Effects of Plasmid Length and Positioned Nucleosomes on Chromatin Assembly in Vitro†

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ABSTRACT: Histone H5 induces extensive nucleosome alignment in vitro, with a 210 ± 5 base pair (bp) average unit repeat, on some of the constructs derived from plasmid pBR327. Plasmid pBR327 itself aligns nucleosomes poorly, even though it possesses a chromatin organizing region which nucleates the alignment reaction [Jeong et al. (1991) *J. Mol. Biol. 222*, 1131–1147]. Examination of various regions of pBR327 chromatin by Southern hybridization revealed no substantial regional differences, suggesting an essentially all-or-none alignment mechanism. Twenty-four pBR327 deletion constructs, with the chromatin organizing region intact, were analyzed for nucleosome alignment in vitro, in addition to the six previously described. Although nucleosome alignment on plasmids of size greater than 5 kb was not affected by small length changes, circular plasmids with total lengths between 2400 and 3600 bp generally permitted alignment only when their lengths were close to integer multiples of 210 ± 3 bp. The measured repeat lengths for the large plasmids and the smaller ones that aligned nucleosomes were all 210 bp, within experimental precision. The failure of two approximately 3.2-kb plasmids to align nucleosomes, even though their lengths were close to 15 × 210 bp, could be attributed to the effects of four strongly positioned nucleosomes that form on pBR327 sequences. Evidence is provided that nucleosome arrays can be quasicrystalline and are capable of transmitting information over a distance of more than 2 kb.

Ordered spaced nucelosomes are the hallmark of chromatin structure. Many questions still exist, however, concerning the generation of such arrays, the precision of the spacing, and the influence of the interactions of histone octamers with particular base sequences on nucleosome alignment. In one extreme model, nucleosomes are essentially randomly arranged along the DNA, with the average spacing simply determined by the average number of nucleosomes deposited per unit length (Kornberg & Stryer, 1988). In the other extreme model, nucleosomes in arrays have precise spacings, almost a crystalline arrangement [see, for example, Kornberg (1981) and Eissenberg et al. (1985) for discussions of the problem]. These two models suggest very different functional consequences. For the latter model, the array can transmit information over a substantial distance. For example, the precise positioning of one nucleosome with respect to the base sequence would be expected to influence nucleosome placement several thousand base pairs away. Also, introducing a defect into a "crystalline" array might destabilize a whole chromatin domain (Weintraub, 1985). The former model would not permit such phenomena.

The ability to efficiently assemble small circular plasmids into chromatin should allow such models to be tested because the DNA length and, consequently, the boundary conditions for the alignment reaction are precisely defined. Chromatin has been efficiently assembled on small plasmids using crude *Xenopus* oocyte (Shimamura et al. 1988; Rodriguez-Campos et al., 1989) or *Drosophilia* embryo (Becker & Wu, 1992) extracts, and these systems tend to support the idea that the average nucleosome spacing is determined largely by the number of nucleosomes on the plasmid. In the oocyte system, the poor resolution of intermediate size nucleosome oligomer lengths excised by micrococcal nuclease (MNase),[1] but good resolution of long and short oligomer lengths, suggested that a population of molecules with different nucleosome spacings was present in the sample, consistent with the measured heterogeneity in plasmid nucleosome number (Rodriguez-Campos et al., 1989). Both the *Xenopus* oocyte and *Drosophila* embryo chromatin assembly extracts contained endogenous core histones, some of which are unusual variants, and the extracts lacked histone H1 (Shimamura et al., 1988; Becker & Wu, 1992), which is known to affect nucleosome spacing (Rodriguez-Campos et al., 1989; Becker & Wu, 1992; Stein & Kunzler, 1983; Stein & Bina, 1984; Stein & Mitchell, 1988). Exogenous histone H1 could be added, resulting in nucleosome spacings greater than 180 bp (the value without H1) (Rodriguez-Campos et al., 1989; Becker & Wu, 1992), but the apparent population of molecules with different spacing periodicities in the sample persisted, and the heterogeneity perhaps increased (Rodriguez-Campos et al., 1989).

Quite different characteristics were observed using a completely defined histone H1-dependent in vitro chromatin assembly system (Jeong et al., 1991). First, histone H5 (or H1) was required in order for regular nucleosome spacings, other than those arising simply from close packing, to occur. Second, the average spacing value appeared to be fixed at 210 ± 5 bp, insensitive to reaction conditions or the linker histone used. Third, a particular region of plasmid pBR327 appeared to be necessary to nucleate the alignment reaction, which could then spread over the entire plasmid. Finally, it seemed remarkable that nucleosomes failed to align appreciably on the parent plasmid, pBR327, whereas excellent alignment with a 210-bp periodicity occurred when a small insertion or several deletions were made that adjusted plasmid lengths to values that were close to integer multiples of 210 bp. Plasmid pBR327 could have generated physiological spacings of 182, 193, or 205 bp, on circular DNA molecules containing 18, 17, or 16

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[1] Abbreviations: bp, base pairs; kb, kilobase pairs; MNase, micrococcal nuclease; COR, chromatin organizing region.

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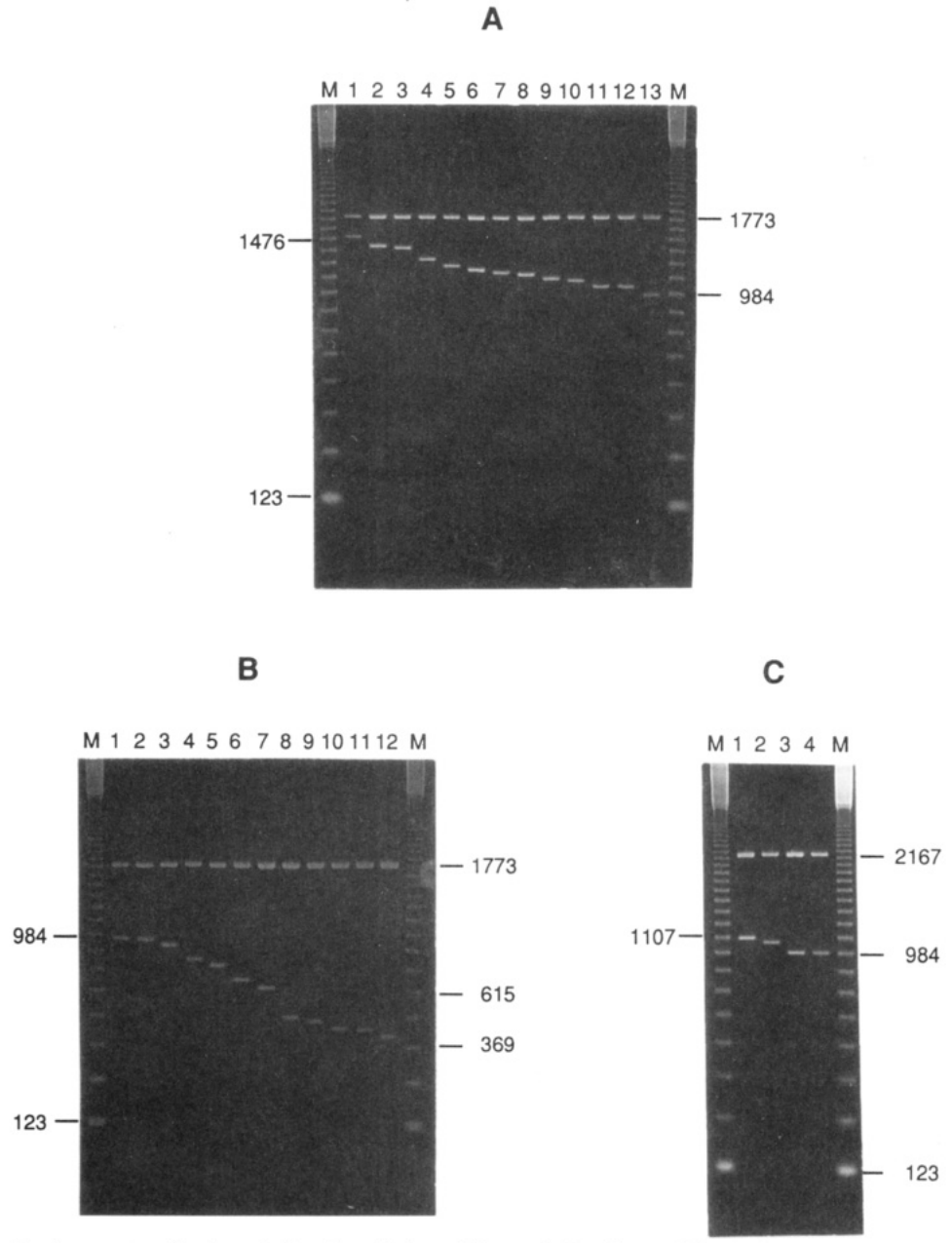

FIGURE 1: Restriction fragments reflecting relative plasmid sizes. The restriction sites used flanked the regions deleted, so that relative sizes could be easily compared and confirmed. (A) Lanes 1–13 show *Hin*dIII + *Alw*NI digests of pBR327, P1, D7, P2, A1, D5, P3, A2, A3, A4, P4, A5, and A6, respectively. The 1773-bp fragment is common to all. (B) Lanes 1–12 show *Hin*dIII + *Alw*NI digests of D4, A7, P5, P6, P7, P8, P9, A8, A9, A10, P10, and A11, respectively. The 1773-bp fragment is common to all. (C) Lanes 1–4 show *Hin*cII digests of pBR327, S1, D7, and S2, respectively. The 2167-bp fragment is common to all. Selected 123-bp ladder marker fragments are indicated.

nucleosomes, respectively, uniformly spaced. In order to investigate this phenomenon further, we have examined the effects of systematically changing the plasmid length by either very small or large amounts. The results largely support a quasicrystalline model and also reveal effects of small plasmid size and of strongly positioned nucleosomes on the overall alignment induced by histone H5.

MATERIALS AND METHODS

Histones, both core and H5, were prepared from chicken erythrocyte nuclei as previously described (Jeong et al., 1991). Nucleosome formation on supercoiled plasmid DNA and nucleosome alignment using histone H5 in a buffer containing 0.15 M NaCl and sodium polyglutamate, as well as micrococcal nuclease (MNase) digestion and electrophoresis, were as previously described (Jeong et al., 1991).

Plasmid Constructions. Deletions of pBR327 were made using exonuclease III and nuclease S1 as described for generating sets of nested deletions (Sambrook et al., 1989) except the exonuclease reactions were carried out at 23 °C instead of 37 °C to slow the rate of nucleotide removal to approximately 100 nucleotides/min. Deletions were made either unidirectionally using pBR327 cut with the restriction enzymes *Aat*II and *Ssp*I or bidirectionally using pBR327 cut with either the restriction enzyme *Pst*I or *Sph*I. Since both *Pst*I and *Sph*I leave 4-base 3′ overhangs, it was necessary to first make the ends of the molecules blunt ended using Klenow fragment of *Escherichia coli* DNA polymerase I following standard procedures (Sambrook et al., 1989). The samples were purified by phenol extraction/ethanol precipitation prior to treatment with exonuclease III. After transformation of either HB101 or DH5α, colonies were screened for various extents of deletion. Constructs of the desired size were then

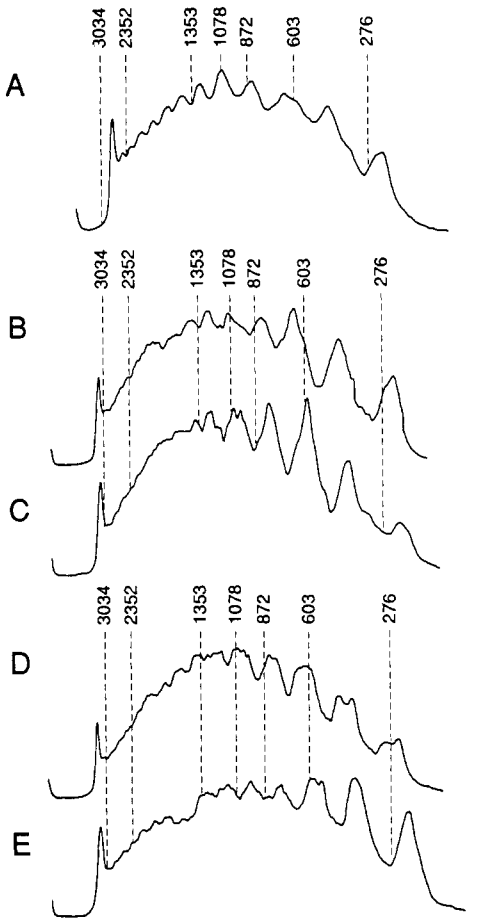

FIGURE 2: Assessment of nucleosome alignment in various regions of plasmid chromatin. Plasmid chromatin was digested with 4.2 units of MNase/μg of DNA for 2 min at 37 °C, and the separated DNA fragments were blotted and detected by Southern hybridization. Densitometer scans of autoradiograms are shown. Chromatin assembled using the length-adjusted plasmid D4 was probed with a DNA fragment corresponding to nucleotides 1690–1947 on the pBR327 map (A). Chromatin assembled using pBR327 (3274 bp) was probed with DNA fragments corresponding to nucleotides 1690–1947 (B), 1268–1486 (C), 375–566 (D), and 3085–3274 (E). Migration positions of marker fragments are indicated.

prepared for nucleosome reconstitution as described (Jeong et al., 1991). The constructs were divided into three clusters on the basis of the deletion origin: A, P, and S, denoting deletions originating from the *Aat*II, *Pst*I, and *Sph*I sites, respectively.

Constructs were sized by analysis of the DNA fragments generated after digestion with various restriction enzymes. The DNA fragments were sized on either 1.5%, 2.0%, or 2.5% agarose gels run in TBE (89 mM Tris–borate, pH 8.0, 2 mM Na_2EDTA) buffer and calibrated using 123-bp ladders (BRL). The 123-bp ladders were run in lanes flanking the restriction fragments to be sized. Additionally, restriction fragments of known size were also used as size markers. Constructs that were deleted from the *Ssp*I site were digested with either *Hin*dIII and *Pst*I, *Alw*NI and *Cla*I, or *Hin*dIII and *Hin*fI. Constructs that were deleted from the *Pst*I site were digested with *Hin*dIII and *Alw*NI, *Hin*dIII and *Hin*fI, *Bsa*I and *Cla*I, or *Dde*I. Constructs that were deleted from the *Sph*I site were digested with *Ava*II or *Hin*cII. Generally, the total construct size was determined with a precision of ±10 bp. Figure 1 shows restriction fragments reflecting the relative sizes of the constructs in each of the three deletion sets.

The anonymous chicken erythrocyte DNA fragment used to increase plasmid size was chosen randomly from clones containing *Bam*HI-digested chicken erythrocyte DNA inserted into the *Bam*HI site of pBR327. This 2.3-kb fragment was subsequently subcloned into the *Bam*HI site of D7, D8, and D1 and also into the *Eco*RI site of pBR327, D1, P2, P8, A10, S2, and D9.

Deletion constructs D7, D8, and D9 were constructed by digestion of pBR327 with restriction enzymes, treatment with the Klenow fragment of *E. coli* DNA polymerase I, where necessary, and religation of the resulting blunt ends. D7 was constructed by deletion of the 118-bp *Ssp*I–*Aat*II fragment of pBR327. D8 was constructed by deletion of the 215-bp *Ssp*I–*Cla*I fragment of pBR327. D9 was constructed by deletion of the 595-bp *Bam*HI–*Nru*I fragment from pBR327. Constructs D8 and D9 were used only in the experiments for which the plasmid size was increased by insertion of a 2.3-kb anonymous chicken genomic fragment. D1 was described previously (Jeong et al., 1991).

Southern Blotting/Hybridization. Hybridization of DNA was performed as described (Jeong et al., 1991) except hybridizations were carried out at 68 °C instead of 65 °C. DNA restriction fragments from pBR327 were used to probe pBR327 and D4. These were the 218-bp *Taq*I–*Taq*I fragment (1268–1486 on the pBR327 map), the 191-bp *Bam*HI–*Sph*I (375–566) fragment, the 257-bp *Alu*I fragment (1690–1947), and the 189-bp *Ssp*I–*Eco*RI (3085–3274) fragment. The DNA restriction fragment used to probe pBR327, and S2 in the indirect end-label experiment was the 189-bp *Eco*RI–*Eco*RV fragment. The DNA restriction fragment used to probe pBR327, P2, and P4 in the indirect end-label experiment was the 189-bp *Ssp*I–*Eco*RI fragment. The 191-bp *Bam*HI–*Sph*I restriction fragment from pBR327 was used to probe constructs containing the 2.3-kb anonymous genomic chicken fragment inserted into the *Bam*HI site. The 191-bp *Bam*HI–*Sph*I and the 188-bp *Eco*RV–*Bam*HI restriction fragments from pBR327 were used to probe constructs containing the 2.3-kb anonymous genomic chicken fragment inserted into the *Eco*RI site. For the indirect end-label analysis, DNA electrophoresis was performed on 5-mm-thick 1.5% (w/v) submarine agarose gels using TBE buffer. These gels were 20 cm long and 15 cm wide, and contained slots of dimensions 1 mm × 6 mm. DNA was loaded at 100 ng/slot, and electrophoresis was at 5 V/cm for 4.5 h. Gels were calibrated using end-labeled 123-bp ladder (BRL) flanking the lanes of interest.

Measurement of Nucleosome Repeat Lengths and Criteria for Determining Nucleosome Alignment. The nucleosome repeat lengths were measured on calibrated gels. Gels were first calibrated using a 123-bp ladder (BRL). The midpoints of MNase ladder bands were then sized and plotted versus band number. The slope of the least-squares line through the points in a plot of oligomer length (for the oligomers greater than dimer, excluding the full-length linear) versus oligomer number gave the repeat length (Thomas & Thompson, 1977). Either the bands were measured directly from photographic negatives or the negatives were densitometrically scanned and measurements were then made from the traces. Generally, the nucleosome repeat was determined with a precision of ±5 bp.

Constructs were considered "aligning" if the MNase ladder bands were visible above the background fluorescence and periodic (some multiple of a unit length). Some constructs had a closely spaced group of bands instead of a single band in some positions. This was due to the presence of preferred MNase cutting sites and should be expected for unique sequence DNA. These constructs were considered "aligning"

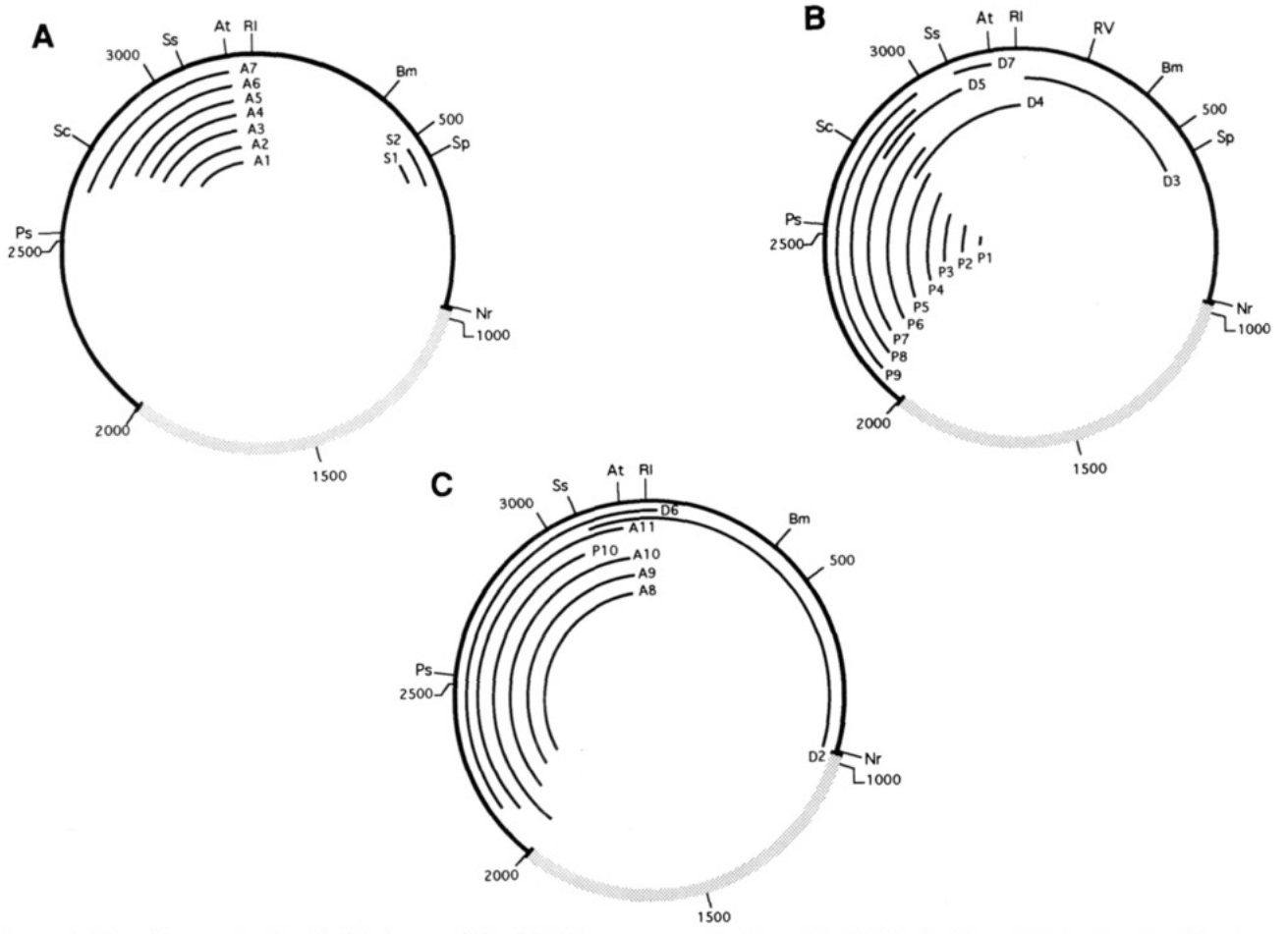

FIGURE 3: Plasmid maps showing the deletions made in pBR327 to construct A1–A7 and S1, S2 (A); P1–P9 and D3, D4, D5, D7 (B); A8–A11, P10, D2, D6 (C). Restriction sites *Eco*RI (RI), *Eco*RV (RV), *Bam*HI (Bm), *Sph*I (Sp), *Nru*I (Nr), *Pst*I (Ps), *Sca*I (Sc), *Ssp*I (Ss), and *AatII* (At) are indicated (B). Construct I1 contained a 301-bp fragment of *E. coli* DNA inserted into the *Eco*RV site. Base pairs are numbered clockwise from the *Eco*RI site. The shaded region on the map contains the COR.

as long as the bands were well resolved from those of other oligomers in the ladder and the center of the group fell within a periodic array. Finally, "aligning" constructs should have MNase ladders which are stable during fairly extensive digestion.

RESULTS

Nucleosome Alignment Is Relatively Poor throughout pBR327 DNA Compared with a Length-Adjusted Construct. Plasmid pBR327 contains an intact chromatin organizing region (COR), a signal which can nucleate the formation of a long and well-ordered spaced nucleosome array when histone H1 (or H5) is added, yet fails to exhibit appreciable overall nucleosome alignment (Jeong et al., 1991). As a first step toward understanding why nucleosome alignment on this plasmid is inhibited, we examined the local alignment of nucleosomes in different plasmid regions by Southern hybridization using short probes. Since this plasmid length is not close to a multiple of the 210-bp nucleosome repeat usually generated when alignment occurs, one possibility was that histone H5 might have spaced nucleosomes properly in the vicinity of the COR but that those distal to the nucleation signal could not be accommodated into the array due to the incorrect boundary conditions. Alternatively, nucleosome alignment might be essentially an all-or-none reaction. Thus, nucleosomes all over the relatively small (3.3-kb) plasmid might communicate with each other, in the presence of histone H5, and sense whether the total space available is adequate for the formation of a single continuous array with a 210-bp repeat. If the space available is not adequate, arrays of appreciable length migh not form anywhere on the plasmid.

The results of this analysis, shown in Figure 2, support the latter possibility. Densitometer scans of DNA fragment lengths excised by MNase from pBR327 "chromatin" show the extent of alignment in four different regions of the plasmid. The extent of alignment in the vicinity of the COR (parts B, and C) was not appreciably better than in other regions, and all regions exhibited significantly poorer ladders in the upper region of the gels than for a length-adjusted construct (tracing A).

Nucleosome Alignment on Small Plasmids Is Strongly Influenced by Small Changes in Plasmid Size. We next sought to directly test the hypothesis that in order or nucleosome alignment to occur in this system, the total DNA length must be close to an integer multiple of the nucleosome repeat generated, a type of boundary effect (Jeong et al., 1991). Thus, the internucleosome spacing would have to be sufficiently precise to distinguish among boundary conditions imposed by the DNA length on circular plasmid molecules. To assess the effects of small, as well as larger, changes in plasmid length on nucleosome alignment in pBR327 "chromatin," exonuclease III digestions to various extents (plus S1 digestion) were performed in several regions of the plasmid. Clones were selected to achieve a variety of plasmid lengths. Care was taken to maintain the intact COR. The locations and the extents of the deletion sets are shown on the plasmid maps in Figure 3. Several deletion constructs prepared using restriction enzymes, D2–D6, from our previous study, and the one

Table I: Summary of Results and Accessible Repeats

construct	length (bp)	observed repeat[a]	accessible repeats
I1	3575	210 ± 4	188.2, 198.6, 210.3
pBR327	3274	N	181.9, 192.6, 204.6, 218.3
S1	3230	N	190.0, 201.9, 215.3
P1	3171	N	186.5, 198.2, 211.4
D7	3156	210 ● 5	185.6, 197.3, 210.4
S2	3154	N	185.5, 197.1, 210.3
P2	3036	N	189.8, 202.4, 216.8
A1	2973	(212 ± 6)	185.8, 198.2, 212.4
D5	2950	210 ± 4	184.4, 196.7, 210.7
P3	2933	210 ± 5	183.3, 195.5, 209.5
A2	2910	N	181.9, 194.0, 207.8
A3	2872	N	191.5, 205.1
A4	2858	N	190.5, 204.1, 219.8
P4	2816	N	187.7, 201.1, 216.6
A5	2812	(210 ● 5)	187.5, 200.8, 216.3
A6	2751	209 ± 5	183.4, 196.5, 211.6
D3	2734	210 ± 5	182.3, 195.3, 210.3
D4	2733	210 ± 4	182.2, 195.2, 210.2
A7	2725	209 ± 5	181.7, 194.6, 209.6
P5	2692	(209 ● 6)	192.3, 207.1
P6	2577	N	184.1, 198.2, 214.8
P7	2556	N	182.6, 196.6, 213.0
P8	2453	N	188.7, 204.4
P9	2401	N	184.7, 200.1, 218.3
A8	2260	202 ± 5	188.3, 205.4
A9	2243	202 ● 5	186.9, 203.9
A10	2207	201 ● 5	183.9, 200.6
P10	2204	(199 ± 6)	183.7, 200.4
A11	2177	N	181.4, 197.9, 217.7
D6	2116	N	192.4, 211.6
D2	2109	N	191.7, 210.9

[a] "N" denotes nonaligning constructs. Parentheses denote marginal alignment. The separation in the table body marks the approximate size range where the observed repeat value changed.

designated D7, have also been included on the maps and in Table I. Supercoiled plasmid was in all cases used for the in vitro chromatin assembly reactions.

The results of the chromatin assembly reactions are summarized in Table I. For the plasmid constructs where nucleosome alignment was successful, by the criteria stated in the Materials and Methods Section, the repeat length was measured and the value is reported. In those plasmid constructs where alignment was poor, an "N" is entered, for nonaligning. Selected gel photographs, representative of the data set, are shown in figure 4. It can be seen that the approximately 117-bp length difference between constructs P3 and P4 from the "P" cluster had a substantial effect on the MNase ladders generated. In P3, 13 bands, which are multiples of 210 bp, were generated in addition to the full-length linear, whereas, in P4, only 4 or 5 well-defined bands were generated. The large difference in the extent of nucleosome alignment between these two constructs is similar to that observed between the larger plasmids, pBR327 (3.3 kb) and I1 (3.6 kb), described previously (Jeong et al., 1991). In the "A" cluster, plasmid A1 (3 kb) permitted fairly good nucleosome alignment, whereas the 63-bp smaller plasmid A2 did not. Similarly, upon further deletion, A6 (lane 2 of A5/A6) aligned nucleosomes well and slightly better than A5 (lane 1), which was 61 bp larger. Also, A5 aligned nucleosomes better than A4, which was only 46 bp (1.6%) larger. It can be seen from Figure 4 that plasmids A3 and A4 generated many bands in the central region of the gel that were not multiples of a unit nucleosome repeat. Therefore, these data suggest that nucleosome alignment is quite sensitive to plasmid size.

The results in Table I also suggest that a purely size-dependent change in the alignment properties of the constructs occurs as the size is decreased below about 2.4 kb (the constructs listed below the separation in the table body). The very small constructs that align nucleosomes have measured repeats of about 200 bp, instead of 210 bp. For example, the 2.2-kb construct A10 aligns nucleosomes well with a periodic 201 ± 5 bp spacing. As shown in Figure 4, an MNase ladder of 11 multiples was generated. Plasmid A11, which is only 30 bp smaller, aligns nucleosomes less well. The precise plasmid size for which the repeat length change from 210 to 200 bp occurs could not be determined from the data available.

These circular DNA molecules with different lengths can be regarded as having different accessible nucleosome repeats for uniformly spaced nucleosomes, defined by the boundary conditions. Thus, for pBR327 (3274 bp), molecules containing 18 evenly spaced nucleosomes would be expected to have a repeat of 182 bp (3274/18). Similarly, molecules containing 17, 16, or 15 evenly spaced nucleosomes would be expected to have repeats of 193, 205, or 218, respectively. However, if these accessible repeats do not coincide (to within a certain tolerance) with a sufficiently precise actual repeat, the formation of a continuous ordered array might not occur. This could provide an explanation for the failure of pBR327 to align nucleosomes, in contrast with constructs having accessible repeats coinciding with observed repeats. The different plasmid sizes lead to a variety of different accessible repeats (listed for each plasmid in Table I), and we can ask whether successful nucleosome alignment correlates with the existence of particular accessible repeats and whether the observed (measured) repeat coincides with a particular accessible repeat.

In order to better reveal the pattern in the data, we have illustrated the accessible repeats for each plasmid diagrammatically, as a function of plasmid size. Figure 5 shows the (calculated) accessible repeats from 180 bp to 220 bp, which almost spans the physiological range for histone H1-containing chromatins (Van Holde, 1989; Widom, 1992). In general, Figure 5 reveals that the aligning plasmids (open circles) had different accessible repeats than the nonaligning plasmids (filled circles). For example, all of the aligning plasmids with lengths greater than 2.4 kb, except the marginal A5 (2.8 kb), had accessible repeats very close to 210 bp. Plasmid A5, which had an accessible repeat of 216 bp, might be a special case, and it is discussed below. The smaller aligning plasmids had accessible repeats around 200 bp. Correspondingly, with two apparent exceptions (plasmids P1 and S2, just under 3.2 kb in size, arrows), the nonaligning plasmids greater than 2.4 kb lacked accessible repeats between about 208 and 213 bp, and the nonaligning plasmids less than 2.4 kb lacked accessible repeats between about 198 and 211 bp. Additionally, one accessible repeat for each of the aligning plasmids matched the observed repeat (crosses), within experimental precision, consistent with the hypothesis proposed.

Boundary Effects Due to Plasmid Length Are Relieved When the Plasmid Size Is Increased. It was next of interest to know the approximate size upper limit for which small length changes affect nucleosome alignment. To address this question, we selected a random clone containing an anonymous 2.3-kb chicken genomic DNA insert in the *Bam*HI site of pBR327 to increase the plasmid size. This construct was assembled into chromatin as above. Figure 6 (lane 1) shows that upon probing the plasmid DNA at the junction between the COR and the insert on a Southern blot a strong 210-bp ladder, containing more than 14 bands, can be detected. Since the >14-band ladder encompasses more than 3 kb of DNA, this result suggests that nucleosome alignment spread, with

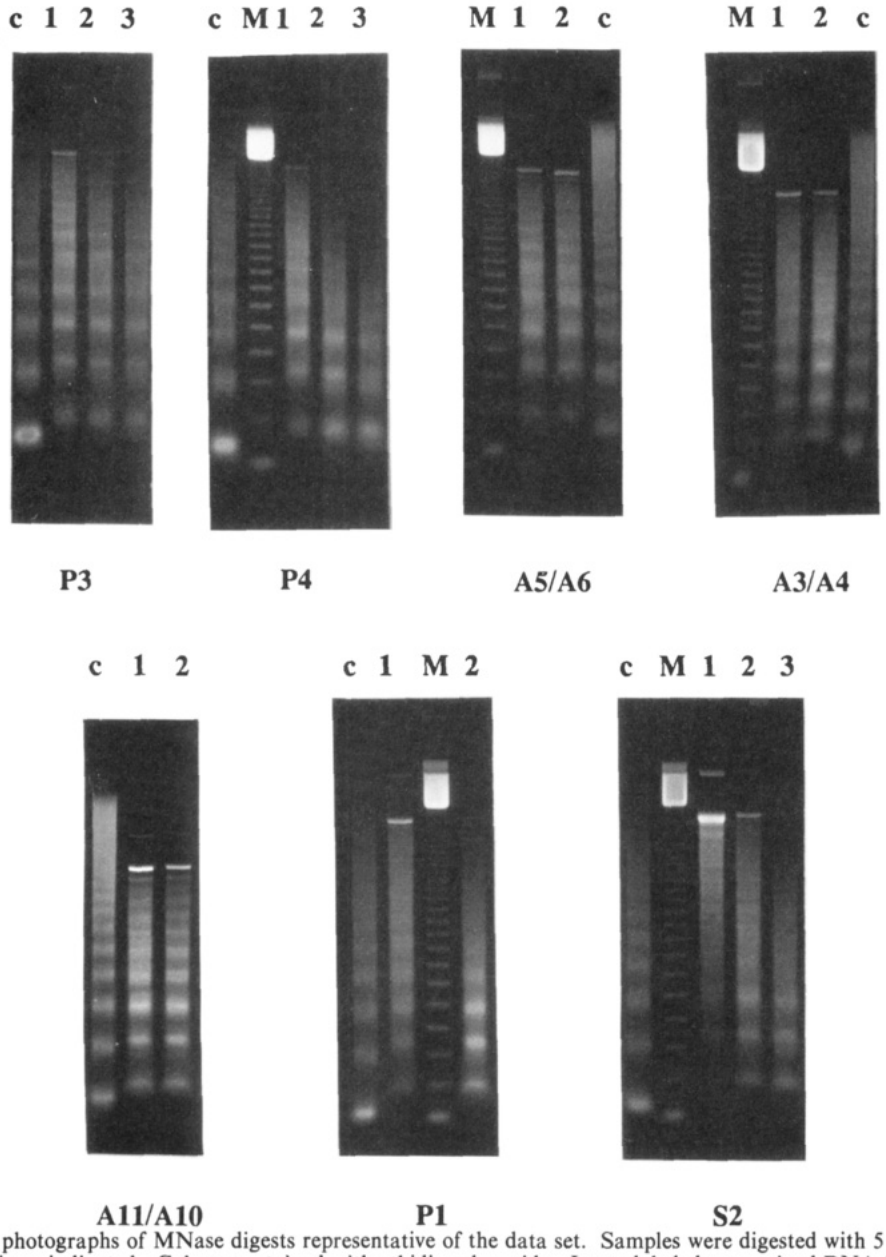

FIGURE 4: Selected gel photographs of MNase digests representative of the data set. Samples were digested with 5 units of MNase/μg of DNA at 37 °C for the times indicated. Gels were stained with ethidium bromide. Lanes labeled c contained DNA fragments from digests of chicken erythrocyte chromatin; lanes labeled M contained 123-bp ladder markers. (P3) Lanes 1–3 show digestion times of 2, 3, and 4 min, respectively. (P4) Lanes 1–3 show digestion times of 2, 5, and 8 min, respectively. (A5/A6) Lanes 1 and 2 show 2-min digests of A5 and A6, respectively. (A3/A4) Lanes 1 and 2 show 2-min digests of A3 and A4, respectively. (A11/A10) Lanes 1 and 2 show 2-min digests of A11 and A10, respectively. (P1) Lanes 1 and 2 show 1- and 2-min digests, respectively. (S2) Lanes 1–3 show 1-, 2-, and 4-min digests, respectively.

the characteristic 210-bp spacing, from the COR (less than 1 kb in extent) over about 600 bp of plasmid DNA and well onto the chicken DNA. Moreover, the control experiment shown in lane 2 shows that when a portion of the (plasmid) COR was deleted from this large construct, no appreciable nucleosome ordering could be detected using the same probe. Thus, the portion of this chicken DNA fragment near the probe does not align nucleosomes on its own. Lanes 3 and 4 show that strong 210-bp ladders, virtually indistinguishable from that of lane 1, were detected when another plasmid region (outside of the COR) was deleted. Here, the deletions made were 118 or 215 bp, showing that nucleosome alignment on a plasmid of this size (5.3 kb) is no longer sensitive to small changes in plasmid length. It should be mentioned that the overall nucleosome alignment on the plasmid, detected by ethidium bromide staining (not shown), was not as good as that detected in the vicinity of the COR, suggesting that the alignment spread for several kilobases pairs, but not over the entire large plasmid.

Nonaligning Constructs Exhibited Good Alignment with a 210-bp Spacing Periodicity When the Plasmid Size Was Increased. The two apparent exceptions to the rule that accessible repeats and actual repeats must coincide for nucleosome alignment to occur (plasmids P1 and S2) indicate that alignment is influenced by more than just plasmid size. One possible explanation is considered here. Because the boundaries of the COR, which is necessary for alignment, were not very well defined in our previous study (Jeong et al., 1991) due to the relatively small number of deletion constructs used, it might have been larger than initially estimated. Thus, poor alignment or different repeat lengths might have arisen from deleting various portions of the COR. This explanation was quite plausible because we have now reclassified deletion constructs D2 and D6, which flank the COR, as nonaligning

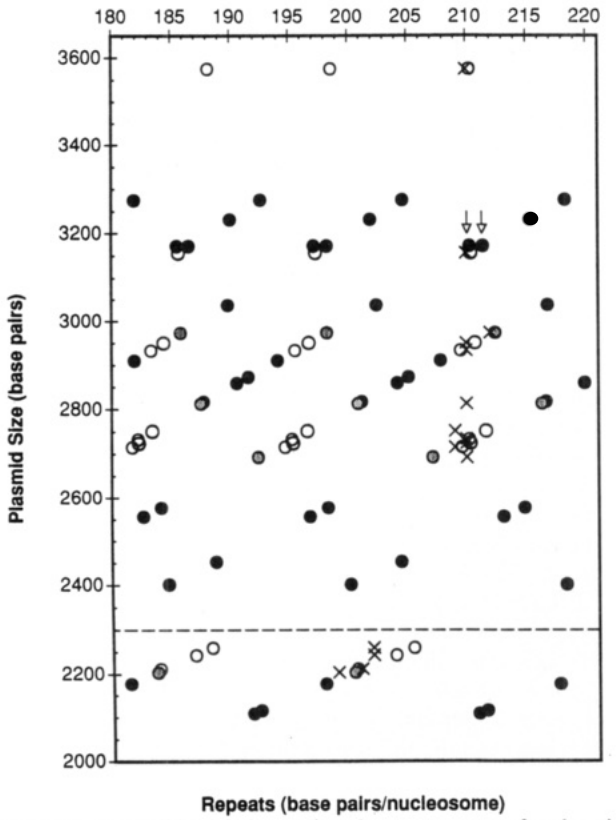

FIGURE 5: Accessible and observed nucleosomes repeats for plasmid pBR327 constructs of varying size. Accessible repeat values (calculated as described in the text) for each of the plasmids listed in Table I were plotted versus plasmid size. Filled circles were used for nonaligning constructs, open circles represent constructs that aligned nucleosomes well, and shaded circles represent marginally aligning constructs. Crosses indicate the measured values of the observed repeats for aligning plasmids. Symbols for constructs S2, A10, and D3 were offset upward by about 15 bp so that they could be distinguished from other constructs with essentially the same sizes. The dashed line marks the approximate plasmid size where the observed repeat value changed.

(on the basis of more strict criteria than used before). Because the inhibitory effects resulting from small changes in plasmid size could be eliminated by using larger plasmids (see above), we could now directly test whether both the inhibitory effects and the repeat length changes that we observed were consequences of deleting portions of the COR or were truly boundary effects of some type.

To perform these experiments, we inserted the same (2.3-kb) anonymous chicken DNA fragment used above into the *Eco*RI site of pBR327 and various deletion constructs in order to considerably increase the sizes of these plasmids. Figure 7A, lane 2, shows that a strong MNase ladder with a 210-bp spacing, extending for more than 3 kb, was detected for the pBR327-derived construct, upon probing a Southern blot in the region of the plasmid between the COR and the insert on the right-hand side of the map (Figure 3). Again, the control construct (lane 1) that lacked an intact COR failed to exhibit appreciable order, using the same probe. Lanes 3, 4, and 5 show the analogous experiments with chicken DNA inserted into P1 and P7, which were nonaligning, and A10, which exhibited a 201-bp spacing periodicity. It can be seen that all three samples exhibited very similar extended ladders, essentially indistinguishable from that of the intact pBR327-derived construct (lane 2) and much better than those obtained on the small plasmids that lacked the chicken DNA insert (see Figure 4 for the P1 MNase digest). These experiments show that the deletions made in P1, P7, and A10 did not

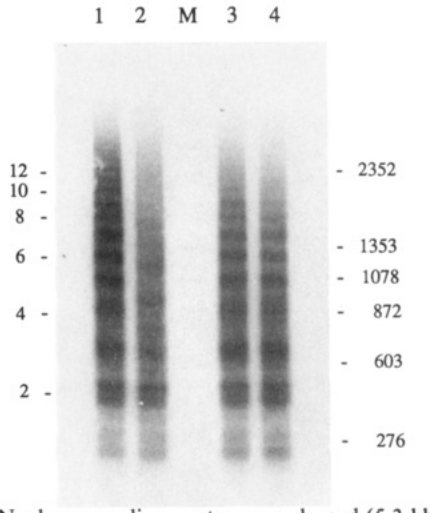

FIGURE 6: Nucleosome alignment on an enlarged (5.3-kb) plasmid is no longer sensitive to small changes in plasmid size. Samples were digested with 5 units of MNase/μg of DNA at 37 °C for 1 min. A Southern blot is shown (probe described in the text) for four different pBR327 constructs, each of which contained the same 2.3-kb insert in the *Bam*HI site. Lane 1, all pBR327 sequences were present, lane 2, a 535-bp portion of the COR was deleted; lane 3, a 118-bp portion of the plasmid DNA was deleted; lane 4, a 215-bp portion of the plasmid DNA was deleted. Nucleosome oligomer multiples are identified on the left. *Hae*III + *Acc*I ΦX174 RF DNA marker fragments in lane M are identified on the right.

invade or influence the COR. Additionally, these results are consistent with our previous estimate for the left-hand boundary of the COR.

We next examined the properties of a size-expanded construct prepared from the nonaligning plasmid S2 and also tested the right-hand boundary of the COR using a large plasmid. Figure 7B, lane 3, shows that deleting 595 bp of plasmid DNA going counterclockwise from the *Nru*I site (see Figure 3) did not inactivate the COR, consistent with our previous estimation of this COR boundary. The strong 210-bp MNase ladder in lane 3 is essentially identical to that for the control construct in lane 2, which contained all of the pBR327 sequences. The S2-derived construct (lane 1) also exhibited a strong MNase ladder, in contrast with S2 itself (Figure 4), but an approximately 110-bp length decrease occurred for all nucleosome oligomer lengths greater than the 3-mer. This phenomenon is consistent with the 123-bp S2 deletion being between the COR and a strongly positioned nucleosome and the propagation of nucleosome alignment through an incorrectly located fixed nucleosome (see below).

Positioned Nucleosomes Influence the Alignment Induced by Histone H5. Since the COR was intact in all of the deletion constructs examined here and its properties were unaltered when boundary effects due to small plasmid size were eliminated, a likely explanation for the two apparent exceptions to the accessible repeat–actual repeat coincidence rule is the influence of strongly positioned nucleosomes. Previously, we identified two positioned nucleosomes in the COR, which appeared to remain fixed when histone H5 induced alignment (Jeong et al., 1991). The phenomena observed here are consistent with the existence of two additional fixed nucleosomes, one located near the *Eco*RV site, giving rise to the shifted MNase ladder described above (Figure 7B, lane 1), and another near the *Pst*I site. A fixed nucleosome near the *Pst*I site would be consistent with the small deletion in P1 (nonaligning) leaving this presumed nucleosome in place, incorrectly situated relative to the two fixed nucleosomes of the COR, while the larger deletion in P3 (aligning) should have removed this nucleosome and its interfering effect.

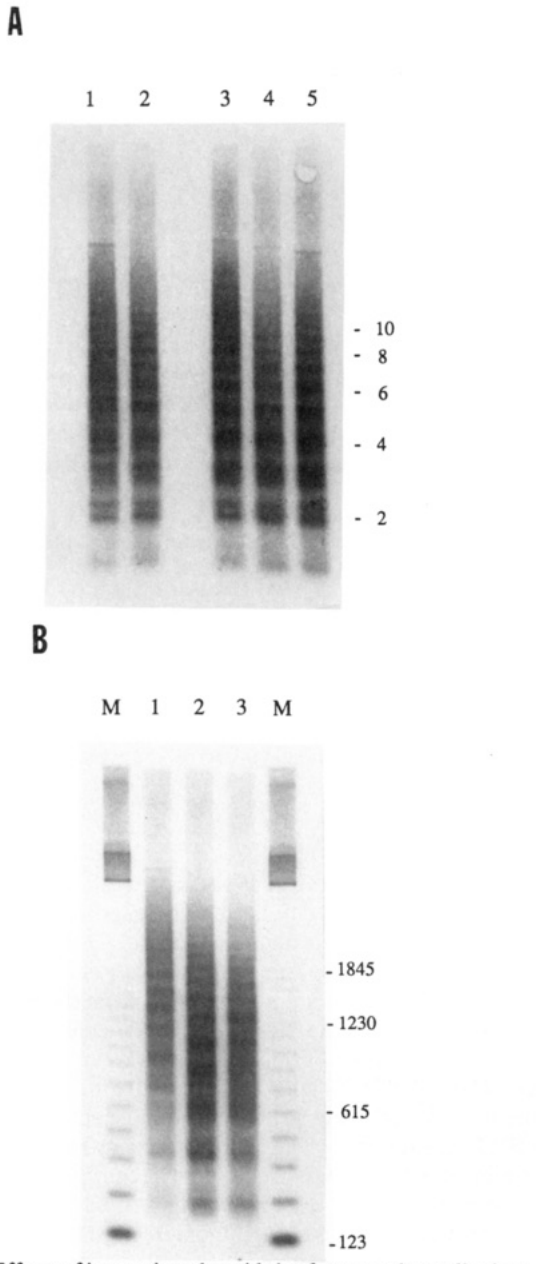

FIGURE 7: Effects of increasing plasmid size for several nonaligning constructs and a construct that generated a 200-bp repeat. Samples were digested with 5 units of MNase/μg of DNA and digested for 1 min at 37 °C. Southern blots (probe described in the text) are shown for various pBR327 constructs that contained the 2.3-kb insert (used in Figure 6) in the *Eco*RI site. (A) Lane 2, pBR327 was used for the insertion; lane 1, pBR327 containing a 535-bp deletion in the COR was used; lane 3, construct P1 was used; lane 4, construct P7 was used; lane 5, construct A10 was used. Nucleosome oligomer lengths are indicated. (B) Lane 1, construct S2 was used for the insertion; lane 2, pBR327 was used; lane 3, pBR327 containing a deletion extending 595 bp counterclockwise from the *Nru*I site was used. Lanes labeled M contained 123-bp ladder markers; selected lengths are indicated.

The existence of a positioned fixed nucleosome in the vicinity of the *Pst*I site in construct D4 is very apparent form the indirect end-label (Nedospasov & Georgiev, 1980; Wu, 1980) analysis performed previously (Figure 7B; Jeong et al., 1991), although this was not commented upon. The region of the plasmid near the *Eco*RV site was too close to the probe to analyze. We have now examined nucleosome positioning on pBR327, P1, P3, and S2 (in the absence of histone H1 or H5) to see if it is consistent with the above hypothesis.

The criteria used to identify a positioned nucleosome was (i) partial protection of an MNase sensitive site(s), observed in naked DNA, in a way consistent with the size of a nucleosome, and (ii) enhancement of cutting at one or both sites immediately flanking the protected region. Uniquely positioned nucleosomes do not necessarily have higher affinities for DNA than nonpositioned nucleosomes. Thus, protection of particular MNase sites need not be complete due to the presence of molecules in the sample that contain lower than the mean nucleosome number.

Figure 8A shows indirect end-label analysis of nucleosome positioning near the *Pst*I site on plasmids pBR327, P1, and P3. In pBR327, the bands corresponding to DNA cleavages that were 790 and 828 bp from the *Eco*RI reference site are partially diminished in intensity in the histone reconstituted sample (R), and cleavage was enhanced at the sites 674 and 842 bp (a 168-bp region) immediately flanking the protected region. This provides good evidence for the existence of a positioned nucleosome near the *Pst*I site, as indicated in the diagram. For the small deletion giving rise to construct P1, the MNase cleavage site 674 bp from the reference site (still present), which defined one edge of the positioned nucleosome in pBR327, was still enhanced, and the sites now at 728 and 795 bp from the reference site were partially protected. Thus, this experiment strongly suggests that a nucleosome was able to form in essentially the same position (from the *Eco*RI site) as in pBR327, despite the 100-bp deletion. In contrast, for the 341-bp deletion (P3), no evidence for nucleosome positioning was detected, as expected, since the entire histone octamer binding site was deleted. These data therefore support our hypothesis for the influence of a positioned fixed nucleosome in this region of the plasmid. Additionally, the shift in the positions of strong MNase binding sites in the naked DNA samples for the two deletion constructs, relative to pBR327, confirmed our previous measurements on the deletion sizes, within experimental error.

Similar results were obtained for construct S2, where the deletion was in another region of the plasmid. Here, the MNase site at 216 bp from the reference site was partially protected, and the flanking sites at 135 and 322 bp were enhanced in S2, as well as pBR327 (Figure 8B). This result strongly suggests that a positioned nucleosome was present near the *Eco*RV site, as shown, consistent with the hypothesis proposed above.

DISCUSSION

We have investigated how boundary conditions influence nucleosome alignment in an H5-dependent in vitro system. On large (>5-kb) plasmids that contain a chromatin organizing region (COR), which nucleates the alignment reaction, an ordered nucleosome array appeared to spread 3–4 kb, with a spacing periodicity of 210 ± 5 bp. Consistent with nucleosomes in the array having fairly precise relative spacings, almost as if in a crystalline arrangement, alignment on small (<4-kb) circular plasmids was quite sensitive to the DNA length and the presence or location of strongly positioned (fixed) nucleosomes. The characteristics of this system can be understood in terms of a requirement that the space available for the aligned array must be close to an integer multiple of the nucleosome repeat generated, leading to the observed coincidence between observed and accessible repeats (Figure 5).

The spacing precision for nucleosomes in ordered arrays can be estimated in a novel way from our data. From Table I and Figure 5, plasmids greater than 2.4 kb in size with accessible repeats deviating from 210 bp by more than 3 bp

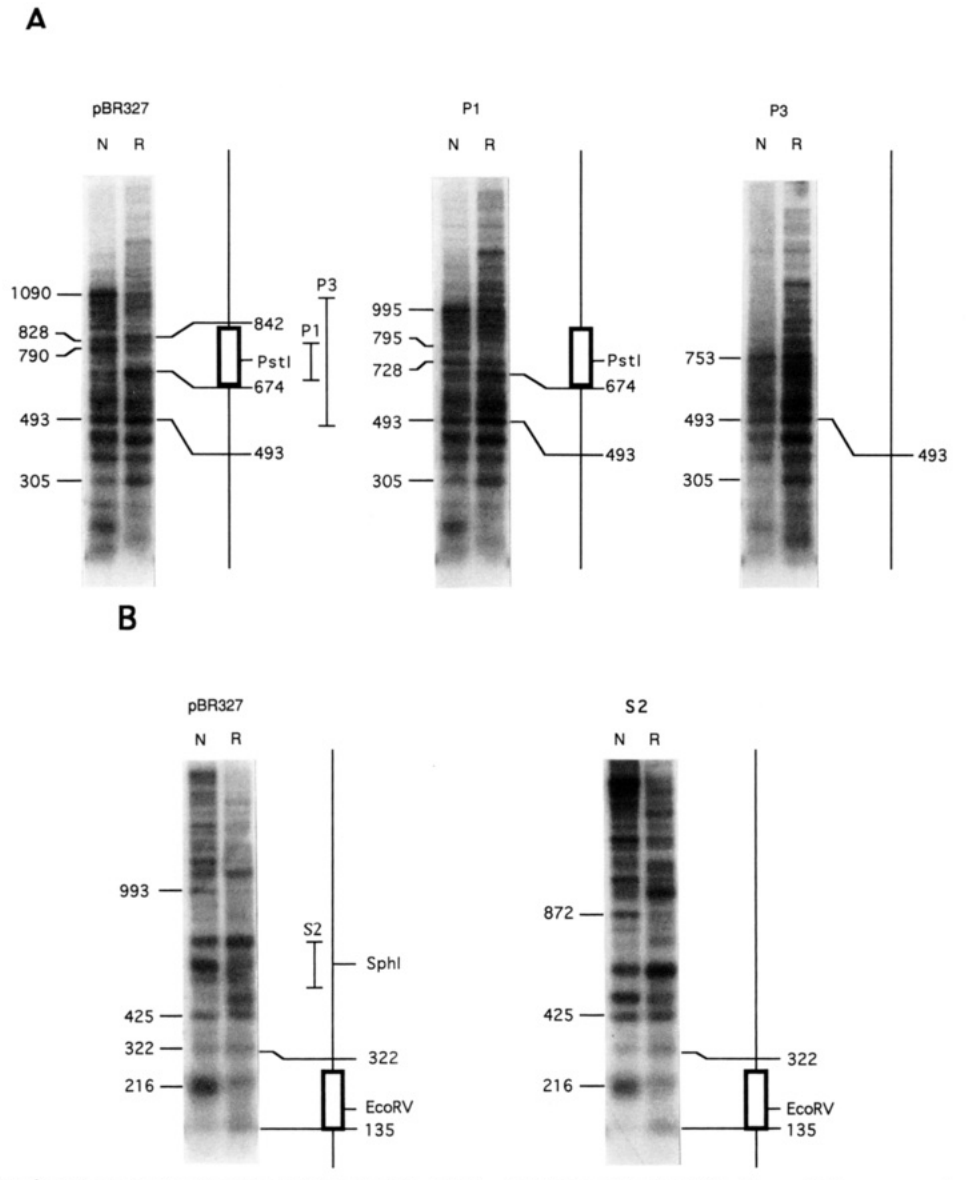

FIGURE 8: Analysis of nucleosome positioning with respect to DNA. (A) Plasmids pBR327, P1, and P3 were analyzed. Reconstituted chromatin lacking histone H5 (R) was digested with 2 units of MNase/μg of DNA for 30 at 37 °C. Naked DNA (N) was digested with 1 unit of MNase/μg of DNA for 90 s. Purified DNA from each sample was then cut to completion with *Eco*RI, the fragments separated, blotted, and hybridized to an appropriate probe (see Materials and Methods). Lengths of selected DNA fragments and the position of the *Pst*I site are indicated. The nucleosome position is indicated by the rectangle, 146 bp in length. Brackets denote the DNA deleted from pBR327 to generate plasmids P1 and P3. (B) Plasmids pBR327 and S2 were analyzed. Reconstituted chromatin lacking histone H5 (R) was digested with 1 unit MNase/μg of DNA for 2 min at 37 °C. Naked DNA (D) was digested with 1 unit of MNase/μg of DNA for 100 s. Purified DNA from each sample was cut to completion with *Eco*RI, the fragments separated, blotted, and hybridized to an appropriate probe (see Materials and Methods). Fragment lengths, the extent of the deletion in pBR327 to generate S2, and the nucleosome position are indicated as in (A).

usually failed to align nucleosomes. Thus, for a 16-nucleosome plasmid, the length variation that can be tolerated for formation of a continuous array is approximately ± 3 bp $\times$ 16 = ± 48 bp. If this ± 48-bp tolerance is regarded as arising from the statistical variation around a mean linker value of 64 bp (210 bp–146 bp) for the individual linkers in a *continuous array*, it follows that the statistical variation in an individual linker is about 48 bp/$\sqrt{16}$ = 12 bp. This value is large enough so that unique nucleosome positioning with respect to DNA or "phasing" would excluded by high-resolution experiments (Engelke & Gottesfeld, 1990; Chipev & Wolfe, 1992). However, this value is small enough so that the precise positioning of one nucleosome in an array would determine the positioning of a nucleosome 2 kb away ($\sim 10 \times 210$ bp) with a variation of only about ± 38 bp ($\sqrt{10} \times 12$ bp), solely on the basis of statistical effects. This value is low enough to ensure that a 10-bp factor binding site is wholly contained in a nucleosome core and inaccessible. Moreover, the statistical variation in a nucleosome positioned 2 kb away from a precisely positioned nucleosome should be low enough to allow a 10-bp site located (on average) in the center of a linker to remain accessible in most cells. There is considerable evidence that the peripheral 20 or so bp at each end of the core particle has properties similar to linker DNA (Simpson, 1991). Specific base sequence effects on nucleosome formation near the factor binding site could restrict positioning further. Therefore, such models for how chromatin structure might control access to DNA are not necessarily in conflict with nucleosome positioning data.

The reason for the approximately 10-bp shortening of the nucleosome repeat for plasmids smaller than about 2.4 kb is not understood, but the phenomenon seems clearly length related. One possibility is that the nucleosome spacing action of histone H5 involves DNA writhing (Stein, 1980; Zivanovic

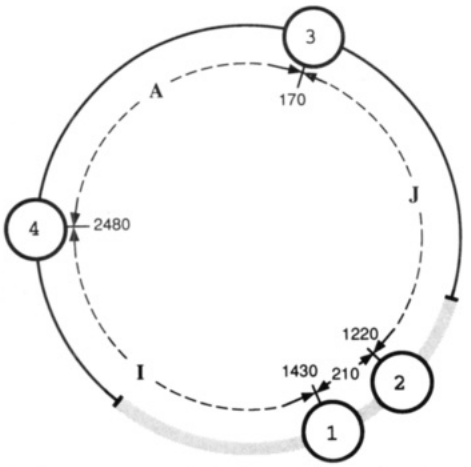

FIGURE 9: Arrangement of the four positioned nucleosomes that form on pBR327. Nucleosomes are denoted by circles occupying 146 bp and are numbered from 1 to 4. The lengths between nucleosome centers indicated by letters are 5.0 × 210 bp for I and J and 4.6 × 210 bp for A. Plasmid map positions of nucleosome centers are also indicated. Nucleosomes 1 and 2, within the COR (shaded region), correspond to those previously designated (Jeong et al., 1991) II and III, respectively.

et al., 1990), which might be partially inhibited on very small plasmids due to either steric or energetic constraints. Interestingly, both the observed repeat and the accessible repeat range over which alignment occurred changed correspondingly, supporting our interpretation of the data. This purely size-dependent change in the repeat provides an explanation for why the 2.1-kb construct D6, examined previously (Jeong et al., 1991), aligned nucleosomes poorly compared to the other constructs examined; all were close to integer multiples of 210 bp. The other previously studied 2.1-kb construct, D2, which gives a fairly good 210-bp MNase ladder at low extents of digestion (Jeong et al., 1991), exhibits strong aberrant bands when digested more extensively (not shown). Hence it should be classified as nonaligning according to the criteria used here. Moreover, construct D2 might be expected to have an unusual propensity for a 210-bp repeat because of the presence of three fixed nucleosomes that are located at positions which are nearly multiples of 210 bp from each other (see below). It is clear that fixed nucleosomes can also dictate boundary conditions, leading to effects similar to those observed upon varying the total DNA length. The relative arrangement of the four positioned nucleosomes on pBR327, consistent with our previous mapping data (Jeong et al., 1991), is shown in Figure 9. The positioned nucleosome covering the *Pst*I site (number 4), as well as two positioned nucleosomes on the COR (numbers 1 and 2) have been demonstrated to remain in place in the presence of histone H5, which induces a massive rearrangement in other nucleosome positions during alignment. The measured positions of these four nucleosomes are largely consistent with how they appear to influence alignment over the plasmid. For example, nucleosome 3 is close to 5.0 × 210 bp away from 2 (distance J) and can remain in place as alignment spreads counterclockwise from the COR (Jeong et al., 1991). Nucleosome 4, however, is incorrectly positioned at 9.6 × 210 bp measuring counterclockwise from 2, and it inhibits alignment unless a length-adjusting deletion removes it or is made in region A. The length-adjusting deletion P1 was not successful because it was made in region I, altering the 5.0 × 210 bp distance between nucleosomes 1 and 4. Similarly, the length-adjusting deletion S2 was not successful because it was in region J, altering the 5.0 × 210 bp distance between nucleosomes 2 and 3.

The presence of four fixed nucleosomes, positioned as shown in Fig. 9, is also consistent with alignment being somewhat less sensitive to plasmid length for a large deletion made in region A than for a P-series deletion of similar size. For example, plasmids A5 and P4 were almost the same length and both possessed accessible repeats of 216 bp; A5 permitted alignment, whereas P4 did not. In A5, most of region A was deleted, but all four fixed nucleosomes were in place and in correct relative positions, measuring counterclockwise from 4 to 3, for a 210-bp array, the repeat observed for this plasmid. In P4, nucleosome 4 was deleted. Thus, A5 may be unusually permissive because only a small region of the plasmid between two fixed nucleosomes has incorrect boundary conditions.

Interestingly, when the nonaligning construct S2 (for which alignment was apparently inhibited by an incorrectly positioned nucleosome 3) was enlarged, the propagation of nucleosome alignment with a 210-bp spacing periodicity appeared to resume after it was disturbed by nucleosome 3. It is not currently known if this phenomenon resulted from a particular juxtaposition of nucleosomes in the array. In contrast, propagation through nucleosome 4 in pBR327 with just a local disturbance did not occur, as shown in the experiment depicted in Figure 2. If regularly spaced nucleosomes were present throughout pBR327 except in the vicinity of nucleosome 4, better MNase ladders should have been detected overall, and the digestion patterns should have appeared less aberrant when blots were probed further away from the *Pst*I site. Instead, nearly the same limited degree of alignment was detected in all regions of the plasmid. Thus, nucleosome 4, which is located at a position that is not close to an integer multiple of 210 bp from the other fixed nucleosomes in pBR327 (measuring counterclockwise from nucleosome 1), appears to inhibit overall nucleosome alignment.

The data presented here suggest that relative nucleosome spacings, resulting from histone H5-dependent (or H1-dependent) alignment are precise enough to give rise to boundary effects that affect chromatin regions several kilobase pairs in extent. Thus, it should be possible for nucleosome arrays to transmit information over such a distance. Also, it is tempting to speculate that boundary effects, similar to those observed here, might affect chromatin structure in vivo. For example, DNA length changes observed in different individuals and in different cells, similar to the triplet expansions recently associated with fragile X syndrome (Oberle et al., 1991; Kremer et al., 1991; Fu et al., 1991), myotonic dystrophy (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992), and spinal bulbar muscular atrophy (La Spada et al., 1991), might in some cases alter the boundary conditions for correct nucleosome alignment, thereby affecting the expression of nearby genes.

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