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Introduction of Superhelical Turns into DNA by Adenoviral Core Proteins and Chromatin Assembly Factors

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The interaction in vitro between adenoviral histone-like proteins and DNA in the presence of chromatin assembly factors was investigated. Viral core protein VII or its precursor pVII was incubated with DNA in the presence of an extract of HeLa cell chromatin, which mediates nucleosome assembly from histones and DNA. We have demonstrated that either protein can introduce superhelical turns into relaxed closed-circular DNA and that the presence of chromatin extract is necessary for the supertwisting effect. A greater density of superhelical turns was produced by pVII than by VII, but neither protein-DNA interaction resulted in the "physiological" amount of supertwisting produced by histones. The inhibition of histone-induced supercoiling by both proteins and the protection of turns in supertwisted starting material are also described. The nucleosome assembly factor, nucleoplasmin, fails to mediate the introduction of superhelical turns by VII or pVII.

Adenoviral DNA, a linear, 35-kilobase molecule, is complexed with virally coded histone-like proteins V (42,000 daltons), VII (18,000 daltons), and X (4,000 daltons) in the core of the virus particle (4, 15, 25). The mass of these proteins together in the viral core is equal to the mass of the DNA. The major core protein, VII, present in 1,070 copies per virion, is a small arginine-rich protein with an amino-terminal basic domain similar to that of histones (19). Protein VII is synthesized in the form of a 20,000-dalton precursor, polypeptide pVII, which associates with newly replicated viral DNA. Proteolytic processing of the 20 N-terminal amino acids, hydrophobic in character, accompanies virion maturation (1, 10, 27, 31). The difference in basicity between pVII and VII is enhanced by the presence of an N-terminal acetyl moiety on pVII (11).

Adenoviral DNA is replicated and transcribed in the nucleus of the infected cell. This has prompted extensive use of adenovirus infection as a simple model system for the study of eucaryotic gene expression, and detailed analysis of viral transcription has been carried out. It has been demonstrated recently that viral cores can be transcribed in vitro and that the products of transcription are markedly different from those generated from deproteinized viral DNA (7). Knowledge of the physical properties of the viral core proteins and of the nature of their association with DNA is necessary to understand the controls of transcription both in vivo and in vitro. Comparisons with the known properties of histones and cellular chromatin may elucidate the relationship between gene expression and chromatin structure.

We have begun the study of viral chromatin structure in vivo at different stages of infection (8). We find that early in a productive infection, parental viral DNA is digested by micrococcal nuclease into a pattern of multimers which is indistinguishable from the nucleosomal repeat of cellular chromatin. Newly replicated viral DNA late in infection, however, yields nuclease digestion products which are distinctly different from the cellular nucleosomal repeat (5, 8). This difference is particularly intriguing in light of the demonstration that only replicated viral DNA serves as a template for late transcription (32).

We have examined the strength of ionic and nonionic interactions between viral proteins and DNA in viral cores and in infected-cell nuclei. Viral proteins pVII and VII are similar to histones in the strength of their electrostatic binding to DNA, although protein-protein interactions may be substantially different (2; M. J. Fedor and E. Daniell, submitted for publication).

To further investigate the functional properties of the core proteins, we have sought in vitro systems in which we could monitor the binding of these proteins to DNA. Recently, several procedures which generate nucleosomes from DNA and histones mixed at physiological ionic strengths have been described. A nucleosome assembly factor described by Laskey et al. (18) will promote formation of histone octamers

which will then associate with DNA. Extracts have been characterized from mammalian (13) and from *Drosophila* cells (22) which will mediate nucleosome assembly, apparently by interaction of RNA with DNA to prevent nonspecific acid-base interactions with histones (23). A variety of polyanions will also perform this function (30).

A convenient assay for assembly of nucleosomes is the detection of superhelical turns introduced into relaxed closed-circular DNA. When DNA and histones associate in vivo or in vitro under conditions which promote proper nucleosome formation, topological constraints are introduced into the DNA by the winding of DNA around the nucleosome core. If the DNA in the complex is relaxed, as it can be in vitro by the action of a nicking-closing enzyme and presumably it is in vivo by a similar activity, then upon deproteinization the intranucleosomal constraints are spread over the entire DNA molecule. The negative superhelical turns which result can be detected and measured by gel electrophoretic separation. If, in an in vitro reassociation between DNA and histone, a relaxed closed-circular DNA (lacking superhelical turns) is used as substrate, then the number of superhelical turns present after association and deproteinization is a measure of the number of nucleosomes formed (12, 17). Several proteins other than histones have the ability to introduce supertwists; a 20,000-dalton DNA binding protein from yeast mitochrondria and protein HU from *Escherichia coli* exhibit this characteristic (6, 29). Other proteins which bind DNA, such as cytochrome *c*, lysozyme, and T4 gene 32 protein, do not introduce supertwists (6). Other types of DNA-protein interactions may also introduce supertwists in DNA in this type of assay; protein molecules may bind to single-stranded segments of DNA to uncoil the double helix or change the DNA helix rotation (16).

In this paper, we investigate the ability of the adenovirus proteins pVII and VII to introduce superhelical turns into DNA when they are used in place of histones in reactions with either extracts of mammalian tissue culture cells or the nucleosome assembly factor described by Laskey. The protection of supercoiled DNA from relaxing (nicking-closing) activity and the effect of VII and pVII on supercoiling by histones are also reported.

MATERIALS AND METHODS

DNA preparation and proteins. Supercoiled simian virus 40 (SV40) DNA was extracted from infected BSC-1 cells by the method of Hirt (14), extracted with phenol and chloroform, and purified by isopycnic centrifugation in CsCl in the presence of 200 μg of ethidium bromide per ml (26). Relaxed covalently closed DNA (F_{Ir}) was prepared by incubation of supercoiled SV40 DNA with chromatin extract (described below) to remove superhelical turns. Relaxed DNA was repurified by either CsCl-ethidium bromide centrifugation or by phenol extraction and ethanol precipitation before use in assays. Histones were the gift of Bob Garcia, University of California, San Francisco, and contain H1 as well as the four core histones. pVII and VII were gifts from Michael Sung, University of California, San Francisco (31), and were greater than 95% pure as judged by sodium dodecyl sulfate-gel electrophoresis.

Chromatin extract. Chromatin extract rich in nicking-closing activity was prepared according to the method of Germond et al. (13) from HeLa cells grown in suspension culture. The extract contained no detectable histones and was free of nuclease activity. The extract also served to mediate the assembly of histones and DNA into beaded chromatin as assayed by electron microscopy and by gel electrophoresis of DNA, which was relaxed in the presence of histone and then deproteinized. Ten- to 20-fold higher concentrations of extract were needed for mediation of histone assembly than those needed for removal of superhelical turns from SV40 F_I.

Preparation of nucleosome assembly factor. Nucleoplasmin was prepared from eggs of *Xenopus laevis* as described by Laskey and co-workers (18, 20). For most experiments, the heated sucrose gradient peak fractions were used with no further chromatographic purification. These preparations were free of histones as judged by gel electrophoresis, but they did contain minor protein species in addition to the prominent 29,000-molecular-weight nucleoplasmin. When nucleoplasmin was further purified on DEAE-cellulose, no difference in results obtained was observed.

DNA-protein associations and gel electrophoresis. Incubations of DNA, protein, and chromatin extract were performed at 37°C in 40-μl reaction mixes in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA–50 to 150 mM NaCl as indicated. Chromatin extract (1 to 5 μl) was mixed with DNA (5 μg/ml) for 1 to 5 min at 37°C before addition of protein. Histones or core proteins were then added in amounts indicated in the figures, and incubation was continued for 1 h. Since different extract preparations had different levels of activity, amounts were used which generated maximal supercoiling with a 1:1 ratio of histone to DNA. Conical Eppendorf tubes were used, and care was taken that the depth of solutions did not exceed the diameter of the surface (13).

Nucleosome assembly mediated by nucleoplasmin was performed in low salt buffer at 22°C as described by Earnshaw et al. (9), at histone-to-DNA ratios of 3:1, with 15 μl of nucleoplasmin peak fraction in 50-μl reaction mixes. When adenoviral proteins were substituted for histones, a wide range of DNA protein ratios was used. After incubation of DNA, nucleoplasmin, and histones or viral core proteins, chromatin extract (described above) was added for a brief time to relieve torsional constraints in the DNA. The concentrations of the extract used in these experiments were completely inactive in nucleosome assembly activity.

After incubation in either type of assembly mix, samples were made 10% sucrose–0.3% sodium dodecyl sulfate–0.05% bromphenol blue and heated at 60°C for 10 min to dissociate protein from DNA. Samples

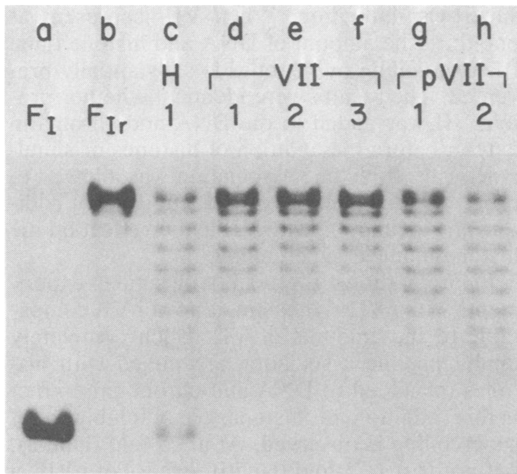

FIG. 1. Introduction of superhelical turns into relaxed closed-circular SV40 DNA (F_{Ir}) by adenovirus core proteins VII and pVII in the presence of HeLa cell chromatin extracts. [^{3}H]thymidine-labeled DNA (0.2 μg) was incubated with histones (lane c), virion protein VII (lanes d, e, and f), or precursor pVII (lanes g and h) at the protein-to-DNA ratios indicated. After deproteinization and electrophoresis of DNA through a 1% agarose gel at 4°C (60 V, 3 h), the gel was stained and photographed and then impregnated with PPO (2,5-diphenyloxazole) and fluorographed. The autoradiograph is reproduced here. Lane a contains fully supercoiled SV40 DNA and lane b the relaxed starting material for these reactions. Starting material incubated with chromatin extract alone appeared identical in this gel system.

were then electrophoresed through 10-cm vertical gels of 1% agarose in 36 mM Tris–30 mM NaH_2PO_4–1 mM EDTA (pH 7.7) to resolve closed-circular DNA molecules with different superhelical densities. Staining, photography, and fluorography of gels have been described previously (8).

RESULTS

Introduction of superhelical turns into relaxed DNA by proteins VII and pVII. Relaxed closed-circular SV40 DNA (F_{Ir}) was incubated with core protein VII, precursor pVII, or histones in the presence of a chromatin extract from HeLa cells. The extract contained a nicking-closing activity so that torsional constraints introduced into the DNA by its interaction with proteins were constantly relieved by the action of the enzyme (see above). After incubation at 37°C, samples were deproteinized and electrophoresed on agarose gels to determine the amount of superhelicity. Figure 1 illustrates that interaction of either VII or pVII with F_{Ir} DNA results in the introduction of superhelical turns. In neither case does the degree of superhelicity approach the limit "physiological" number of turns introduced by histones at the optimal ratio of protein to DNA. The small, but reproducible, number of superhelical turns induced by incubation with VII is not dependent on the ratio of protein to DNA (lanes d, e, and f). Ratios from 0.5 to 5.0 give about the same amount of induction, in contrast to the sharp optimum exhibited by histones (13; E. Daniell, unpublished data). The number of supercoils introduced by pVII is greater than that introduced by VII, but it does not exceed the amount seen with a 2:1 ratio of protein to DNA (lane h).

Several parameters of the supercoiling interaction were investigated. The ionic strength optimum for pVII-DNA interaction was 150 mM NaCl, the same as for histone and DNA (13). In contrast, supercoiling by VII was more consistent and reproducible if incubations were performed at 50 mM NaCl. Supercoiling by either viral protein was dependent on the order of addition of components of the mixture. As is the case with histones (13), the chromatin extract had to be mixed with DNA before addition of protein to produce any superhelical turns. If DNA and protein were mixed in the absence of extract, no supertwists were introduced, even when extract was subsequently added and incubated with the DNA for several hours.

Protection of supercoiled DNA from the action of nicking-closing enzyme. We examined the ability of pVII and VII to protect preexisting turns in DNA from the action of nicking-closing enzyme. The rationale for this investigation was to see whether the degree of superhelicity in DNA at the end point of these DNA-protein interactions was dependent on the conformation of input DNA. Bina-Stein and Singer (3) demonstrated that histone H1 protects DNA from relaxation by enzymes, although it induces no supercoiling in fully relaxed DNA. This behavior of H1 contrasts with the interaction of the four nucleosomal histones with DNA, which yield a degree of superhelicity that is dependent on the histone-to-DNA ratio. At a given ratio, approximately the same amount of supercoiling results from relaxation of superhelical DNA as from introduction of turns into initially relaxed DNA.

Since the chromatin extract contained DNA-relaxing activity as well as nucleosome assembly activity, the protocol for these experiments had an added complication. Our investigation of supercoiling of initially relaxed DNA indicates that DNA and extract must be mixed before protein is added. We therefore established conditions for mixing DNA and extract under which supercoiled DNA does not become appreciably relaxed, but which allow introduction of superhelical turns into relaxed DNA by histones added subsequently. These conditions consisted of a 1-min incubation at 4°C.

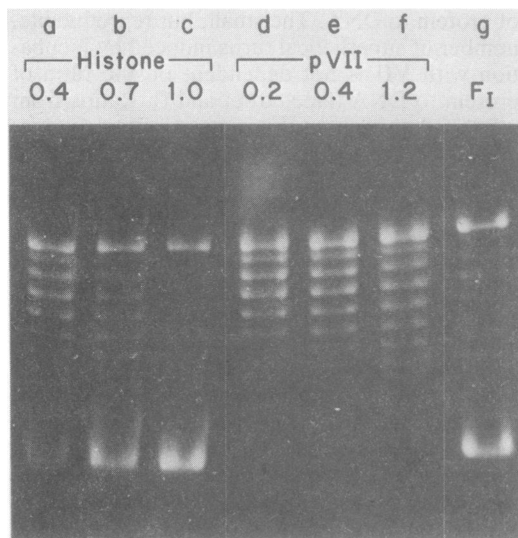

FIG. 2. The protection of superhelical turns in supercoiled SV40 DNA by histones and by pVII. SV40 F_I DNA (0.2 μg) was incubated with chromatin extract for 1 min at 4°C (lane g), and then histone (lanes a, b, and c) or pVII (lanes d, e, and f) were added at the protein-to-DNA ratios indicated. Samples were deproteinized and electrophoresed, and the gel was stained and photographed.

Figure 2 shows the protection of superhelical turns by histones and by pVII. For a given ratio of either pVII or histone to DNA, the number of superhelical turns remaining in DNA after incubation is about the same as the number introduced with relaxed starting material. (Compare Fig. 1 and Fig. 2). This behavior suggests interactions that do not depend on the initial conformation of DNA.

In contrast, Fig. 3 shows that protein VII protects superhelical DNA from relaxation to a far greater extent than it introduces turns. At a protein-to-DNA ratio of 1, for example, the DNA is almost completely protected from relaxation. As shown in Fig. 1 and in the rightmost lane of Fig. 3, very few turns are introduced into relaxed DNA at this ratio. This behavior is similar to that observed for histone H1 (3). We have confirmed that H1 introduces no superhelical turns under our experimental conditions and that it fully protects initial supertwists at a protein-to-DNA ratio of 0.4 (data not shown).

Inhibition of histone-induced supercoiling by viral core proteins. Because histones introduce many more superhelical turns into the DNA substrate than do core proteins, we were able to investigate the effect of the viral core proteins on nucleosome assembly by these same electrophoretic methods. Figure 4 illustrates that protein VII inhibits nucleosome assembly as assayed by supertwist induction. When VII is present at one-third the amount of DNA and histone (lane f), supercoiling by histones is substantially prevented. The results were identical whether protein VII was added to the DNA and chromatin extract before the addition of histones or simultaneously. If histone association was allowed to proceed in the absence of VII, subsequent addition and incubation with VII had no effect on the histone-induced supercoiling.

Figure 5 shows the results of similar experiments with pVII. When amounts of pVII comparable to the amounts of VII which completely inhibit histone association are mixed with histones (or added to DNA and chromatin extract before addition of histones), no inhibition of supercoiling is observed. At a 3.5-fold (lane b), but not at a 1.75-fold (lane i), excess of pVII, a diminution of supercoiling is seen.

Interaction of viral core proteins with nucleoplasmin from *Xenopus laevis* oocytes. We investigated the ability of the nucleosome assembly factor (nucleoplasmin), described by Laskey and co-workers, to mediate interactions between DNA and the adenovirus core proteins.

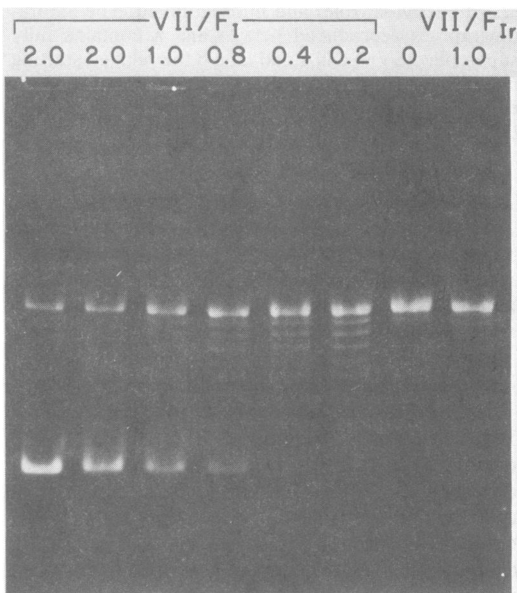

FIG. 3. The protection of superhelical turns in supercoiled SV40 DNA by virion protein VII. SV40 F_I DNA (0.2 μg) was incubated with chromatin extract and various amounts of protein VII as described in the legend to Fig. 2 for pVII and histone. Chromatin extract was omitted from the sample shown in the leftmost lane, and the resulting pattern is identical to the supercoiled starting material used for the other reactions. In the rightmost lane is shown, for comparison, a sample in which relaxed DNA was the starting material.

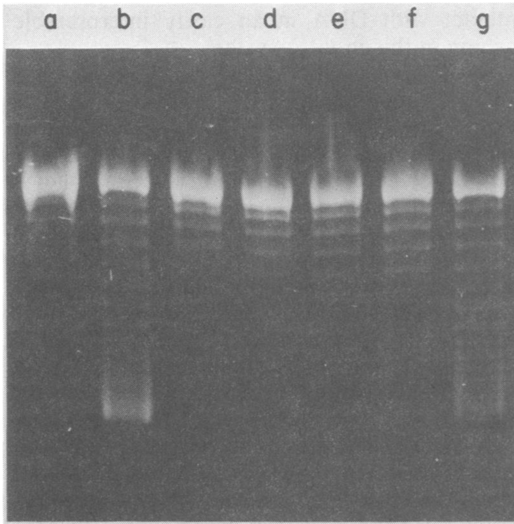

FIG. 4. Inhibition of histone-induced supercoiling of SV40 DNA by protein VII. Relaxed SV40 DNA (F_{Ir}) was incubated with chromatin extract and a mixture of histone and protein VII. Histone-to-DNA ratios were held constant at 1, while the ratio of VII to DNA was varied. Lane a, Incubation with chromatin extract alone (starting material); lane b, chromatin extract and histone; lanes c through g, histone and protein VII at protein VII-to-DNA ratios of 3, 2, 1, 0.6, and 0.3, respectively.

First, VII and pVII were substituted for histones in reaction mixes containing DNA and nucleoplasmin, following the same rationale as in the experiments described above, using chromatin extract. Using the introduction of superhelical turns as an assay, we were unable to detect any effect of the core proteins on DNA superhelical density in the presence of nucleoplasmin. We then investigated the inhibitory effect of protein VII on histone association and supercoil induction in this system. We found that protein VII has no inhibitory effect on nucleosome assembly and supercoiling of DNA when histones, nucleoplasmin, and VII are incubated for 30 min or more before the addition of DNA (data not shown). If DNA and VII are mixed before the addition of histones and nucleoplasmin, supercoiling is completely inhibited, even if nucleoplasmin is present in the DNA-VII mixture. Thus, protein VII does not interfere with the interaction between histone and nucleoplasmin, and preformed nucleosome cores can assemble onto DNA in the presence of VII. Nucleoplasmin does not prevent protein VII from binding to DNA in a way which inhibits the introduction of superhelical turns by histones. These experiments suggest that nucleoplasmin and protein VII do not interact strongly, although they are, respectively, acidic and basic proteins.

DISCUSSION

We have shown that a chromatin extract capable of promoting nucleosome assembly from histones and DNA at physiological ionic strength also mediates the interaction between DNA and adenoviral core proteins. Addition of the proteins in the presence of extract results in the introduction of superhelical turns into closed-circular DNA. If proteins are mixed with DNA before addition of extract, no supercoiling is observed, probably because DNA and protein form an insoluble complex. Our previous demonstration that the core proteins themselves precipitate at these concentrations in the absence of urea (Fedor and Daniell, submitted for publication) supports this interpretation. These results suggest that the chromatin extract we used prevents DNA from being precipitated by these proteins and at least partially solubilizes the proteins themselves.

The experiments described here focus on one parameter of DNA binding, the ability of proteins to introduce superhelical turns into DNA. We chose to investigate the adenoviral core proteins in this way because these proteins are similar to histones in physical properties, including the strength of their electrostatic binding to DNA (Fedor and Daniell, submitted for publica-

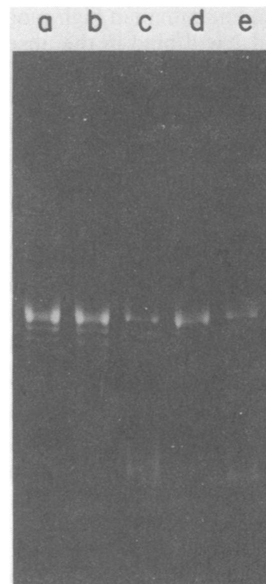

FIG. 5. Inhibition of histone-induced supercoiling of SV40 DNA by pVII. Incubations were performed as described in the legend to Fig. 4, but with various amounts of protein pVII. pVII-to-DNA ratios: lane a, 7; lane b, 3.5; lane c, 1.75. Lane d, starting material; lane e, chromatin extract and histone, no pVII.

tion). The use of SV40 DNA in these experiments is justified by considerable evidence that histone-like proteins do not associate with specific sequences in DNA; nucleosomes, for example, form equally efficiently on bacteriophage DNA as on eucaryotic DNA (24). It has not been determined whether adenoviral DNA is supercoiled in vivo, although apparently supercoiled DNA has been seen in preparations of adenovirus DNA-protein complexes released from virions (28).

It is clear that precursor pVII, the moiety which binds to DNA in vivo in the infected cell nucleus, induces supercoiling. The superhelical density induced by pVII does not reach the physiological number of turns which are present in native SV40 supercoiled (F_I) DNA and which can be introduced in vitro by histone association. At a given ratio of protein to DNA, the degree of superhelicity introduced into initially relaxed DNA is the same as that remaining when protection of turns in supercoiled DNA is examined. This is in contrast to results with protein VII discussed below and is similar to the behavior of histones. The salt optimum for introduction of turns is 150 mM for pVII as it is for histones, and it is essential that the DNA and chromatin extract be mixed before addition of the protein. Histones and the DNA-binding protein HM from yeast share this requirement for chromatin extract and dependence on its addition to DNA before protein (6), whereas *E. coli* protein HU and mammalian high-mobility-group proteins 1 and 2 will bind in the absence of any factors to change the linking number of the DNA (16, 29). It must be emphasized that these results do not suggest that DNA winds around complexes of pVII in the manner that DNA winds around histone octamers. Other types of interactions result in the formation of supercoils in this assay system (16).

Protein VII introduces a small but consistent number of superhelical turns in a portion of the relaxed circular DNA in a reaction mix. This induction is dependent on the presence of chromatin extract. At a given molar ratio of protein to DNA, VII protects supertwists in F_I DNA to a far greater extent. Similar observations have been made with histone H1 (3). It is possible that protein VII precipitates much of the DNA in solution even in the presence of chromatin extract. This hypothesis would account for the protection of supercoils from nicking-closing enzyme as a nonspecific event. In support of this notion, the presence of low amounts of VII inhibits the introduction of superhelical turns into DNA by histones. Since protein VII does not normally bind to DNA in vivo but is formed by cleavage of previously bound pVII, it is perhaps not surprising that the protein fails to interact with DNA in an easily interpretable fashion in this in vitro system.

Several types of evidence indicate that pVII and VII interact differently with DNA. It has been suggested, based on structural studies, that pVII binds to DNA with lower affinity than VII or with an altered conformation (11, 31). Studies of infection with mutant virions containing pVII (21) show that such virions are not properly uncoated in vivo. Our in vitro supercoiling assays support the general conclusion that these two proteins differ in their interaction with DNA.

Nucleoplasmin fails to mediate an interaction between viral histone-like proteins and DNA as revealed by a supercoiling assay. Nucleoplasmin interacts with histones in the absence of DNA to form histone octamers (nucleosome cores) which then can associate with DNA. This interaction results in a protection of positive charges on histones, demonstrated by the loss of the ability to bind to negatively charged surfaces (9). Preliminary studies reveal that nucleoplasmin also protects charges on protein VII, but we have no evidence that it then facilitates DNA-protein association. The active nucleosome assembly component in the chromatin extract we employed is RNA (23). This RNA may have a more general role than nucleoplasmin in facilitating interactions between DNA and basic proteins, as suggested by the observation that this extract mediates supertwisting by basic proteins such as yeast HM and adenovirus pVII. (No investigation of the interaction of HM with nucleoplasmin has been reported.) It is not known whether RNA or other polyanions function in vivo in adenovirus-infected cells to assemble pVII-DNA complexes. It is possible that minor core proteins V and X are important in viral chromatin assembly, but preliminary studies reveal that they do not increase the level of supercoiling by pVII and VII in our assay system (J. L. Burg, unpublished data).

The fact that superhelical turns are introduced into DNA by pVII is of interest in comparing the properties and functions of this protein with those of histone-like proteins in other systems. Yeast mitochrondria and bacteria each contain a single protein (HM and HU, respectively) which introduces superhelical turns into DNA; in each case the proteins are implicated as having a role in structural organization of a chromatin lacking histones. pVII is thus one of an interesting class of histone-like proteins which compact DNA in similar, yet distinct, ways.

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