areas under peaks were measured with a Numonics graphics calculator.

To measure the sizes of small DNA fragments, samples were electrophoresed at 4°C through 10% acrylamide gels (13 by 17 cm) in tris(hydroxymethyl)aminomethane borate-ethylenediaminetetraacetate buffer [0.09 M tris(hydroxymethyl)aminomethane borate, pH 8.3, 2.5 mM ethylenediaminetetraacetate], as described by Maniatis et al. (30). DNA patterns were visualized by fluorography of gels after treatment with EN³HANCE (New England Nuclear Corp.). Fragments of a plasmid pBR322 derivative generated by endonuclease *Msp*I, which ranged in size from 9 to 1,000 base pairs, served as size markers (a gift from R. Saiki). All of these fragments that were smaller than 150 base pairs migrated in a single diffuse band on 1.4% agarose gels.

RESULTS

Nucleosome-sized repeating unit in parental viral DNA early in infection. HeLa cells were infected with Ad5 or Ad3 labeled with [^3H]thymidine. At 3 h after infection, cells were harvested, and nuclei were isolated and treated with micrococcal nuclease for different lengths of time. Table 1 shows the fractions of the labeled viral DNAs which adsorbed to cells and penetrated to the nuclei. The DNAs purified from these nuclei were electrophoresed on 1.4% agarose gels. Total DNA was visualized by ultraviolet fluorescence after staining with ethidium bromide, and input viral DNA was visualized by fluorography of the gels. Figure 1 shows the results of such an experiment. The mock-in-

TABLE 1. Fraction of input [^3H]thymidine-labeled viral DNA that adsorbed to and penetrated cells

Expt	Virus	Cells	Fraction adsorbed to cells	Fraction in nuclei
1	Ad3	HeLa	0.4	0.3
	Ad5	HeLa	0.15	0.1
	Ad3	BHK-21	0.03	0.01
2	Ad3	HeLa	0.5	0.35
		HeLa (+ butyrate) ^a	0.46	0.27

^a Cells were treated with 7.5 mM sodium butyrate for 24 h before infection.

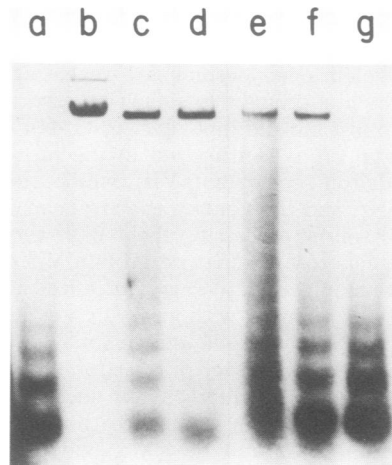


FIG. 1. Micrococcal nuclease digestion of parental [^3H]thymidine-labeled Ad5 DNA in HeLa cell nuclei. Cells were infected with ^3H -labeled Ad5 virions and harvested 3 h (lanes b through d) or 24 h (lanes e and f) after infection. Isolated nuclei were treated with micrococcal nuclease (60 U/ml) for the following times: lane b, no digestion; lanes c and e, 1.5 min; lane f, 5 min; lane d, 8 min. HeLa cell DNA from [^3H]thymidine-labeled mock-infected nuclei digested for 4 min was used as a marker (lanes a and g). The preparations were the same preparations used for Tables 1 and 2 experiment 1. Samples were electrophoresed through a 1.4% agarose gel for 4 h at 50 V and 4°C . The gel was dehydrated with methanol, impregnated with PPO, dried under a vacuum, and fluorographed for 10 days at -80°C with an intensifying screen.

fect samples in the fluorograph, which consisted of [^3H]thymidine-labeled DNA from uninfected nuclei, provided a size comparison. The sizes of the viral fragments were indistinguishable from the sizes of cellular sequences. The patterns produced by labeled cellular and parental viral DNAs electrophoresed into higher-resolution acrylamide gels were also indistinguishable (data not shown).

A high-molecular-weight fraction of the viral DNA associated with nuclei remained even after extensive digestion (Fig. 1, lane d). Samples were electrophoresed through 0.5% agarose with appropriate size markers to confirm that this resistant DNA was full-length viral DNA (data not shown). As the time of nuclease treatment was increased, this full-length fraction remained unaffected, whereas the nucleosome-sized pieces were degraded further (Table 2). Table 2 also shows that the amount of resistant DNA varied from preparation to preparation (Table 2, experiment 2). Apparently some preparations of labeled purified virus included particles which reached the nucleus but the viral DNA was not

uncoated and remained protected from nuclease digestion by viral capsid proteins.

We also investigated the protection of parental DNA from nuclease at late times after infection. Figure 1 shows that the same cell-like nucleosomal repeat was detected in HeLa cells 24 h after infection with [³H]thymidine-labeled Ad5 (Fig. 1, lanes f through h). Thus, it appeared that most if not all of the viral sequences which assumed a conformation yielding a cellular type of micrococcal nuclease repeat remained in that conformation throughout infection. The proportion of viral DNA which was protected fully from micrococcal nuclease was somewhat smaller at 24 h than at 3 h, but the difference was less than twofold (Table 2). Apparently some parental DNA resided in the nucleus in a nuclease-resistant form throughout infection.

Ad3 adsorbs to and penetrates hamster cells, and early viral gene products are synthesized; however, no viral DNA synthesis or late gene expression occurs (22a, 40). Figure 2c shows that the nucleosomal repeat was detected in BHK-21 cells infected with [³H]thymidine-labeled Ad3. The fraction of DNA that remained undigested after nuclease treatment was similar to the fraction in permissive cells (Table 2), although the proportion of infecting DNA that reached the nucleus was much smaller in nonpermissive cells (Table 1).

Early repeat in butyrate-treated cells. When HeLa cells are treated with *n*-butyrate for 24 h, cellular DNA synthesis and cell division cease (23). Histone synthesis also ceases, but

TABLE 2. Fraction of input viral DNA protected from micrococcal nuclease

Expt ^a	Virus	Cells	Length of digestion (min) ^b	Fraction precipitable ^c	Fraction full length ^d
1	Ad3	HeLa	2	0.7	0.1
			8	0.35	0.1
	Ad3	BHK-21	3	0.4	0.12
			8	0.3	0.09
	Ad5	HeLa	2	0.7	0.07
			8	0.3	0.09
Ad5	HeLa (24 h postinfection)	2	0.75	0.05	
		5	0.25	0.05	
2	Ad3	HeLa	3	0.6	0.5
		HeLa (+ butyrate)	3	0.6	0.4

^a Same experiments as in Table 1.

^b Reaction mixtures contained 60 U of micrococcal nuclease per ml and approximately 10⁷ nuclei per ml.

^c Fraction of starting material (labeled viral DNA in the preparation of isolated nuclei) which remained trichloroacetic acid precipitable after nuclease treatment.

^d These fractions were obtained by measuring the areas under curves in densitometer scans of fluorographs. They represent the fractions of total viral DNA in the nuclei which were not affected by nuclease treatment.

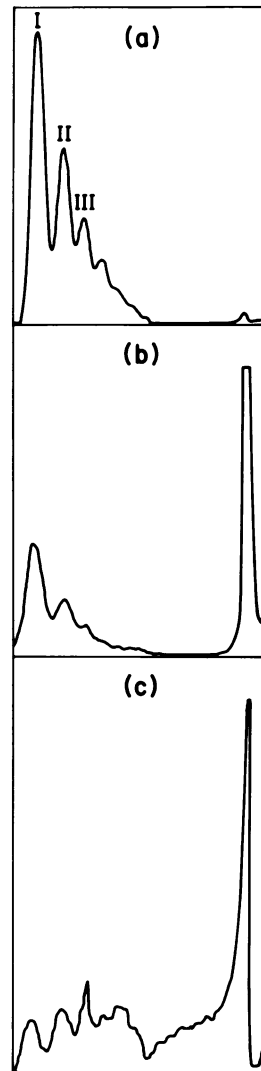


FIG. 2. Comparison of micrococcal nuclease digestion patterns of infecting viral DNA in BHK-21 cells and in HeLa cells with the cellular pattern. Nuclease digestion, electrophoresis, and fluorography were performed as described in the legend to Fig. 1. Tracings from adjacent lanes on a single gel were made with a Joyce-Loebl microdensitometer. The Ad3 (BHK-21) and Ad3 (HeLa) preparations were the same preparations used for Tables 1 and 2, experiment 1. A total of 10⁴ cpm of tritium was loaded per slot (10 µg of total DNA in the HeLa preparation and 30 µg in the BHK-21 preparation). (a) Uninfected HeLa cells labeled with [³H]thymidine. (b) HeLa cells infected with labeled Ad3. (c) BHK-21 cells infected with labeled Ad3.

cells remain viable and continue the synthesis of other proteins. Adenovirus infection of cells blocked in this way by butyrate proceeds nor-

mally, with no detectable stimulation of either cellular DNA or histone synthesis at any point (12). Therefore, we determined whether the nucleosomal repeat pattern (possibly a result of histones replacing core proteins on viral DNA) was generated in butyrate-treated cells. Figure 3 shows a typical example of such an experiment. A repeating unit of the same size as the cellular repeat was detected in butyrate-treated cells, but in four separate experiments this unit was less sharply defined than the repeating unit in untreated cells. A band of digested viral sequences which were smaller than the cellular monomer was also observed. This material, which appeared only in some preparations, did not necessarily represent a single size class of DNA; in 1.4% agarose gels, DNA less than 140 base pairs long accumulated in this region of the gel. The submonomer DNA may have represented the digestion of DNA which was uncoated to form virion cores but not converted to a nucleosomal configuration.

Digestion of viral chromatin late in infection, blotting, and hybridization to detect total viral sequences. Nuclei isolated from infected cells were treated with micrococcal nuclease. The total DNA was extracted from these nuclei, deproteinized, electrophoresed on 2% agarose gels, and blotted onto diazotized paper (2). Nuclease-resistant fragments of cellular and



FIG. 3. Micrococcal nuclease digestion of parental Ad3 DNA in cells treated with *n*-butyrate. Cells were infected with ^3H -labeled Ad3 virions and harvested 3 h after infection. The preparation of Ad3 was the same preparation used for Table 2, experiment 2. Cells to be infected were treated with 7.5 mM *n*-butyrate for 24 h before infection (lane b) or were left untreated (lane c). Nuclei were treated with micrococcal nuclease for 3 min in both cases. The DNA from uninfected cells (lanes a and d) was the same DNA used for Fig. 1. A total of 2.3×10^4 cpm of tritium was loaded into each slot. The gel was fluorographed for 14 days.

viral origin were visualized independently by hybridizing the corresponding ^{32}P -labeled DNAs to the blot. When such an experiment was performed late in an Ad5 infection of HeLa cells, we observed a distinct difference between cellular and viral DNAs (Fig. 4). Visualization of cellular sequences by ethidium bromide staining revealed fragments which were multiples of approximately 180 base pairs, as expected. Longer digestion times produced progressively more monomer DNA. The viral probe also exhibited protected material in a limit digest, but there were no discrete multimers; rather, there was a broad distribution which gradually decreased in size with time of digestion. This band was more diffuse than the cellular monomer even at the longest times of digestion tested. This pattern of digestion, with a broad limit digest band smaller than 160 base pairs, was similar to the pattern obtained when viral cores were digested with micrococcal nuclease (44; Fedor and Daniell, unpublished data).

There is a large amount of single-stranded adenoviral DNA in cells during the late phase of infection, and this DNA is mostly in replicative intermediates (20, 48). Therefore, we treated such samples extensively with S1 nuclease to assure that partially degraded single-stranded DNA was not obscuring a more discrete pattern. This produced no difference in the agarose gel pattern of either cellular or viral DNA in the size range from 100 to 2,000 base pairs.

The Ad5 genome was digested with endonuclease *Xho*I, which generated six fragments. Restriction fragments were eluted from gels, nick-translated, and used individually as probes against blots of the infected-cell micrococcal nuclease digests. The patterns of hybridization were the same as the pattern seen when unfractionated viral DNA was used as a probe, showing that all sequences were protected equally (data not shown).

Micrococcal nuclease digests of viral DNA in virus-cell interactions where packaging is reduced. We investigated infections in which viral DNA synthesis proceeded efficiently but in which production of mature virus particles was reduced to determine whether different aspects of viral chromatin structure were revealed when packaging of DNA was inhibited. Three temperature-sensitive mutants with lesions in late functions were investigated. One of these (H5ts19) forms assembly intermediates at the nonpermissive temperature, whereas the other two form no detectable particles (15). Some processing of the core protein precursor pVII to its mature form is found in cells infected with H5ts19 at the nonpermissive temperature, whereas H5ts18 and H5ts1 synthesize pVII but

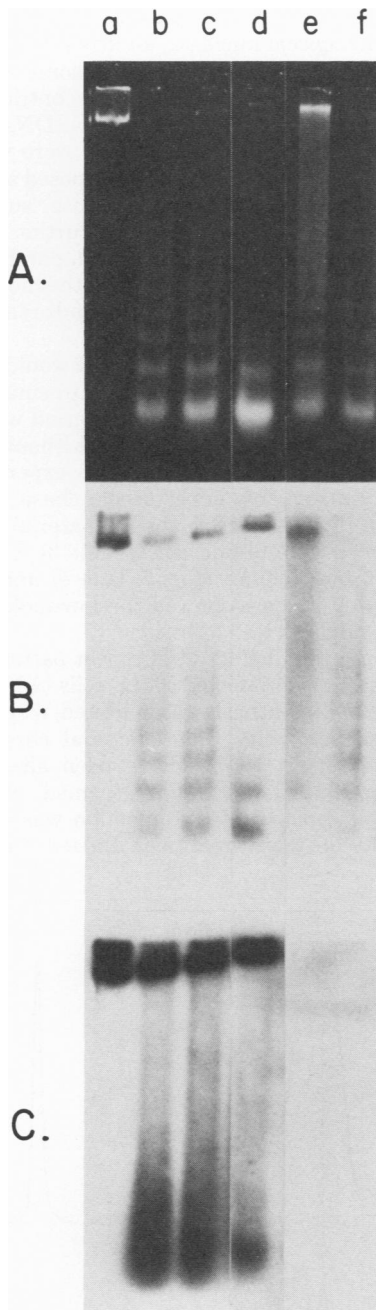


FIG. 4. Micrococcal nuclease digestion of Ad5-infected cell nuclei late in infection. Cells were harvested 22 h after infection with Ad5 (20 PFU/cell), and isolated nuclei were treated with micrococcal nuclease (60 U/ml) for different times. Lanes a through d, Ad5-infected cells; lanes e and f, uninfected cells; lane a, no digestion; lanes b and e, 1-min digestion; lanes c and f, 2-min digestion; lane d, 5-min digestion. (A) DNA was extracted, electrophoresed on 2% agarose gels for 3 h at room temperature

show no processing (15, 17; Daniell, unpublished data). Micrococcal nuclease treatment of nuclei infected with these mutants at the permissive temperature produced a limit digest that exhibited a broad band of fragments smaller than 160 base pairs. No multimers were discerned. The proportion of DNA which remained in the fully protected fraction was decreased a great deal in these mutants; this observation was consistent with the hypothesis that the DNA which was completely resistant to nuclease was a result of formation of complete virus particles.

We also investigated the infection of monkey cells (BSC-1) which were semipermissive for human adenovirus. As with the temperature-sensitive mutants, very little DNA was protected from the nuclease completely, but the time course of digestion and the array of products in the 100 to 2,000-base pair size range were indistinguishable from the results with the permissive infections.

Digestion of viral chromatin labeled late in infection: discrete protected fragments revealed. Because adenoviruses generally shut off host cell DNA synthesis slowly and with variable efficiency, it has been difficult to study newly replicated adenoviral DNA with the certainty that the label is going solely into viral sequences. This problem has been overcome by the discovery that adenoviruses can productively infect cells in which cellular DNA synthesis has been blocked by *n*-butyrate (12). Butyrate-treated HeLa cells infected with Ad5 were labeled for 0.5 to 2 h by adding [³H]thymidine (5 μ Ci/ml) to the medium between 18 and 26 h postinfection. Isolated nuclei were treated with micrococcal nuclease, and the DNA was extracted and subjected to electrophoresis as described above.

Figure 5 shows an ultraviolet-fluorescent photograph and a fluorograph of one such preparation. A series of bands were present in the [³H]thymidine-labeled viral DNA which did not correspond to the cellular nucleosomal repeat in the adjacent lane. Figure 6 shows representative tracings from which fragment size measurements were obtained.

Extensive measurements were made to determine the sizes of the viral protected fragments. Fragments of simian virus 40 DNA digested with

(5 μ g of DNA per slot), stained, and photographed. The DNA was blotted onto diazotized paper. The blot was hybridized first with ³²P-labeled Ad5 DNA (C) and then washed extensively and hybridized with ³²P-labeled HeLa DNA (B). The probes were labeled by nick-translation (35) to a specific activity of 10⁷ cpm/ μ g. Autoradiography was performed for 24 h (Ad5 probe) or for 3 days (HeLa probe).

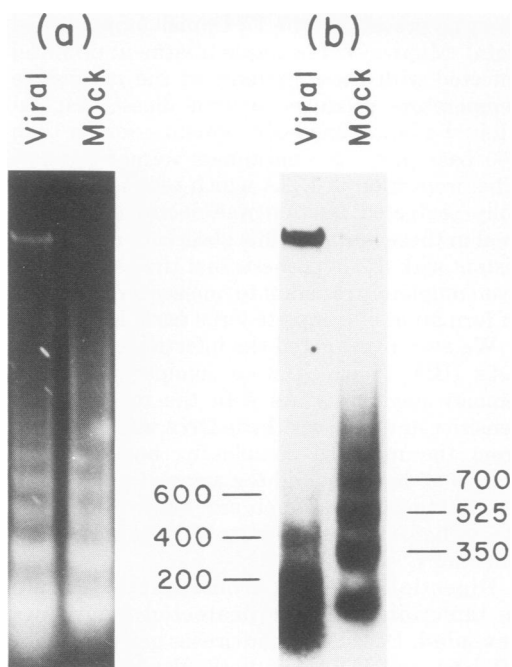


Fig. 5. Micrococcal nuclease digestion of Ad5 chromatin labeled late after infection of cells treated with *n*-butyrate. HeLa cells were treated with 7.5 mM *n*-butyrate for 24 h before infection with Ad5, labeled for 2 h at 20 to 22 h after infection in the presence of butyrate, and harvested immediately. Nuclei were treated with nuclease for 4 min, and the DNA was extracted and electrophoresed on 1.4% agarose gels for 4 h at 4°C. (a) Ultraviolet-fluorescent photography of stained gel. (b) fluorograph (2 days). A total of 1.5×10^5 cpm of tritium was loaded into the viral slot, and 1×10^5 cpm was loaded into the mock-infected slot. The mock sample was the same sample used for Fig. 1, lanes a and g.

endonuclease *Hinf*I were used as size markers (Fig. 7). Measurements were obtained both by determining distances from the origin on each autoradiograph directly and by tracing with a densitometer and determining distances between peaks. On these 1.4% agarose gels, the linear relationship between electrophoretic mobility and log molecular weight did not hold below 170 base pairs. Indeed, fragments smaller than 150 base pairs ran together at a front (see above). For this reason, we could not deduce an accurate size for the smallest viral band in this gel system. The larger bands were centered at 200, 400, and 600 base pairs and were about 40 to 50 base pairs wide in samples from nuclei treated for 2 min with micrococcal nuclease (60 U/ml) (Fig. 5 and Fig. 7, lanes c and g). Occasionally a tetramer of this repeat was detected at 800 base pairs. Cellular (HeLa) DNA was digested into multimers of 175 base pairs under these conditions.

When cellular chromatin was digested mildly with micrococcal nuclease, an array of seven or eight multimers of the unit nucleosome was observed in a single preparation. In contrast, the kinetics of digestion of labeled viral DNA were such that when higher multimers were visible, the lower bands were much overexposed and the 200-base pair "monomer" and diffuse "submonomer" bands coalesced (Fig. 5). Further digestion (8 min, 60 U/ml) reduced the apparent sizes of both the viral submonomer and the monomer (Fig. 7, lanes e and h). The two bands remained after a 16-min digestion.

To determine whether the label would accumulate in the form that gave rise to small protected fragments, a 2-h labeling period was followed by a 2-h chase in medium without label. Figure 7 shows the results of this experiment; the only observable effect of the chase was to increase the fraction of labeled material which was inaccessible to nuclease (Table 3). Labeled material both before (Fig. 7, lane e) and after (lane i) a 2-h chase showed the low-molecular-weight bands.

Because the distinct viral repeat pattern was observed in Ad5-infected HeLa cells only when cellular DNA synthesis was inhibited, we considered the possibility that late viral chromatin structure might in fact have been altered in butyrate-treated cells. This seemed unlikely since the outcome of the infection was not affected by butyrate treatment. Indeed, the viral

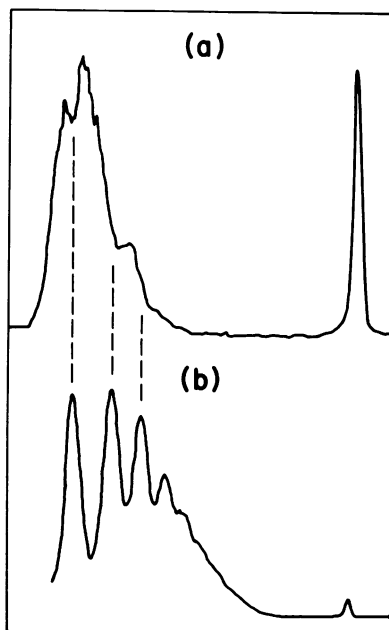


Fig. 6. Tracings of Fig. 5b. Line a, Viral; line b, HeLa.

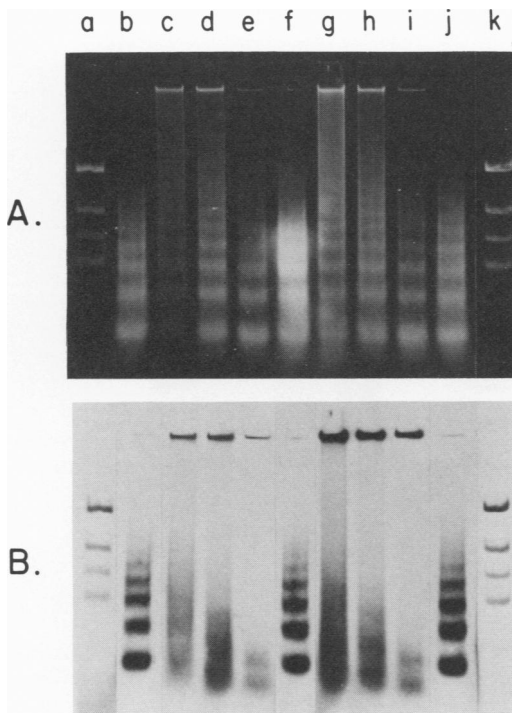


FIG. 7. Comparison of digestions of viral chromatin labeled with and without a chase period in unlabeled medium. HeLa cells pretreated with *n*-butyrate were infected with Ad5 and were labeled from 20 to 22 h after infection. One plate was chased in unlabeled medium for 2 h before harvest and micrococcal nuclease digestion, and the other was harvested and treated immediately. Electrophoresis was carried out on 1.4% agarose gels for 3 h at room temperature. (A) Ultraviolet-fluorescent photograph of ethidium bromide-stained gel. (B) Fluorograph. Lanes b, f, and g, mock infected, from a 1-day exposure; lanes a, c through e, and h through k, 4-day exposure, no intensifying screen; lanes a and k, simian virus 40 DNA digested with endonuclease *Hind*III; lanes c through e, cells harvested immediately after labeling (digestion for 1, 3, and 8 min, respectively); lanes g through i, 2-h chase period before harvest of cells (1, 3, and 8 min of digestion, respectively).

repeat was detected in Ad5-infected HeLa cells in the absence of *n*-butyrate if the DNA was labeled very late in infection (28 h) and if the multiplicity of infection was very high (300 to 400 PFU/cell) (data not shown). In the detailed size analysis of low-molecular-weight fragments discussed below, virus-specific fragments were observed superimposed on the cellular pattern in DNA from cells labeled without butyrate.

Measurement of viral DNA fragments generated by micrococcal nuclease. To determine more precisely the sizes of fragments produced from viral chromatin by micrococcal nuclease, samples were electrophoresed through

10% polyacrylamide gels (Fig. 8). The lanes in Fig. 8A contained the same samples which were electrophoresed on agarose gels to produce Fig. 5 and 7; the infected cells (Fig. 8A, lanes b, c, and e) were labeled in the presence of butyrate so that only viral sequences were labeled. The monomer and submonomer viral bands consisted of a heterogeneous array of fragments between 30 and 230 base pairs long which were also present when the samples were electrophoresed under denaturing conditions (data not shown). The increase in the mobility of the bands in agarose gels with increasing digestion (Fig. 7, lanes d and e) was due to the increase in the number of smaller fragments in the distribution. To confirm our analysis of the DNA distribution on two different gel types, the DNA was eluted from slices of agarose gels by using the ethidium bromide staining pattern of cellular DNA as a marker; this DNA was electrophoresed on acrylamide gels and visualized by fluorography. The submonomer regions from agarose gels were resolved into labeled bands containing fragments which were 30 to 140 base pairs long. Viral monomer material was present in the gel slices between cellular monomer material and dimer material.

To obtain the samples shown in Fig. 8B, cells were labeled late in infection, but they were labeled in the absence of *n*-butyrate so that the label entered both cellular DNA and viral DNA. In these samples (Fig. 8B, lanes a and b), the closely spaced bands of viral DNA were interspersed with the cellular fragments. Increasing digestion of cellular chromatin (Fig. 8B, lanes c and d) increased the amount of the monomeric subunit at the expense of the multimeric forms, trimmed the dimer to a smaller size, and generated discrete submonomer bands, as first observed by Axel et al. (4, 47). These effects of digestion were also observed in the cellular DNA of infected cells whereas the distribution of viral

TABLE 3. Nuclease digestion of viral DNA labeled late in infection

Prepn	Length of digestion (min) ^a	Fraction precipitable ^b	Fraction of label in full-length DNA ^c
Label	1	0.94	0.04
	2	0.82	0.04
	8	0.37	0.02
Label + chase	1	0.97	0.09
	2	0.87	0.10
	8	0.50	0.17

^a Reaction mixtures contained 60 U of micrococcal nuclease per ml.

^b See Table 2, footnote c.

^c See Table 2, footnote d.

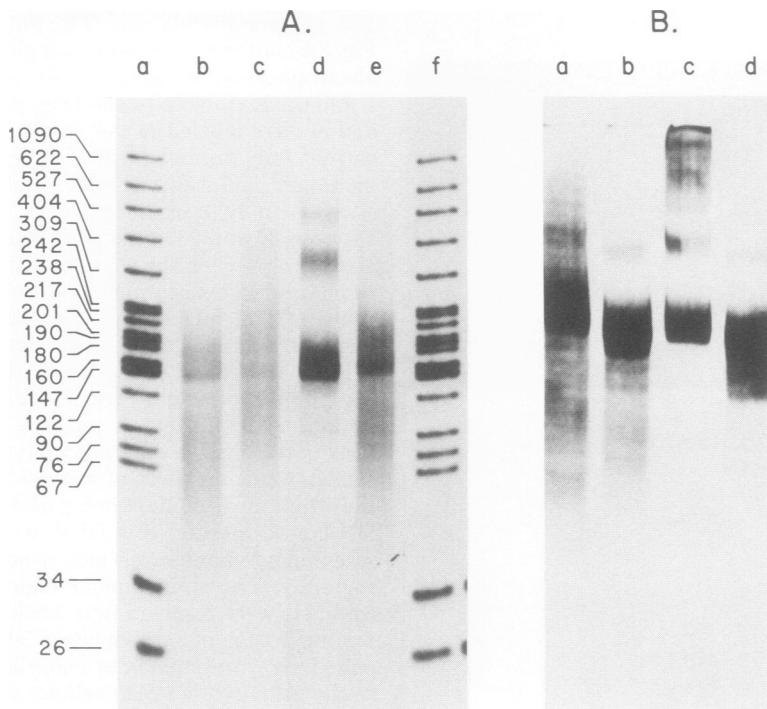


FIG. 8. Products of micrococcal nuclease digestion of chromatin in infected and uninfected cells run on 10% polyacrylamide gels. (A) Samples were from infected cells labeled in the presence of butyrate (lanes b, c, and e) and from uninfected cells (lane d), with restriction fragments as size markers (lanes a and f). The samples were the same as those used in experiments shown in other figures, as follows: lane b, Fig. 7, lane c; lane c, Fig. 7, lane d; lane d, Fig. 5 (mock infected); lane e, Fig. 5 (viral). (B) Infected (lanes a and b) and uninfected (lanes c and d) cells were labeled in the absence of butyrate, and nuclei were treated for 1 min (lanes a and c) or for 4 min (lanes b and d) with micrococcal nuclease.

fragments was skewed toward smaller sizes, as noted above. The viral bands containing fragments smaller than 100 base pairs differed in size by about 5 base pairs; in the region from 100 to 200 base pairs these bands were spaced about 20 base pairs apart.

DISCUSSION

The structures of adenovirus DNA-protein complexes at different times in infection have been probed by micrococcal nuclease digestion, revealing clear differences between viral chromatin early in infection and viral chromatin late in infection. Of particular interest is the repeating pattern which is detected when digestion of viral DNA labeled late in infection is studied. This repeating unit differs in size from the cellular multimers; parental infecting adenoviral DNA exhibits a repeat pattern more similar to the cellular nucleosomal repeat than to the late-labeled viral repeat.

A possible interpretation of these observations is that after infection histones replace adenovirus core protein VII on viral DNA as particles

are uncoated. Late in infection, newly synthesized viral protein pVII associates with newly synthesized adenoviral DNA, producing a structure which exhibits different kinetics of digestion and sizes of protected fragments than histone-DNA complexes.

An early viral repeating structure which mimics the cellular structure has been reported by other workers in Ad2 (38, 44). Our results are similar. Viral DNA and cellular DNA in nuclei early in infection are digested with similar kinetics. The size difference between cellular and early viral nucleosomes reported previously was not evident in our data; cellular and viral bands remained in register up to hexamers of the unit fragment (Fig. 1). The source of this discrepancy is not clear. It should be noted that in this study we used [^3H]thymidine instead of ^{32}P to label virus particles and that we studied Ad3 and Ad5 instead of Ad2. However, it seems unlikely that there is a significant difference between Ad2 and Ad5, which are very closely related.

The persistence of the cell-like repeating pattern in parental viral DNA even late in infection

is intriguing. Parental DNA in some form must be the template for the first round of DNA replication. Perhaps the fraction of parental DNA which assumes a nucleosomal configuration is distinct from the fraction that serves as a replicative template. Alternatively, we can imagine mechanisms through which the nucleosomal structure of part of the template could be preserved during replication.

The fact that the viral DNA assumes a nucleosome-sized repeat in butyrate-treated cells is interesting, as little or no histone synthesis occurs in these cells (12). There may well be a sufficient histone pool which accumulates on incoming viral genomes; the sizes of the histone pools in butyrate-treated cells have not been determined. In five separate infections, the early viral repeat pattern in butyrate-treated cells was less sharply defined than the pattern in untreated cells in viral DNA. This may be a function of a lack of available histones or of the highly acetylated nature of histones in butyrate-treated cells. No differences in the cellular repeat in butyrate-treated cells have been observed in ethidium-bromide stained gels, except for a slightly faster appearance of monomer than in normal cells, as observed by Simpson (41). Finally, input Ad3 DNA in nuclei of BHK-21 cells has a nucleosomal configuration. These cells are nonpermissive for Ad3 and exhibit early viral ribonucleic acid synthesis and protein synthesis but no viral DNA synthesis (22a, 40). This supports the hypothesis that the early phase of infection is not altered in these cells, but a block occurs at the onset of viral DNA replication.

A unique repeating structure was shown by micrococcal nuclease digestion of viral chromatin which had been replicated within 4 h of nuclear isolation. No repeating pattern was detected by blotting and hybridization, which detect total viral sequences. There are two possible explanations for this difference. The first is a technical effect of the blotting protocol. Hybridizations to blots are performed with a ^{32}P -labeled probe, whereas tritium is used in the late label. The high energy of ^{32}P decay produces broader bands than tritium. Since the bands are quite broad and show considerable background even in the late-label (tritium) experiment, the added effect of ^{32}P and the extra steps of blotting could obscure the repeat. Of course, the cellular repeating pattern can be detected by these procedures (Fig. 4), and a nucleosomal repeat was clearly present in intracellular simian virus 40 DNA in experiments performed in an identical fashion (data not shown). Perhaps the adenoviral bands are simply too diffuse to detect in this way.

A more interesting explanation is that adeno-

viral DNA exists in a variety of states in cells late in infection and only recently replicated DNA occurs in the repeating structure. Different products may be generated from other structures, all hybridizing to a probe in the blotting experiments. For example, we found that the multimers generated from input viral sequences appear on gels in between the bands of late-labeled viral DNA. However, we would expect to be able to ignore the contribution of parental DNA in these experiments since its concentration is so small compared with that of progeny DNA. Breakdown products of incomplete virus particles may be another source of DNA which obscures the repeat pattern of newly replicated sequences.

It is probable that the repeat unit of roughly 200 base pairs which we have observed in DNA labeled late in infection results from complexes between the DNA and viral core proteins. An oligomer of pVII, perhaps in association with minor core proteins V and X, could be the basis for protection of the DNA monomer fragment, by analogy with histone octamers of cellular chromatin. We have found that pVII can introduce superhelical turns into closed circular DNA after association *in vitro* (J. L. Burg and E. Daniell, unpublished data). This is a property that is shared by histones and by other proteins which have a role in chromatin structure (9, 21, 36). The introduction of superwinds results from torsional constraints imposed by the protein either by coiling of DNA around nucleosomes (in the case of histones) or by unwinding of the double helix or a change in helix rotation (26). The complicated array of fragments visualized on high-resolution acrylamide gels may be derived from more extensive digestion of viral multimers, which are analogous to nucleosomal submonomer bands. Alternatively, they may be produced from completely different structures, such as single-stranded viral DNA in association with the viral single-stranded DNA-binding protein.

In a recent report (8), which was published while this paper was in preparation, Brown and Weber describe micrococcal nuclease digestion of Ad2 chromatin late in infection that was probed by blotting procedures similar to those described here. The results of these authors and our results are very similar; no repeating unit was detected, and the limit digest material appeared at the same position in these gels as in gels obtained from digestion of viral cores.

Herpes simplex virus DNA-protein complexes extracted from infected cells late in infection do not contain histones or any evidence of nucleosomal structure (33). Blotting techniques similar to those which we used have been used to show that the DNA of Epstein-Barr virus in producer

cells is non-nucleosomal (39). Comparisons of viral nonhistone chromatin from adenovirus and other virus types with cellular chromatin may indicate which features of DNA-protein complexes are essential for their function(s) in eucaryotic cell nuclei.

The observed difference between early and late viral chromatin raises the possibility that changes in chromatin structure are important in transcriptional control of viral gene expression. Analyses of the structure and protein components of viral DNA-protein particles generated by nuclease digestion will contribute to our understanding of this problem.

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LITERATURE CITED

1. **Aboud, M. M., and M. S. Horwitz.** 1979. The DNA polymerases associated with the adenovirus type 2 replication complex: effect of 2'-3'-dideoxythymidine-5'-triphosphate on viral DNA synthesis. *Nucleic Acids Res.* **6**:1025-1039.
2. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. U.S.A.* **74**:5350-5354.
3. **Anderson, C. W., P. R. Baum, and R. F. Gesteland.** 1973. Processing of adenovirus 2-induced proteins. *J. Virol.* **12**:241-252.
4. **Axel, R., W. Melchior, B. Sollner-Webb, and G. Felsenfeld.** 1974. Specific sites of interaction between histones and DNA in chromatin. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4101-4105.
5. **Black, B. C., and M. S. Center.** 1979. DNA-binding properties of the major core protein of adenovirus 2. *Nucleic Acids Res.* **6**:2339-2353.
6. **Brison, O., C. Keding, and J. Wilhelm.** 1977. Enzymatic properties of viral replication complexes isolated from adenovirus type 2-infected HeLa cell nuclei. *J. Virol.* **24**:423-435.
7. **Brown, D. T., M. Westphal, B. T. Burlingham, U. Winterhoff, and W. Doerfler.** 1975. Structure and composition of the adenovirus type 2 core. *J. Virol.* **16**:366-387.
8. **Brown, M., and J. Weber.** 1980. Virion core-like organization of intranuclear adenovirus chromatin late in infection. *Virology* **107**:306-310.
9. **Caron, F., C. Jacq, and J. Rouviere-Yaniv.** 1979. Characterization of a histone-like protein extracted from yeast mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* **76**:4265-4269.
10. **Corden, J., H. M. Engelking, and G. D. Pearson.** 1976. Chromatin-like organization of the adenovirus chromosome. *Proc. Natl. Acad. Sci. U.S.A.* **73**:401-404.
11. **Daniell, E.** 1976. Genome structure of incomplete particles of adenovirus. *J. Virol.* **19**:685-708.
12. **Daniell, E.** 1980. Cells inhibited with n-butyrate support adenovirus replication. *Virology* **107**:514-519.
13. **Daniell, E.** 1981. Subgenomic viral DNA species synthesized in simian cells by human and simian adenoviruses. *J. Virol.* **37**:620-627.
14. **Daniell, E., and T. Mullenbach.** 1978. Synthesis of defective viral DNA in HeLa cells infected with adenovirus type 3. *J. Virol.* **26**:61-70.
15. **Edvardsson, B., S. Ustacelebi, J. Williams, and L. Philipson.** 1978. Assembly intermediates among adenovirus type 5 temperature-sensitive mutants. *J. Virol.* **25**:641-651.
16. **Everitt, E., L. Lutter, and L. Philipson.** 1975. Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirus type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology* **67**:197-208.
17. **Everitt, E., S. A. Meador, and A. S. Levine.** 1977. Synthesis and processing of the precursor to the major core protein of adenovirus type 2. *J. Virol.* **21**:199-214.
18. **Fareed, G. C., and D. Davoli.** 1977. Molecular biology of papova viruses. *Annu. Rev. Biochem.* **46**:471-552.
19. **Felsenfeld, G.** 1978. Chromatin. *Nature (London)* **271**:115-122.
20. **Flint, S. J., S. M. Berget, and P. A. Sharp.** 1976. Characterization of single-stranded viral DNA sequences present during replication of adenovirus types 2 and 5. *Cell* **9**:559-571.
21. **Germond, J. E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon.** 1975. Folding of the DNA double helix in chromatin-like structures from simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1843-1847.
22. **Green, M., M. Piña, and R. C. Kimes.** 1967. Biochemical studies on adenovirus multiplication. XII. Plaquing efficiencies of purified human adenoviruses. *Virology* **31**:562-565.
- 22a. **Groff, D. E., and E. Daniell.** 1981. Early RNA of adenovirus type 3 in permissive and abortive infections. *J. Virol.* **40**:620-624.
23. **Hagopian, H. K., M. G. Riggs, L. A. Schwartz, and V. M. Ingram.** 1977. Effect of n-butyrate on DNA synthesis in chick fibroblasts and HeLa cells. *Cell* **12**:855-860.
24. **Harpst, J. A., J. F. Ennever, and W. C. Russell.** 1977. Physical properties of nucleoprotein cores from adenovirus type 5. *Nucleic Acids Res.* **4**:477-490.
25. **Honda, B. M., D. L. Baillie, and E. P. M. Candido.** 1975. Properties of chromatin subunits from developing trout testis. *J. Biol. Chem.* **250**:4643-4647.
26. **Javaherian, K., L. F. Liu, and J. C. Wang.** 1978. Nonhistone proteins HMG₁ and HMG₂ change the DNA helical structure. *Science* **199**:1345-1346.
27. **Kedinger, C., O. Brison, F. Perrin, and J. Wilhelm.** 1978. Structural analysis of viral replicative intermediates isolated from adenovirus type 2-infected HeLa cell nuclei. *J. Virol.* **26**:364-379.
28. **Kit, G., and E. Daniell.** 1978. Adenovirus core protein synthesis in the absence of viral DNA synthesis late in infection. *J. Virol.* **27**:74-80.
29. **Kornberg, R. O.** 1977. Structure of chromatin. *Annu. Rev. Biochem.* **46**:931-954.
30. **Maniatis, T., A. Jeffrey, and H. Van deSande.** 1975. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry* **14**:3787-3794.
31. **Mirza, M. A. A., and J. Weber.** 1977. Genetic analysis of adenovirus type 2. VII. Cleavage-modified affinity for DNA of internal viral proteins. *Virology* **80**:83-97.
32. **Mirza, M. A. A., and J. Weber.** 1979. Uncoating of adenovirus type 2. *J. Virol.* **30**:462-471.
33. **Pignatti, P. F., and E. Cassai.** 1980. Analysis of herpes simplex virus nucleoprotein complexes extracted from infected cells. *J. Virol.* **36**:816-828.
34. **Prage, L., and U. Pettersson.** 1971. Structural proteins of adenoviruses. VII. Purification and properties of an arginine-rich core protein from adenovirus type 2 and type 3. *Virology* **45**:364-373.
35. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P.**

- Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-251.
36. **Rouviere-Yaniv, J., M. Yaniv, and J.-E. Germond.** 1979. *E. coli* DNA binding protein HU forms nucleosome-like structure with circular double-stranded DNA. *Cell* **17**:265-274.
37. **Russell, W. C., K. McIntosh, and J. J. Skehel.** 1971. The preparation and properties of adenovirus cores. *J. Gen. Virol.* **11**:35-46.
38. **Sergeant, A., M. A. Tigges, and H. J. Raskas.** 1979. Nucleosome-like structural subunits of intranuclear parental adenovirus type 2 DNA. *J. Virol.* **29**:888-898.
39. **Shaw, J. E., L. F. Levinger, and C. W. Carter, Jr.** 1979. Nucleosomal structure of Epstein-Barr virus DNA in transformed cell lines. *J. Virol.* **29**:657-665.
40. **Shimojo, H., and T. Yamashita.** 1968. Induction of DNA synthesis by adenovirus in contact-inhibited hamster cells. *Virology* **36**:422-433.
41. **Simpson, R. T.** 1978. Structure of chromatin containing extensively acetylated H3 and H4. *Cell* **13**:691-699.
42. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
43. **Sung, M. T., M. A. Lischwe, J. C. Richards, and K. Hosokawa.** 1977. Adenovirus chromatin. I. Isolation and characterization of the major core protein VII and precursor pro-VII. *J. Biol. Chem.* **252**:4981-4987.
44. **Tate, V. E., and L. Philipson.** 1979. Parental adenovirus DNA accumulates in nucleosome-like structures in infected cells. *Nucleic Acids Res.* **6**:2769-2785.
45. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzoyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. U.S.A.* **76**:3683-3687.
46. **Weinmann, R., H. J. Raskas, and R. G. Roeder.** 1974. Role of DNA-dependent RNA polymerases II and III in the transcription of the adenovirus genome late in productive infection. *Proc. Natl. Acad. Sci. U.S.A.* **71**:3426-3430.
47. **Weintraub, H., and F. Van Lente.** 1974. Dissection of chromosome structure with trypsin and nucleases. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4249-4253.
48. **Winnacker, E.-L.** 1978. Adenovirus DNA: structure and function of a novel replicon. *Cell* **14**:761-773.