Sequence-specific Interaction of Histones with the Simian Virus 40 Enhancer Region in Vitro*

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DNA fragments containing either one or both of the 72-base pair (bp) elements which constitute the SV40 enhancer and the three adjacent 21-bp repeats were associated with histone octomers from chicken erythrocytes in vitro. Both fragments formed complexes with electrophoretic mobilities of nucleosomes containing the appropriate length of DNA. Analysis of DNase I cutting of uniquely end-labeled complexes suggests that the fragment containing a single 72-bp element forms a positioned core particle. Control experiments show that positioning is not due to the 21-bp repeats or to end effects. The fragment with a tandem repeat of the 72-bp element also does not associate randomly with histones. The data are consistent with formation of a core particle on one or the other of the repeated enhancer sequences. We discuss possible functional consequences of such nucleosome positioning.

The SV40 viral genome is packaged with cellular histones into chromatin with a structure similar to that of the host cell (1). The viral DNA contains a 72-bp1 direct repeat sequence adjacent to an imperfect triple 21-bp repeat near the origin of replication (Fig. 1). The 72-bp repeat sequence increases transcription from viral promoters about 100 times (2, 3); it is the prototype "enhancer" element. Such elements increase transcription from eukaryotic promoters in an orientation- and position-independent fashion (for review, see Ref. 4). The origin and enhancer regions apparently lack canonical nucleosomes in a fraction of native SV40 minichromosomes (5); this may be related to the DNase I sensitivity of these regions of the chromosome (6, 7). The enhancer sequences are necessary for this characteristic nucleosome sensitivity of this chromatin region (7) and others have suggested that phasing of nucleosomes might contribute to the DNase I digestion pattern (6).

Nucleosome phasing in vitro has been thought to occur on a number of eukaryotic and viral genes (for review, see Ref. 8) although this is still a matter of contention. In vitro studies of the reassociation of SV40 DNA with histones have produced apparently conflicting results. In one study the origin of replication region was found to be a nonfavored site for nucleosome formation (9); in another, the origin was shown to be capable of interacting with histones (10). In other in vitro associations, positioned nucleosomes have been demonstrated following association of histones with short DNA fragments containing the lac operator (11), a sea urchin 5 S ribosomal RNA gene (12), or a cloned segment of Escherichia coli DNA (13). Not all short DNA fragments form such positioned nucleosomes; certain mutants of the 5 S sequence abolish phasing.3 Using methodology similar to that previously employed for investigation of the histone complex with the 5 S gene (12), we have studied the interactions of histones with the SV40 enhancer region.

EXPERIMENTAL PROCEDURES

Plasmid pL-1 (14) containing the replication origin, 72-bp enhancer repeats, 21-bp repeats, and the early gene promoter of SV40 was kindly provided by Dr. H. Okayama (National Institutes of Health). Plasmid pLSph-1 contains only a single 72-bp element; it was generated by digestion of pL-1 with SpH1 and recircularization. Plasmid pLSph-2 was generated by digestion of plasmid pLSph-1 with restriction endonuclease NcoI, followed by digestion with E. coli exonuclease III for 5 min, and then treatment with S1 nuclease followed by recircularization (15). Core particles were prepared from chicken erythrocytes by published methods (10) except that 0.65 M NaCl was used for stripping of the lysine-rich histones.

Plasmids pL-1 and pLSph-1 were digested with either HindIII or NcoI restriction endonuclease and end-labeled by either filling in the 3' end using the large fragment of DNA polymerase I or phosphorylating the 5' end after treatment with calf intestinal alkaline phosphatase. The labeled material was then digested with the other restriction endonuclease and a uniquely end-labeled fragment isolated by preparative polyacrylamide gel electrophoresis. Plasmid pLSph-2 was digested with either BamHI or HindIII, followed by 3' end-labeling.

Labeled fragments were added to a large excess of chicken erythrocyte core particles in 0.6 M NaCl, 10 mM Tris/Cl, pH 8.0, 1 mM EDTA and dialyzed for 5 h at 23 °C against the same buffer. Dialysis was then continued overnight at 4 °C against 0.1 M NaCl, 10 mM Tris/Cl, pH 8.0, 1 mM EDTA. Samples were adjusted to contain 3 mM CaCl2 and 10 mM MgCl2, and digested with DNase I as indicated. Digestions were terminated by addition of EDTA and sodium dodecyl sulfate to concentrations of 25 mM and 1%, respectively. DNA was purified by phenol and chloroform extractions and analyzed on 8%

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The abbreviation used is: bp, base pair.

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particles relative to core particle DNA. Similarly, the E2 complex with histones migrates on electrophoresis with a mobility like that of the 240-bp 5 S rRNA gene histone complex (12); the latter is known to contain a single nucleosome. In both cases, we estimate that approximately 95% of the DNA is associated with histones using the described octomer transfer protocol.

As we have done previously with the complex of the sea urchin 5 S gene with histones (12), we used DNase I digestion of these complexes to ask whether the association of histones with DNA was specific or random. If interactions were random, one would expect to see a pattern of nucleosome cutting sites for the complex that is substantially like that observed for protein-free DNA, since any given DNA sequence is statistically available for cutting as frequently as any other sequence. In fact this is observed for certain deletion mutations of the 5 S gene.\(^2\) Specific positioning of a nucleosome leads to a pattern of cutting sites spaced at about 10-bp intervals alternating with regions which are resistant to nuclease action (12, 15, 18, 20). Such a pattern must span at least 120 bp if a positioned core particle is present; protection of the terminal 10 bp of the 146-bp core particle is less well-defined with longer DNA molecules than with isolated core particles.

Fig. 2A shows mapping data comparing free DNA with the histone complex for the E1 fragment. Fig. 3 schematically shows the positions of the major cutting sites for E1 and E2 complexes with histones. In either strand of the E1 complex, cutting sites occur at intervals of about 10 bp, beginning about 20 bp from the end of the DNA segment in the complex. Between these are regions where access of the nuclease to the DNA is largely blocked. When the E1 fragment is analyzed after 3' end-labeling the HindIII site, there are cutting sites for its complex with histones at bases 16 and 17; 27 and 29; 40; 51 and 52; 62 and 64; 72, 73, and 74; 83; 94, 95, and 96; 105; 113; 128; 137; and 148. The bands in the digest of the complex at bases 64, 73, 113, and 137 are not cut in the naked DNA control. Regions between the cutting sites in the complex are highly protected from the nuclease; note the naked DNA cut sites at bases 24, 39, 48, 49, 55, 56, 60, 65, 66, 71, 74, 79, 87, 88, 101, 107, 108, 109, 120, 124, and 132. Complementary data are obtained when cutting of the other strand is analyzed for fragment E1 as a histone complex, compared with its digestion as protein-free DNA (Fig. 2A, lanes 3 and 4).

Note that the protected regions map to similar loci on the DNA segment when located from either end of the fragment. Cutting sites at a given locus in one strand are displaced slightly (1–3 bases) to the 3' side of the cutting site in the other strand; this displacement of cutting sites is characteristic of the cutting of DNA in core particles by DNase I. The relative susceptibilities of the various cutting sites are not those observed previously for nucleosome core particles containing random sequence DNA (15, 19, 20) but are more reminiscent of the cutting of one strand (C) in the phased 5 S nucleosome core particle (12). Taken together, these observations strongly suggest that the E1 fragment associates with histones in a specific manner to form a positioned nucleosome in vitro. In Fig. 3 the position such a nucleosome might occupy is indicated; the location is based on the assumption that the core particle span of 146 bp is centered on the region where periodic cutting occurs.

Fig. 2B presents similar data for the E2 fragment. Lanes 1 and 2 are comparable mapping data to those in lanes 1 and 2 of Fig. 3.
of Fig. 2A for the shorter DNA segment. In general, there is a similar pattern of cut sites and noncut regions for both fragments although there are differences which we discuss below. Mapping of cutting in the top strand of the E2 complex from the right end (Fig. 2B, lane 6) or the left end (Fig. 2B, lane 4) again shows the pattern of cut and resistant regions with periodicities of about 10 bases. Again, sites which can be mapped from both ends of the complex occur in similar locations. This pattern begins about 20 bp from either end of the DNA.

Comparison of the cutting patterns for the E1 and E2 fragments when mapped from either the 3' HindIII or 3' NcoI sites can be made most easily in the schematic representation in Fig. 3. While the same sites which are cut in E1 are also cut in E2, near the ends of the DNA segment there are additional cut sites in the E2 fragment; these are strong cutting sites in naked DNA that are largely blocked in the E1 complex. Two examples are the sites near 55 and 65 bases in lane 2, Fig. 2A and lane 2, Fig. 2B. The cutting patterns of E1 and E2 complexes are highly similar in the region from 100 to about 160 base pairs from the left end of the fragment, although this region contains entirely different DNA sequences in the two fragments. The total length of the E2 fragment possessing the 10-bp periodicity of cutting sites is about 190 bp. While it might be suggested that this reflects the presence of two nucleosomes on this DNA, note that the mobility of the complex is similar to that of a complex containing the same length of DNA and a single nucleosome (Fig. 1B). As discussed below, we feel that the digestion pattern likely reflects the presence of two populations of positioned core particles.

In order to make certain that the nuclease digestion patterns observed were not due to the 21-bp repeats or positioning of a nucleosome being determined by effects related to the end of the DNA molecule, we constructed the deletion mutant pLSph-2. The plasmid has a deletion which removes the adenovirus early region TATA box, the 21-bp repeats, and 32 bases of the 72-bp repeat. The 210-bp BamHI-HindIII fragment (E-32) was isolated, associated with histones, and digested with DNase I.

Fig. 4A shows the mapping data for this fragment as free DNA and as a complex with histones; an extended (HindIII-BamHI) E1 fragment is shown for comparison. When both fragments are 3' end-labeled at the HindIII site, the digestion patterns of the free DNA samples are identical to base 57, the point of the deletion. The DNase digestion patterns of free DNA are very different beyond the deletion. In contrast, the E1 and E-32 histone complexes give remarkably similar patterns of digestion. There are cutting sites occurring at approximately 10-bp intervals; these occur within 1 to 2 bases of each other in each fragment, including the region with different sequences. Note also that at positions beyond base 150, the patterns of digestion of E-32 DNA alone or complexed with histones are identical.

When E-32 is 3' end-labeled at the BamHI site, the DNase I patterns of E-32 free DNA and the E-32 fragment associated with histones are again consistent with a nucleosome positioned between bases 10–20 and 150–160 from the HindIII site (Fig. 4A). The digestion patterns of the free DNA and DNA associated with histones are almost identical until approximately 60 bp from the BamHI end. Beyond this there appears a pattern of DNase cutting of the E-32 histone complex at 10-bp intervals. The patterns become identical about 20 bp from the end of the fragment (Fig. 4B). The identical patterns of cutting of the two samples for about 60 bp from the BamHI end make the possibility of "end effects" as determinants of positioning highly unlikely. In order to address the possibility of end effects occurring only at the HindIII site, we 3' end-labeled the E1 fragment at the NcoI site, and the HindIII end was either filled in using the large fragment of DNA polymerase I or the 5' HindIII overhang removed with S1 nuclease. This results in fragments which differ by 4 bp in length at the HindIII end. Digestion patterns for these two DNA-protein complexes were essentially identical (not shown).

**DISCUSSION**

The data presented clearly demonstrate a nonrandom association of histone octomers with a 166-bp DNA fragment which contains a single copy of the 72-bp SV40 enhancer element. A region of at least 120 bp where cutting is different from that of protein free DNA is present; within that region, cut sites and noncut regions alternate, each with a periodicity of about 10 bp. The simplest interpretation of such data is that the DNA sequence interacts in a specific fashion with the histones to form a "positioned" or "phased" nucleosome in vitro. The apparent discrepancy between the length of the protected region and the length of core particle DNA, 146 bp, likely results from a weaker interaction of the ends of this DNA fragment with the histone octomer than is present for the segment nearer the center of the core particle.

Interpretation of the data for the E2 fragment is not as straightforward as for the E1 DNA. Clearly, a pattern indicative of nonrandom interaction of histones with the DNA is present. The observation of periodic cutting extending over about 190 bp of DNA might suggest that two nucleosomes form with one or both having a portion of the normal DNA binding region unoccupied. However, the electrophoretic mobility of the E2 complex strongly suggests that only one octomer of histones is bound to the DNA. Another possibility
is that a series of core particles form on the E2 sequence, spaced at approximately 10-bp intervals. This could explain the observation that overall cutting, even at susceptible sites, is less frequent in the central region of the fragment than at the ends by a simple statistical argument.

We feel that a simpler interpretation of the data is the existence of two populations of complexes, each containing a single histone octomer positioned over one or the other of the 72-bp enhancer elements (Fig. 3). This interpretation is supported by the observation of more DNA-like cutting in the end regions of the complexes of E2 and histones than for those of E1. The presence of a central region where overall cutting, even at susceptible sites, is less than in the flanking areas is also consistent with this interpretation; in this region, either positioned nucleosome would protect the DNA. If, indeed, a nucleosome can position over either enhancer sequence in the same location, cutting sites in the overlapping core particle region would be the same for either nucleosome, since the length of the enhancer sequence is nearly 7 times the helical repeat length of DNA in solution. Hence, the region where one or the other nucleosome covers DNA has single cut sites without DNA-like cutting in the protected sections (Fig. 3).

The control experiment using E1 DNA either filled or trimmed at the HindIII site argues against end effects as determinants of in vitro nucleosome positioning. Similarly, the 21-bp imperfect repeats do not appear to determine the core particle location since deletion of these elements in E-32 did not alter the position of histone-DNA interactions. The "signal" specific for interaction of the histone octomer with SV40 enhancer region DNA thus appears to reside in the first 60 bp of the sequence distal to the HindIII site. This contrasts with the results obtained in an analysis of mutant DNA fragments derived from the Lycopsis variegatus 5 S rRNA gene, where sequences located both in the center and one wing of the core particle were found to be critical for in vitro positioning. It is apparent that the currently available data are not sufficient to define the features of DNA sequence or structure which are sufficient to allow formation of a positioned core particle.

While other features of chromatin composition and structure might alter the positions of nucleosomes in vitro from those observed upon in vitro association of DNA and histones, it is certainly possible that a similarly positioned nucleosome might exist in native SV40 chromatin in vivo. Indeed, for another case, the in vitro positioned 5 S rRNA gene nucleosome (12), we have recently found a similar position for the histone binding when the DNA sequence was assembled in vivo into chromatin in a yeast plasmid (21). If nucleosomes positioned on the SV40 enhancer in a similar fashion to that observed here, there are interesting consequences in terms of exposure of potential regulatory sequences. The consensus core enhancer sequence (4) occurs at position 29–38 from the left end of E1. This is a noncut region of the histone complex; hence the minor groove of DNA is facing the octomer, since DNase I cuts by approach to the minor groove. The enhancer DNA major groove thus faces solution components. Further, the core enhancer sequence is located near the end of the nucleosome core particle. The binding site for the 5 S regulatory protein, TFIIA, is also located near one border of the

in vitro phased 5 S core particle (12). When H1 is present, such sequences will be firmly bound in the chromatosome (22) and likely unavailable for interaction with other components. On the other hand, in the presence of H1, such end regions of the core particle DNA interact relatively weakly with the histone octomer and can dislocate from their position on the histone core much more readily than the remainder of the DNA segment (23). Thus, both the core enhancer and 5 S regulatory sequences may be positioned in a location readily modulated by known in vitro structural alterations of chromatin.

If such a phased nucleosome were to interact with a regulatory element and this led to histone dissociation, torsional stress would be introduced into the DNA. Recently, the 5 S gene has been shown to be under such stress when transcriptionally active (24). An interesting possibility arises thereby for the promoter switch found in SV40 transcription. The early region TATA box is more AT-rich than that of the late region (25); the early promoter might be expected to undergo strand separation under torsional stress more readily than the late. This could provide the entry point for RNA polymerase to transcribe early genes. Later, T antigen binding might block propagation of the unwinding stress and thereby switch transcription to the later region.

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