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PREFERENTIAL NUCLEOSOME PLACEMENT ON pBR322 RESTRICTION FRAGMENTS

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Summary: Two restriction fragments of DNA containing the regulatory feature GTG/CAC were experimentally associated with core histones. The reconstituted DNA-histone complexes consisted of different forms of mononucleosomes. Lambda exonuclease and Fnu4HI were used to probe the structure of each distinct nucleoprotein complex. For each of the DNA fragments, one form of particle was produced that showed preferred placement of the core octamer on the DNA. The GTG/CAC base triplets may play some role in determining the final histone core positions in these reconstitutes.

Departure from the regular structure of B-DNA has been reported for a variety of DNA molecules (1). Base-dependent irregularities in shape occur along the backbone of certain DNA sequences (2-4). DNA-protein recognition and binding is accommodated by a complementarity between protein domain and local conformation of the target DNA (5-8). Folding of DNA in the nucleosome requires a decided degree of bending, which includes kinks in the normal path of the DNA helix (9-11).

A number of specific DNA sequential features have recently been identified as possible transcriptional control elements which may operate through chromatin structure. One of these is the CAC/GTG triplet, which has been proposed to be the site of a local structural variation or "molecular notch" on the basis both of nmr studies and an analysis of its frequency of occurrence in a number of prokaryotic and eukaryotic regulatory regions (12-15). It appears as a specific feature of the GCCACACACCC (16,17) and GGCCACGTGACC (18,19) promoter elements in the mouse beta-globin genomic

Abbreviations: CE chicken erythrocyte; **BSA** bovine serum albumin; **bp** base pairs; **nt** nucleotides

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region, and in the 5' upstream region, the CAC frequency is 3.9%, or 2.5 times the statistical frequency of random occurrence. The CACCC box element has very recently been clearly shown to be a strong promoter site in this system (20). Reconstitution experiments suggest that DNA sequence alone can direct DNA-histone core alignment (21-23). We present evidence that CAC triplets may also play a role in preferential nucleosome placement, possibly as a favored kink site in the irregular winding of DNA on the histone octamer (11).

METHODS

Plasmid DNA and End-Labeling Plasmid pBR322 was obtained from **Escherichia coli** RR1 as described (24) with purification on a Sephacryl S-1000 column (Pharmacia). The 298 bp restriction fragment from HinfI digestion of pBR322 was isolated (25). This DNA was end-labeled with both [a32P]-dATP and [a32P]-TTP (NENuclear) using the Klenow fragment (25). The 298 bp fragment was secondarily restricted with either BstNI or HhaI, and the single end-labeled DNA recovered (25).

CE Mononucleosome Preparation Chicken blood was mixed with 3.8% Na citrate (Pel Freez Biologicals). H1-depleted mononucleosomes were prepared as described (26). Mononucleosomes were dialyzed against the reconstitution buffer and stored at 4°.

Reconstitution End-labeled DNA was mixed with an excess of H1-depleted CE mononucleosomes at 2M NaCl and 0.1 mg/ml BSA (V). Five ug of CE mononucleosomes were used in a reconstitution volume of 40 to 60 ul. NaCl was removed in 3 to 5 hours at 4° using a microdialyzer (BRL) and linear gradient dialysis. Samples were dialyzed an additional 2 hours against 0.01M Tris (8.0), 1mM EDTA (8.0), 1.4 mM 2-mercaptoethanol, 0.1 mg/ml BSA (V) at 4° and resolved in native nucleoprotein gels (26).

Nuclease Digestion Free DNA and complexes were treated with lambda exonuclease (NEBiolabs) under conditions specified by the supplier, using sheared salmon sperm DNA [0.2 ug/U enzyme] as a carrier. Fnu4HI (NEBiolabs) digestion was also performed as recommended, in the presence of 0.6 ug/U enzyme pBR322 carrier DNA. All DNA was analyzed in 6M urea denaturing gels (25). Gels were dried and autoradiographed using intensifying screens at -70°.

RESULTS

Two defined DNA fragments from pBR322 that contain GTG/CAC triplets in two distinct arrangements have been purified. **In vitro** reconstitution of this DNA with core histones was performed using salt gradient dialysis. Reconstitutes were electrophoresed in nucleoprotein gels and isolated, which allowed separate analysis of each reconstituted particle that was resolved (27). Comparison of the DNA digestion patterns from lambda exonuclease and

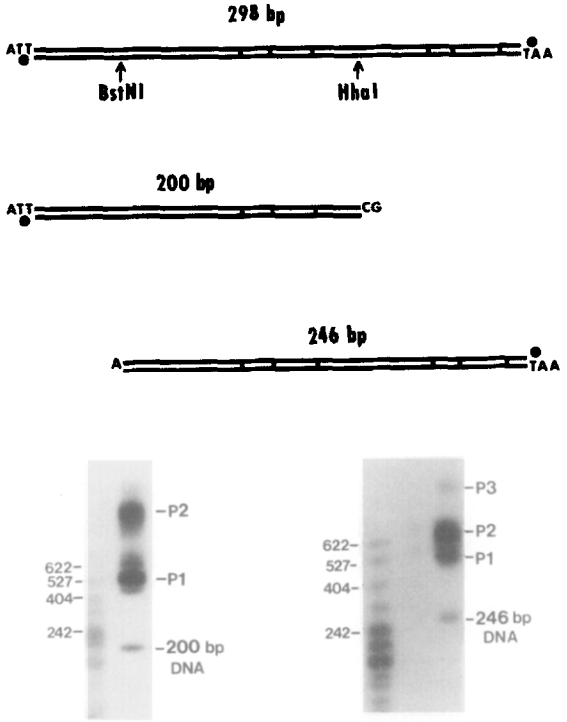

Figure 1. Secondary Fragments and Gel Electrophoresis of the DNA-Histone Particles. Locations of the GTG/CAC triplets are marked. DNA label is indicated with a dot. Nucleoproteins were resolved on 4% polyacrylamide gels. Marker DNA is an end-labeled pBR322-HpaII digest.

Fnu4HI treatment of the naked DNA versus the histone-bound DNA gave regional information about core histone boundaries and histone protection of the DNA.

Hha I restriction of the 298 bp DNA generated the single end-labeled 200 bp fragment, which had three triplets located at its unlabeled end (Fig. 1). Reconstitution of excess core histones with this DNA produced two distinct particles, P1 and P2 which were separated by electrophoresis (Fig. 1). The residual unbound 200 bp DNA migrated well ahead of the histone-bound DNA particles (Fig. 1).

The 200 bp DNA and the histone bound DNA were treated with lambda exonuclease. This enzyme degrades double-stranded DNA from both 5' ends in a highly processive manner (28). The nuclease clearly has some sequence selectivity even on free DNA, and a characteristic limit digest appeared as a pattern of prominent bands indicating preferential cleavage at certain

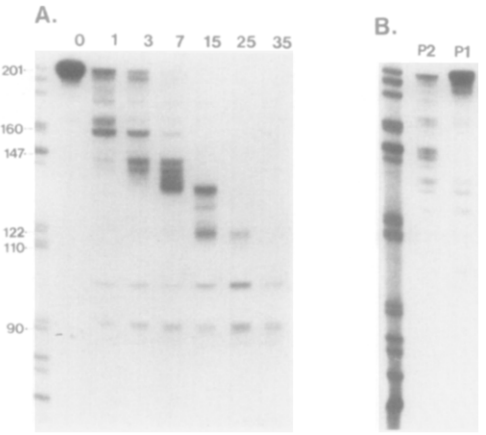

Figure 2. Lambda Exonuclease Digestion of Free vs. Histone-Bound 200 bp DNA. DNA and particles were treated with [5U/ug DNA]. Purified DNA was electrophoresed on an 8% denaturing gel. Lane headings refer to digestion time. Particles were digested for 30 min.

bases. Naked 200 bp DNA was rapidly degraded by lambda exonuclease to a limit digest by 30 minutes (Fig. 2A).

The reconstitutes, under the same limit digest conditions, produced DNA fragments of distinct lengths (Fig. 2B). P1 DNA was largely undegraded, and migrated mainly as 185 nt to 200 nt fragments. This suggested that P1 was bound with histones at the non-labeled 5' end. P2 DNA was digested more readily than P1 DNA. Major sites of inhibition to the exonuclease on P2 occurred at 185, 175, 165, and 145 nts from the 3' label.

Fnu4HI cleaves the 200 bp DNA at three sites (Fig. 3). After 30 minutes, most of the free 200 bp DNA was degraded to the 16 nt fragment (Fig. 3A). However, the particles were cut with differential frequency at the three sites (Fig. 3B). On P1, site V was cut most frequently, followed by site X. There was very little cutting at W. This is consistent with histone protection at the internal regions of the DNA. A core histone octamer bound at the unlabeled end of this DNA could consistently protect the W site. Digestion of P2 was not as complete as that with P1. Both sites V and X were cleaved, but not to the same extent as they were on P1. More importantly, W was cut more frequently on the P2 particle. A variety of internal core positions could make W more accessible on these P2 particles.

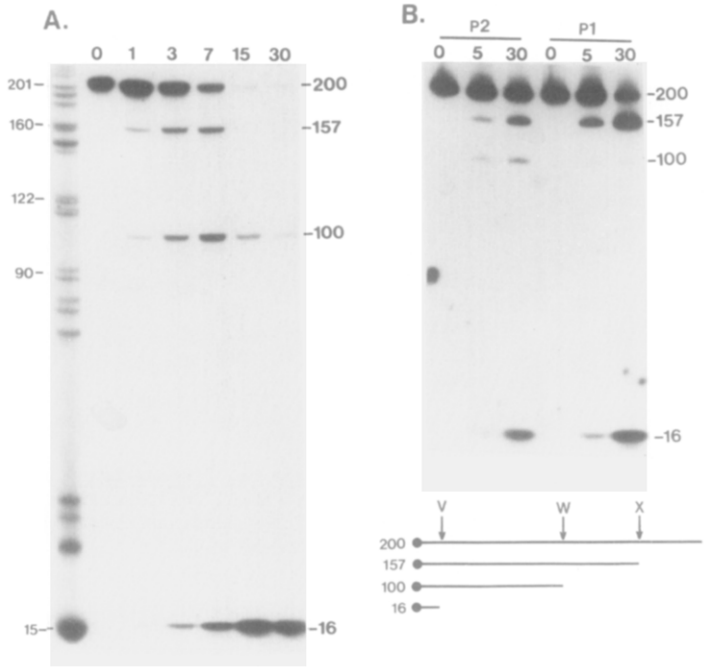

Figure 3. Fnu4HI Treatment of Free 200 bp DNA vs. P1 and P2 Complexes. DNA and particles were digested at [2U/ug DNA]. Restriction sites V, W, and X are shown. 8% denaturing gel.

Secondary restriction of the 298 bp DNA with BstNI generated the 246 bp fragment, which was left with three triplets at the labeled end and three more nearer the 5' end (Fig. 1). This DNA formed three unique complexes upon reconstitution. P3 was a minor, slow migrating particle. The majority of the DNA resolved as two faster migrating particles, P2 and P1 (Fig. 1).

Lambda exonuclease treatment of the 246 bp free DNA produced a limit digest by 20 minutes (Fig. 4A). Limit digest of the P3 particle left DNA ranging in size from 200 nt to 246 nt (Fig. 4B). This indicated an inhibition to digestion at the 5' end of the labeled strand. P1 particle DNA was protected from enzymatic digestion at either end, but the greater proportion of these complexes showed histone octamer binding at the labeled end. Digestion of P2 left a series of DNA bands fairly evenly distributed in length from 246 nt to nearly 125 nt. Major bands occurred at 235, 185, 170, and 165 nts that were unique to the P2 digest. This implied core binding at a number of specific sites on the 246 bp DNA molecule.

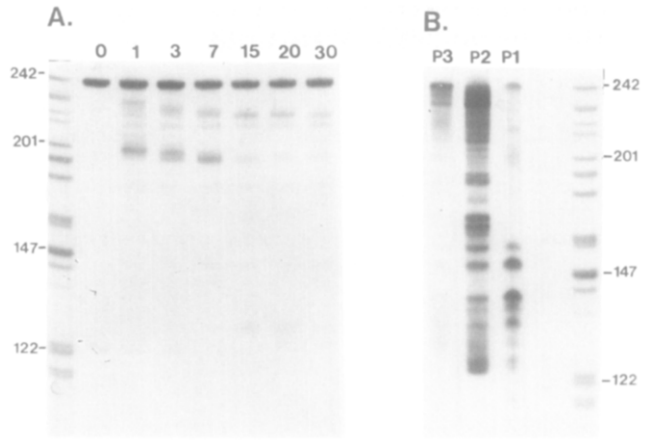

Figure 4. Lambda Exonuclease Digestion of Free vs. Histone-Bound 246 bp DNA. DNA and particles were treated at [4U/ug DNA]. 6% denaturing gel. Particles were digested for 20 min.

Fnu4HI makes 4 restriction cuts within the 246 bp DNA (Fig. 5). By 30 minutes of digestion, the labeled DNA was cleaved to 20 nts (Fig. 5A). In

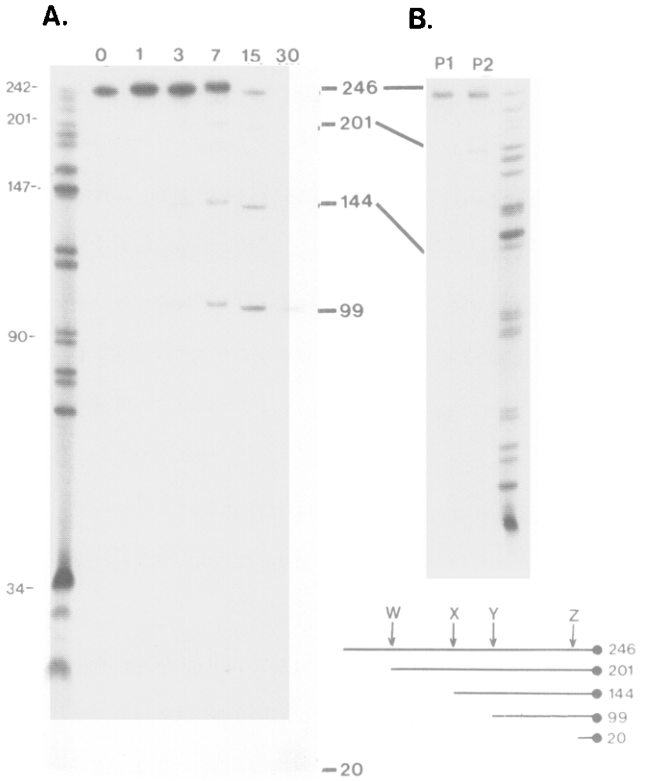

Figure 5. Fnu4HI Treatment of Free 246 bp DNA vs. P1 and P2. DNA and particles digested at [2U/ug DNA]. 6% denaturing gel. Particles were digested for 30 min.

contrast, restriction site Y was protected on both the P1 and P2 particles, even after 30 minutes (Fig. 5B). The bulk of P1 DNA remained intact with some cutting at W and X. P2 DNA was generally not digested, but did show a higher frequency of cutting than P1 at the W and X sites. Different core positions along the sequence on P1 and P2 could provide greater exposure of restriction site X on P2.

DISCUSSION

Analysis of each DNA-histone reconstitute that was resolved showed histone core position to be responsible for differences in electrophoretic mobility. Lambda exonuclease digestion was valuable in detecting histone boundaries along the DNA. Using a restriction enzyme to probe chromatin structure yields information about restriction site accessibility (27,29,30). Fnu4HI site distributions in the two DNA fragments were used to measure this accessibility.

The 200 bp DNA is shorter than the size expected for octameric dimer formation (31). Reconstitutes from the 200 bp fragment produced two electrophoretically distinct particles. The slower of these, P2, appeared to contain a range of internal histone binding sites with core borders concentrated near the triplet-containing end. The faster P1 showed clear preference for octamer binding at the triplet-containing end.

Reconstitutes from the 246 bp fragment were resolved into three nucleoprotein bands. P1 contained a single octamer that covered the triplets on either half of the DNA. Particle P2 was similar in its digestion pattern to the P2 particle from the 200 bp fragment. P3 most likely had excess histones bound since it was protected at one end, as was P1, but migrated much more slowly in the gel (27,32).

These results are highly suggestive that GTG/CAC sites facilitate nucleosome placement. Even in the P1 particles where end effects predominate (27), octamer placement seems to be almost exclusively at the triplet end. Future work will extend these results using additional DNA fragments and other enzymatic digestion methods.

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