A Protected Region Upstream and Limited Nucleosomal Positioning Downstream of the Transcription Initiation Region of the Yeast 35S Ribosomal Gene†

D. Lohr

ABSTRACT: To locate nucleosome positions around the transcription initiation site of 35S rDNA in yeast, single and double cleavage site data for staphylococcal nuclease and single cleavage site data for DNase I were obtained by methods involving some modifications of the usual indirect end-labeling procedure. Results show that immediately upstream of the initiation site there is an ~225 base pair (bp) stretch of chromatin which is quite resistant to nuclease digestion. The nucleosomal domain begins on the coding sequences, at the downstream end of the resistant region, with the major locus 30 bp and a minor locus 5 bp downstream of the initiation site in growing cells. The location of the major locus is corroborated by analysis within the coding sequences, although results within the gene are less convincing because of a strong similarity of chromatin and naked DNA digestion profiles. The staphylococcal nuclease cleavage maps were tested by their ability to reproduce chromatin digest profiles and to explain the presence or absence, depending on which parts of the region were used as probes, of specific bands within the pattern. The site of the major upstream locus of the nucleosomal domain differs in growing and stationary cells. This shift changes the accessibility of ~25 bp of DNA and may be associated with the expression of these genes.

Whether there is specific location of nucleosomes on particular DNA sequences is an important, current question. Most methods of locating nucleosomes [cf. Igo-Kemenes et al. (1982)] depend on an enhanced accessibility of nucleosomal spacer DNA to endonucleases: staphylococcal nuclease, DNase I, and restriction endonucleases. For this approach to succeed, the nuclease must show a steric preference for the digestion of DNA which is a nucleosome spacer in chromatin, regardless of its base sequence.

While there have been a number of suggestive results [cf. Igo-Kemenes et al. (1982)], there is still some uncertainty because none of the methods are unambiguous. For example, DNase I simultaneously nicks DNA in *both* core particle and spacer domains (Noll, 1977; Lohr & Van Holde, 1979) but in general has not been shown to make double-stranded cuts preferentially in spacer DNA. While staphylococcal nuclease shows a general preference to cleave in spacer DNA, it also has significant affinity for specific DNA sequences (Dingwall et al., 1981; Horz & Altenburger, 1981), which complicates interpretation of digestion results for specific regions of chromatin. There are agents which fragment DNA chemically, but many of these agents may also show DNA sequence preferences [cf. Cartwright & Elgin (1982)].

Staphylococcal nuclease and DNase I digestion analyses have yielded a great deal of information about bulk nucleosome structure [cf. McGhee & Felsenfeld (1980)], but most of the data have been obtained by using a somewhat different approach than is used in the nucleosome location experiments: analysis of extensive as well as limited digestions; mapping sites at short range rather than long range. The latter modification, in particular, increases the detail and helps minimize the confounding effects of multiple cleavages, which tend to increase with longer DNAs. To test whether this (bulk chromatin) approach can yield useful data about nucleosome locations, chromatin in the region around the transcription initiation site of the yeast 35S rDNA gene was analyzed. A different kind of analysis (Lohr, 1983) detected a nonnucleosomal to nucleosomal structural transition in this region, suggesting the possibility of limited nucleosomal positioning. The data presented here confirm this and suggest locations for the first nucleosome below the nonnucleosomal region. The approach also yields some unexpected data about the chromatin structure of the 5′ upstream flanking sequences.

Materials and Methods

Yeast cells (strain D585-11c) were grown, nuclei were isolated, staphylococcal nuclease and DNase I nuclear chromatin digestions were performed, and DNA was extracted as in Lohr et al. (1977a). Staphylococcal nuclease and DNase I digestions of deproteinized nuclear DNA were done at the same DNA concentrations and temperature and buffer conditions as for nuclear chromatin digestions but at lower enzyme concentrations. Deproteinized DNA digestions were also done at 10 °C with similar results.

Core particle DNA was obtained from nuclear chromatin digests by isolation on gels according to Maxam & Gilbert (1980). DNA obtained from nuclease digestion of chromatin or deproteinized DNA or core particle DNA was restricted with various restriction endonucleases (described in the text), under the conditions recommended by the supplier. λ phage DNA was included in all restriction endonuclease digestions to judge for completeness of the restriction digest. When judged complete, disodium ethylenediaminetetraacetic acid (Na_2EDTA)[1] was added in excess and the DNA extracted with isoamyl alcohol/chloroform, 1/24.

DNA was electrophoresed on 2.8–3.3% polyacrylamide/0.5% agarose nondenaturing gels or on 5.0–5.8% polyacrylamide/0.4% agarose/7 M urea denaturing gels as described (Lohr, 1983). These DNA profiles were electrophoretically transferred (Stellwag & Dahlberg, 1980) to diazobenzyloxymethyl (DBM) paper (Alwine et al., 1979). These DBM papers were treated and hybridized as described in Alwine et al. (1979).

† From the Chemistry Department, Arizona State University, Tempe, Arizona 85287. *Received March 2, 1983.* This research was supported by U.S. Public Health Service Grant GM27623.

[1] Abbreviations: bp, base pairs; EDTA, ethylenediaminetetraacetic acid; DBM, diazobenzyloxymethyl; kb, kilobase.

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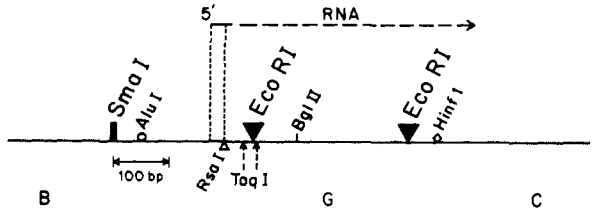


FIGURE 1: Restriction map of the 35S rDNA gene near the transcription initiation site. Parts of *Eco*RI B and C and all of *Eco*RI G are shown with the restriction sites used in this work. The more downstream transcription initiation site [at (Δ)], which is 50 bp upstream from *Eco*RI B/G, was chosen as the zero point. Downstream directions (to the right) are positive; upstream directions are negative. Although 35S rRNA initiates at the two positions shown, 0 and ~−30 bp (Bayev et al., 1980), the initiation site at 0 bp seems to be more prevalent (Klemenz & Geiduschek, 1980). Note that a *Rsa*I site at −30 bp is not shown.

Probes for hybridization were made from various *Eco*RI fragments of the cloned yeast ribosomal genes. These RI fragments were isolated from gels (Maxam & Gilbert, 1980) and radioactively labeled by (1) 5′-^{32}P end labeling with [γ-^{32}P]ATP and polynucleotide kinase after calf alkaline phosphatase treatment or (2) repair of single-strand ends by [α-^{32}P]dATP/[α-^{32}P]dTTP and reverse transcriptase according to Goodman (1980). If necessary, these labeled DNA molecules were redigested with the appropriate restriction endonuclease and the desired probes isolated from gels exactly as described (Maxam & Gilbert, 1980).

Autoradiograms were exposed to X-OMAT X-ray film at −70 °C with intensifying screens present. Films were scanned on a Joyce-Loebl microdensitometer.

Results

In yeast, rDNA consists of about 100 tandem, ~9 kb, repeats of divergently transcribed 5S and 35S genes. Figure 1 shows a restriction map of the 35S gene near the transcription initiation site.

Single Cleavage Site Mapping. To locate individual cleavage sites in chromatin, nuclei were digested to various extents with staphylococcal nuclease or DNase I; the DNA was purified and then cut with a restriction endonuclease. To estimate the contribution of DNA sequence–specific digestion, chromatin and deproteinized DNA digestion courses were compared. To minimize hybridization signals from fragments generated by two nonspecific nuclease cuts, in which one end is close enough to the restriction site used for mapping that the fragment has homology with the hybridization probe, ≤50 bp probes were generally used in this work.

All cleavage sites will be located relative to the transcription initiation site which lies 50 bp upstream of the RI B/G site. Cleavage sites at $-x$ bp lie upstream of the initiation site while sites at $+x$ bp lie downstream, within the coding sequences of the gene. Upstream is to the left and downstream is to the right of the map in Figure 1.

(A) Staphylococcal Nuclease. (1) Upstream from RI B/G. Cleavage sites upstream of the initiation site were located from the RI B/G site. In this region of chromatin, three cleavage sites, which are also hypersensitive (not shown), dominate the pattern at all times of digestion (Figure 2A). Because, by the nature of the technique, any cleavage prevents detection of cleavages more distant on that DNA molecule from the reference restriction site, any weaker cleavage sites toward the coding sequence from the −220 bp site (band 3, Figure 2A) could strongly decrease the signal from this site. However, even in digests that contain predominantly mono- and dinucleosome length DNA (track 7, Figure 2A), the bands corresponding to the −280 and −220 bp sites dominate strongly. The lack of cleavage sites suggests that there may be some physical barrier to digestion in the region downstream of the −220 bp site.

There is a decrease in hybridization efficiency with a decrease in the size of DNA, but this does not explain the lack of intensity noted at lower sizes because in this DBM system the efficiency decrease only becomes serious below 100 bp (determined with ^{32}P end-labeled PM2 restriction fragments hybridized to the same piece of DBM).

Most of the bands in chromatin digests have corresponding bands in naked DNA digests. However, the similar relative intensities in the various bands across the deproteinized DNA profile suggest a more uniform utilization of cleavage sites than in chromatin. In particular, the strong chromatin triplet (−220, −280, and −310 bp) is prominent early in naked DNA digests, but bands corresponding to upstream and downstream cleavage sites are of approximately equal importance. By later times, bands corresponding to sites −50 to −190 bp are dominant. The presence of strong naked DNA cleavage sites in the region (downstream from −220 bp) which is not readily cleaved in chromatin confirms that the lack of digestion noted must arise from protection by chromatin features. Whether the prominence of the chromatin sites from −220 to −310 bp results from a strong feature intrinsic to the sites themselves or from the protection of sequences in the adjacent region cannot be discriminated.

(2) Downstream from SmaI. To analyze the transcription initiation region, staphylococcal nuclease cleavage sites were located relative to the *SmaI* restriction site. At limited extents of digestion, there are several strong bands at sizes which locate the corresponding cleavage sites within the coding region of the gene (band 6 and above in the gel track 2, Figure 2B). Two of these strongly cleaved sites (bands 6 and 7, Figure 2B) are present throughout and in middle and late extents of digestion are the major and sole cleavage sites, respectively. They map to +5 and +30 bp (Figure 3).

Again, most of the chromatin cleavage sites have correspondent naked DNA sites. However, early in digestion, there is a rather uniform distribution of bands throughout the deproteinized DNA profile, including the region upstream from the +5 bp chromatin site (Figure 2B, track 1). Later in digestion, naked DNA cleavage is mainly confined to this region (track 5). The region which corresponds to the +5 and +30 bp chromatin sites shows weak intensity (Figure 2B) either early (track 1) or late (track 5) in digestion. The prominence of the +5 and +30 bp sites in chromatin digests must result from a chromatin structural feature.

The lack of cleavage in chromatin upstream of the +5 bp site, where there are naked DNA cleavage sites, again suggests that chromatin structure restricts cleavage in the upstream flanking region of the gene. The protected region defined here (−150 to +5 bp) overlaps with the region defined by mapping upstream from RI B/G (−220 to −50 bp; previous section) to locate the nonnucleosomal, protected upstream region between +5 and −220 bp.

Since the chromatin on the RI G DNA sequences (and downstream) is nucleosomal (Lohr, 1983), there must be a nucleosomal to nonnucleosomal transition near RI B/G (Lohr, 1983). The differences between the chromatin cleavage map of the 5′ end of the coding region and the upstream flanking region also suggests this transition. There are a number of cleavage sites (+75, +100, and +130 bp, in addition to the +5 and +30 bp sites discussed above) downstream of the transcription initiation site, but there is absence of cleavage

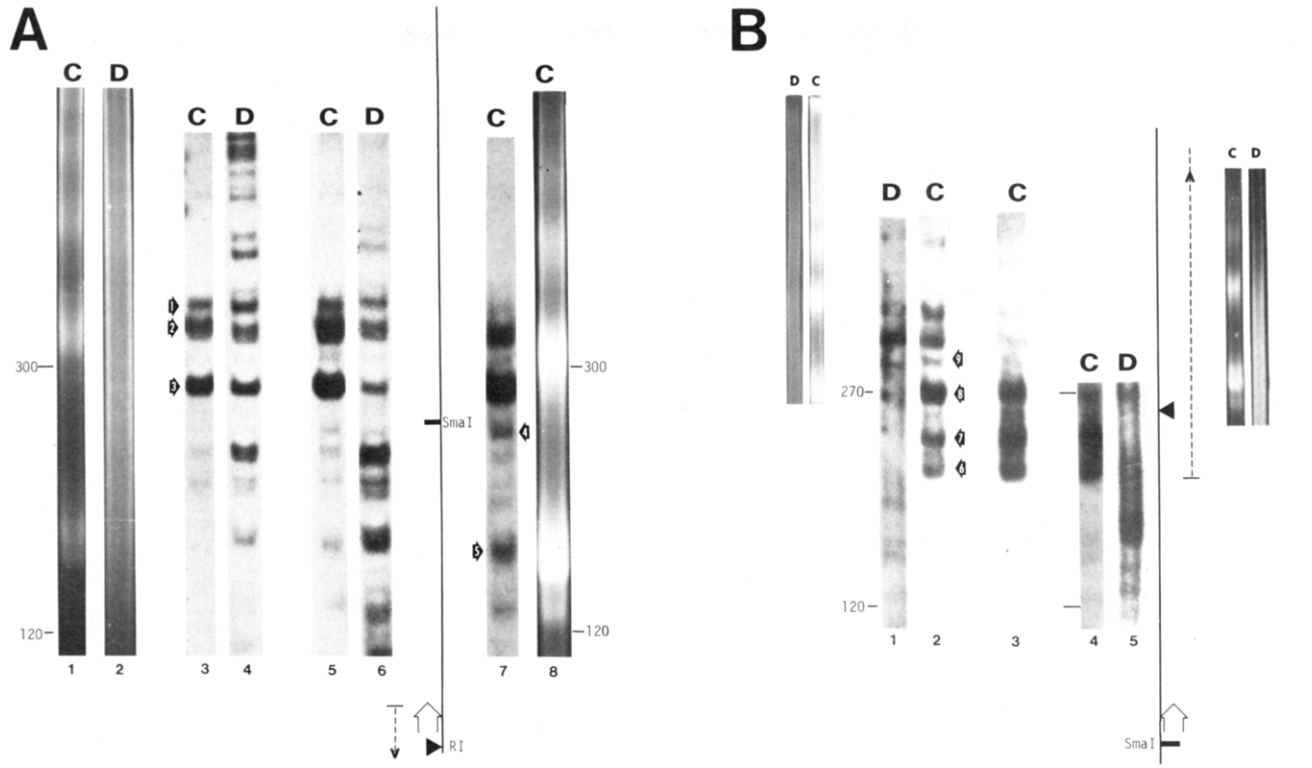


FIGURE 2: Mapping single staphylococcal nuclease cleavage sites near the transcription initiation site of yeast 35S rDNA. DNA from time courses of staphylococcal nuclease digestion of nuclear chromatin (C) or deproteinized DNA (D) was recut with *Eco*RI or *Sma*I, electrophoresed on composite agarose/polyacrylamide gels, transferred to DBM paper, and hybridized with the appropriate small probes. All DNA sizes shown were determined by inclusion of ^{32}P end-labeled PM2–*Hae*III restriction fragments in the hybridization mix to allow visualization of unlabeled PM2–*Hae*III restriction fragments which are included on every gel. A map is also shown (drawn to scale) to aid in locating the sites. *Eco*RI (▼), located at +50 bp, and *Sma*I (—), located at −200 bp in the coordinate system, are shown. The dashed line shows the 35S rRNA, which is transcribed in the direction shown by the arrow. The large arrow shows the hybridization probe; the end point is correctly located relative to the bands shown on the autoradiogram. (A) The RI recut DNA patterns were hybridized with *Rsa*I–RI of RI B (≃50 bp) to map upstream from the *Eco*RI B/G site. Tracks 1 and 2 show the ethidium bromide photographs of the tracks whose autoradiograms are shown in tracks 3 and 4. Tracks 5 and 6 show two more extensive digests, matched for overall extent of digestion. Tracks 7 and 8 show a more extensive digest: (7) the autoradiographic profile; (8) the ethidium bromide photograph. Track 7 is deliberately overexposed to show that bands at smaller sizes in the gel are minor. The locations of DNA 120 and 300 bp in size are shown. Bands 1–5 refer to the sites shown in Table III. (B) The *Sma*I recut DNA patterns were probed with the *Sma*I–*Alu*I subfragment of RI B (≃50 bp) to locate sites downstream toward the transcription initiation site. The two insets show the ethidium bromide photographs of the chromatin (C) and DNA (D) profiles whose autoradiograms are shown in tracks 1 and 2 and tracks 4 and 5, respectively. Track 3 shows an intermediate extent of chromatin digestion. Track 4 was deliberately overexposed to show the absence of bands at sizes smaller than the band numbered 6. The locations of 120 and 270 bp in the gel are shown. Bands 6–9 refer to sites shown in Table III.

upstream of that site (track 2, Figure 2B). Second, some of these cleavage sites, +75, +100 (Figure 2B, bands 8 and 9) and +130 bp, correspond closely in relative frequency of cleavage to the correspondent naked DNA sites (Figure 3).

The chromatin cleavage sites around RI B/G are candidates for the upstream locus of the coding sequence nucleosomal domain. Chromatin cleavage sites should occur at multiples of nucleosome lengths of DNA downstream from such a locus. This requirement is fulfilled best by the +30 bp site, for there is another chromatin site at +180 bp. Also, the naked DNA sites at +230 to +305 bp, which are decreased in cleavage frequency in chromatin (Figure 3), would fall well within the second core particle on such a frame. From sites at +5, +75, and +100 bp, there are only minor chromatin sites 150–170 bp (approximately one nucleosome) away, and the corresponding bands are less prominent in the chromatin than in the deproteinized profile. Mapping from RI G/C also shows chromatin cleavage sites which are most consistent with the frame from +30 bp (Figure 3). It is not possible to test for a spacer predicted (from +30 bp) to lie on or near the RI G/C site because there are no appropriate restriction sites in the adjacent region to use to make a probe to allow cleavage site mapping around the RI site.

The RI C region and some of the RI G region show a tendency for naked DNA cleavage sites to occur at nucleosome intervals and near postulated nucleosome spacers, which renders the cleavage site mapping approach unconvincing. However, both RI G and RI C are clearly organized in nucleosomes (Lohr, 1983) so that the cleavage sites mapped in this section of chromatin do reflect digestion in a demonstrated nucleosomal region. Thus, the presence of a nucleosome structure does not necessarily cause large changes in the cleavage pattern of chromatin from that of deproteinized DNA.

(*B*) *DNase I.* DNase I digests of chromatin are useful in the study of localized features, because pattern information is shorter range (10.5 nucleotide incremental ladder vs. the ~170 bp incremental staphylococcal nuclease nucleosomal ladder). Also, at least in the 35S gene, DNase I shows less DNA sequence-specific cleavage than staphylococcal nuclease (Lohr, 1983; D. Lohr, unpublished observations).

Single cleavage site mapping of DNase I nicking sites has not been attempted. However, it should be possible since DNA which has been digested with DNase I will still be mainly double stranded, although nicked at various places, and should be cleavable by restriction enzymes. Denaturation of the restricted DNA releases the nicked fragments, which have a restriction site at one end and a heterogeneous other end, reflecting the spectrum of DNase I nicking sites. To assess the contribution of DNA fragments with two DNase I pro-

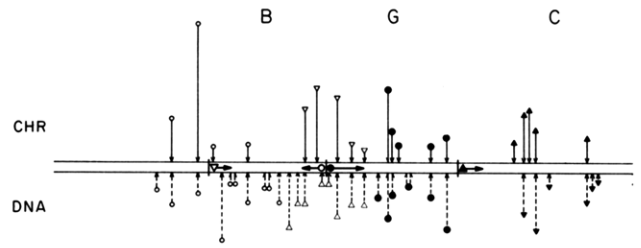


FIGURE 3: Staphylococcal nuclease cleavage site map near the 35S rRNA transcription initiation site for chromatin and deproteinized DNA. Autoradiograms from Figure 2 were scanned on a Joyce-Loebl microdensitometer and band sizes determined by comparison to PM2–*Hae*III calibration curves. The position of a cleavage site is shown by the arrowhead. The length of the arrow is proportional to the height of the peak in the densitometer scan. Peak height was used for simplicity, but since it is the area of a peak that is directly related to DNA concentration, those peaks with increased widths, which generally result from closely spaced bands, were increased in height to reflect their increased area. The same intensity scale is used throughout. The symbol at the tail of the arrow identifies the hybridization probe and restriction enzyme site used to locate the particular cleavage site: *Sma*I–*Alu*I (▽); *Rsa*I–RI (○); RI–*Bgl*II (●); RI–*Hin*fI (▲). Probe and restriction site are shown between the two lines. Chromatin sites are shown above the line (solid lines) and deproteinized DNA sites below the line (dashed lines). The data here are from an "extensive" digestion (cf. Figure 2A, track 7). To concentrate on the major features, any bands that constitute 5% or less of the total intensity are not shown. Since the goal is to analyze the average structure of the ~100 repeats, such a 5% discrimination level should not cause loss of important information. The loss of information is generally less than 10% of the total. The data must be considered semiquantitative because they were not corrected for the efficiency of transfer and hybridization and for the statistical effect of cleavages closer to a restriction site preventing detection of more distant cleavages. However, since the chromatin and naked DNA data were obtained by mapping similar-sized DNA bands from the same restriction sites, these corrections are not likely to change the relative relationship of the two profiles. Thus, the data give a useful picture of the cleavage patterns on chromatin and on naked DNA. The relative intensities of the chromatin and of the naked DNA profiles could not be normalized to one another, so that in the comparison of chromatin to naked DNA, it is the relative intensities of the sites within the profile from a given hybridization probe which are important.

duced ends, the same amount of unrestricted DNA is electrophoresed next to the restricted DNA, and only those bands in the restricted pattern which are unique or markedly increased in intensity compared to the unrestricted profile are considered for use in locating DNase I digestion sites.

Comparison of the cleavage patterns in the two directions from RI B/G shows a clear difference (Figure 4, tracks 1 and 2); a regular pattern downstream within the first core particle and a nonuniform pattern with limited cleavage in the 5′ upstream flanking region. This difference is consistent with the structural transition noted previously in this region. DNase I digestion of naked DNA does not give bands for this DNA (Lohr, 1983; D. Lohr, unpublished observations).

Within the first core particle on the gene, the pattern details are interesting. The greater intensity in the +*Taq*I panel (track 2) and the sharpness of the bands both suggest that the bands shown reflect individual DNase I digestion sites. The bands from individual DNase I nicking sites should be sharper than normal ladder bands because the latter contain contributions from several pairs of individual sites, which adds width to the band since the DNA length between nicking sites varies within the core particle (Lutter, 1978).

There also appears to be two ladder patterns, at least in certain regions. The probe should detect the pattern on only one strand, the 5′ (from the upstream side) strand. Using lengths from the 5′ end of the core particle to the various internal nicking sites determined in vitro (Lutter, 1979) and

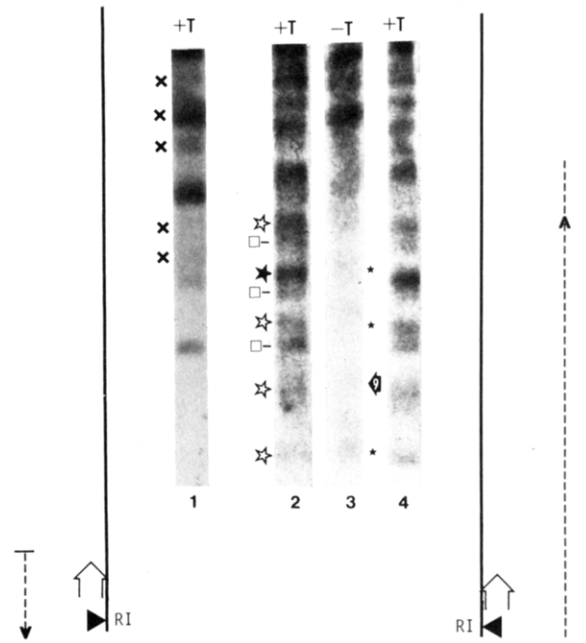


FIGURE 4: Mapping DNase I cleavage sites near the transcription initiation site of yeast 35S rDNA. DNA from DNase I digestions of nuclear chromatin was recleaved with *Taq*I (+T), each sample was electrophoresed on a denaturing gel alongside an unrestricted sample, and the DNA was transferred to DBM paper and hybridized with (1) *Alu*I–RI of RI B (≃200 bp) or (2–4) RI–*Bgl*II of RI G (≃80 bp). Because the two *Taq*I sites lie so close to RI B/G (15 bp upstream and 4 bp downstream), the use of probes with an RI end does not introduce cross-hybridization. The normal DNase I ladder bands (denoted by small solid stars) are shown in track 3 (−T), which is the unrestricted sample corresponding to track 2. Band number 9 is labeled. Band number refers to the 14 bands produced by digestion of core particle DNA; i.e., band number 9 is $^{9}/_{14}$th of a full core particle length of DNA. The two ladder patterns whose sizes are shown in Table I are denoted by (☆) and (□), respectively. Electrophoresis is from top to bottom. Schematic maps of the gene, the 35S transcript (dashed line), and the probe used (large arrow) are shown for each of the two directions of mapping. The *Alu*I–RI probe was used to analyze the pattern upstream from the *Taq*I sites for technical reasons. This probe is large enough that fragments with two DNase I ends are probably also being detected. Such bands can be identified by comparison with the unrestricted profile, where they will be present in equal intensity [shown by (×) for track 1]. Even without this correction, the conclusion that the patterns in the upstream and downstream directions are different is valid because while a large probe could lead to an anomalously large number of bands, it could not produce an artifactually small number. More extensive DNase I single cleavage site mapping studies in this region are in progress.

including the effect of *Taq*I interruption at +54 bp on this strand, one can calculate what DNA sizes should be produced by DNase I cleavage in this first core particle, if its upstream locus is at +5 or +30 bp. The nucleosome locus at +30 bp gives good agreement of expected and observed sizes (Table I) with all the bands in one of the ladders, and if one places the core particle origin at +32 bp, there is exact agreement. Furthermore, band 13, the strongest DNase I cleavage site in core particle DNA (Lutter, 1978), corresponds exactly to the strongest band in the pattern (Figure 4, track 2; solid star). The origin of the other ladder pattern is not clear.

In briefer digests (track 2), the regular pattern seems to continue to a band 15 (i.e., one beyond the number in a core particle), and then there is the expected discontinuity, due presumably to spacer DNA, but later digests show the discontinuity after band 14 as expected (Figure 4, track 4).

Double Cleavage Site Mapping with Staphylococcal Nuclease. (A) Nucleosomal Length DNA. The previous techniques map single cleavage sites. Any two single staphylococcal nuclease chromatin cleavage sites which are separated

Table I: DNase I Band Sizes Observed on the Putative First Nucleosome of the 35S rDNA Gene[a]

	predicted sizes (nucleotides)		obsd sizes (nucleotides)	
site	from +5 bp	from +30 bp	pattern 1	pattern 2
10		81.9	84	
11		(92)	94	
12		102.7	105	101
13	87.5	112.5	114.5	
14	98	123	125.5	121 132.5

[a] Predicted sizes were calculated by using the values of Lutter (1979) for the length (in nucleotides) of a site from the 5′ end of the first core particle, assuming this end is at either +5 bp or +30 bp, minus the effect of *Taq*I cleavage at +54 bp on this 5′ strand. The predicted sizes for site 14 are calculated by assuming the DNase I cutting pattern continues to the end of the core particle. For site 11 (shown in parentheses), the predicted site was taken to be halfway between the sizes from sites 10 and 12, which is necessary since site 11 is too weakly cleaved in vitro to be accurately determined. Observed sizes, calibrated against PM2–*Hae*III fragments, were determined from densitometric scans of autoradiograms (cf. Figure 4). Only data for site 10 and beyond are shown for the frame from +30 bp and for sites 13 and 14 for the frame from +5 bp because DNA ≤80 b was run off the gel.

by one or more integral multiples of nucleosome DNA length suggest the presence of a nucleosome(s), provided the cleavage sites are present together on the same molecule. However, if there is heterogeneity in the DNA population, the distributions of cleavage sites among the heteromorphs cannot be determined from the single cleavage site mapping technique. Heterogeneity could be particularly significant in repeated genes.

A way to examine the pairwise distribution of cleavage sites is to isolate nucleosomal length DNA, then cleave it with a restriction endonuclease, and analyze the number and size of DNA fragments produced, thus locating the staphylococcal nuclease cleavages which produced the DNA. Since the core particle is a distinctive structure and is associated with a rather defined length of DNA (146–148 bp), it has been used in such experiments [cf. Levy & Noll (1979)]. Figure 5 shows the results of a *Taq*I (site at ~+54 bp) cleavage of core particle DNA, probed with RI–*Hae*III (≃40 bp) of RI G, which will lie within the first nucleosome on the 35S coding sequence, whether it begins at +5 or +30 bp. Core DNA from a nucleosomal frame beginning at +5 bp would be expected to yield an ~100 bp fragment while the DNA from a frame beginning at +30 bp would yield an ~125 bp fragment, after *Taq*I restriction. *Taq*I redigestion produces two new bands, a major one of ~125 bp and minor one of ~90 bp (Figure 5A, track 2), confirming the existence of these two nucleosome frames. Restriction of dinucleosome length DNA also yields results corroborating the nucleosome frame from +30 bp (not shown).

Some core particle rDNA remains at its original size after restriction. Incomplete restriction digestion does not seem likely because λ phage DNA, present during restriction digestion, was completely cleaved. Also, several different core DNA digestions gave the same result. The residual core DNA could arise from a nucleosome frame which begins close to the *Taq*I site; for example, a nucleosome frame beginning at +50 or +75 bp would not be affected by *Taq*I redigestion and shares some sequence homology with the probe used. There could also be heterogeneity within the rDNA repeated genes, a possibility suggested previously (Bayev et al., 1980), so that some of the rDNA repeats lack a *Taq*I site.

(*B*) *Prediction of Fragment Patterns.* Nonnucleosomal chromatin structure around the origin of transcription was initially suggested by the unique pattern of fragments produced

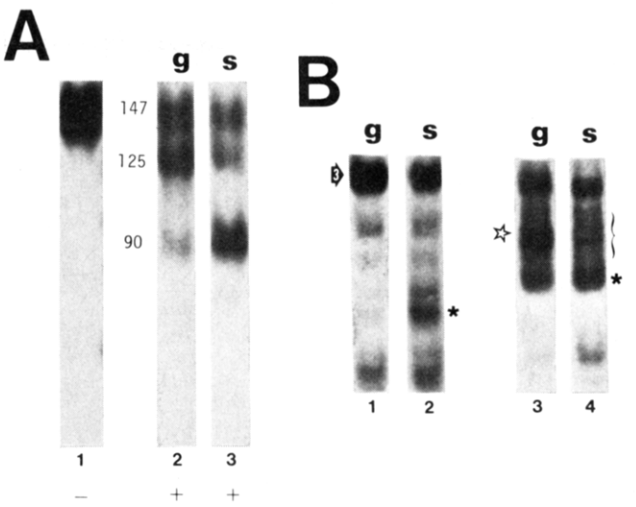
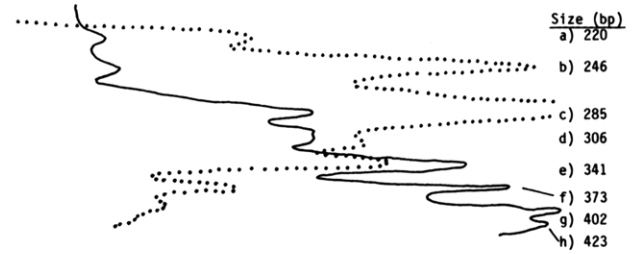


FIGURE 5: Comparison of single and double cleavage site mapping results for growing phase and stationary chromatin. (A) DNA from several extents of digestion (none more extensive than track 8, Figure 2A) was pooled; core particle length DNA was isolated by gel electrophoresis as described by Maxam & Gilbert (1980), recut (+) or not cut (–) with *Taq*I, electrophoresed on a composite polyacrylamide/agarose gel, transferred to DBM paper, hybridized with RI–*Hae*III (≃40 bp) of RI G. Track 2 of (A) shows DNA isolated from growing phase cells (g) while track 3 shows DNA isolated from stationary phase cells (s). The numbers next to the tracks refer to the size of the band in base pairs. Electrophoresis is from top to bottom. (B) Autoradiograms are from the same experiments shown in Figure 2A (tracks 1 and 2) and 2B (tracks 3 and 4), but the comparison is of growing phase chromatin (tracks 1 and 3, g) to stationary phase chromatin (tracks 2 and 4, s). Bands which are more prominent in the stationary phase are denoted by asterisks. Band 3 (Table III) is shown for orientation.

Table II: Sizes of the DNA Fragments Produced by Staphylococcal Nuclease Digestion in the *Sma*I Region of Chromatin[a]

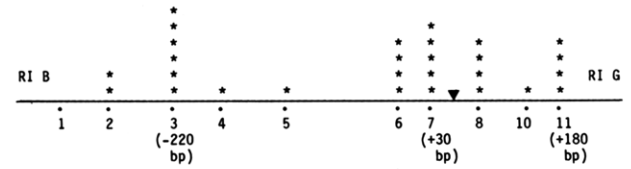


[a] DNA sizes were calculated by calibration with scans of PM2–*Hae*III restriction fragment patterns present on the same piece of DBM paper, as described previously (Lohr, 1983). Densitometer traces from two extents of staphylococcal nuclease digestion [brief (–); more extensive (· · ·)] are shown, with the peaks correctly aligned with the sizes in the table.

by staphylococcal nuclease digestion of the *Sma*I–RI region of chromatin (Lohr, 1983). Densitometric analysis shows the features of this pattern (Table II). Early in digestion, there are strong bands at 285 and 306 bp and very strong peaks at 341, 373, 402, and 423 bp. With increasing digestion, the 220 and 246 bp peaks increase, the 285 bp peak becomes the maximum, and the larger bands generally decrease in intensity.

To test how well the single cleavage site map (Figure 3) describes the digestion of this region of chromatin, the data were used to calculate expected peak sizes and relative intensities. This simple calculation (Table III) reproduces the observed patterns and the digestion-dependent intensity shifts (Table II) reasonably well, which argues that the cleavage site data (Figures 2 and 3) do give an accurate and rather complete description of the digestion of this region of chromatin. The calculation also shows that the anomalous digestion pattern

Table III: Prediction of the Patterns Produced by Staphylococcal Nuclease Digestion of the *Sma*I–RI Region of Chromatin[a]



early			late		
peak size (bp)	bands contributing to peak	expected frequency	peak size (bp)	bands contributing to peak	expected frequency
341	2–8	12	246	3–7	30
	3–10	15		4–8	8
	1–7	6		3–8	24
285	3–8	20	285	2–6	8
	2–6	6		5–11	4
306	3–9	10	220	3–6	24
	1–6	4		4–7	5
	2–7	9	341	2–8	10
373	1–8	8		3–10	8
	2–9	6	306	2–7	10
402	2–10	9			
	1–9	4			
246	3–7	15			
220	3–6	10			

[a] A portion of the cleavage map around the RI B/G site (▼) is reproduced. The various prominent cleavage sites from Figure 3 are numbered. Their approximate relative cleavage frequencies (in an extensive digest, cf. Figure 3) are shown by asterisks, the stronger the cleavage site the greater the number of asterisks. The DNA lengths between the various single-cleavage sites were used, two at a time, to calculate the size of the DNA band that staphylococcal nuclease cleavage at those two sites would produce. The expected frequency of such a band is the product of the frequencies of cleavage at each of the individual cleavage sites. In general, several pairs of sites will yield DNA bands of appropriate length to contribute to a particular peak in the profile. Peaks are listed in order of decreasing expected frequency, from top to bottom in the table. Calculations for two extents of digestion, corresponding to the extents of digestion in the two densitomer scans in Table II, are shown. The expected values for the "early" part of the table were calculated from a cleavage map like Figure 3 but made from data involving a less extensive digest.

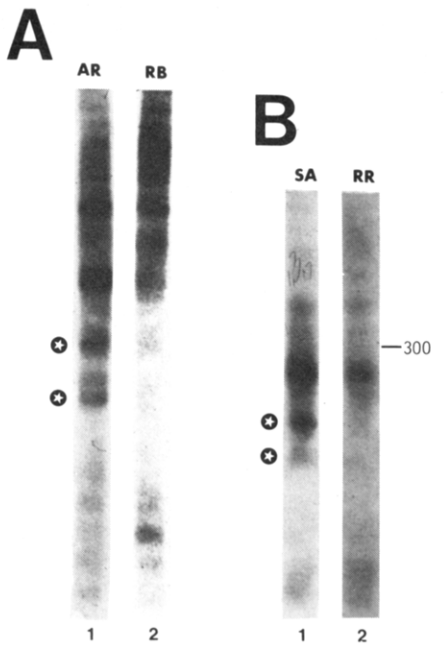


FIGURE 6: Staphylococcal nuclease profiles around the transcription initiation site of 35S rDNA. (A) DNA from a staphylococcal nuclease digestion of chromatin was electrophoresed, transferred to DBM paper, and hybridized with *Alu*–RI of RI B (AR, track 1) or RI-*Bgl*II of RI G (RB, track 2). The main bands which differ in the two profiles, at 220 and 246 bp, are denoted by open stars within circles. Electrophoresis is from top to bottom. (B) Another DBM profile of staphylococcal nuclease digested chromatin was hybridized with *Sma*I–*Alu*I (SA, track 1) or RI–*Rsa*I (RR, track 2). The main bands which differ in the two profiles, at 220 and 246 bp, are denoted by open stars within circles. The RR probe would have <20 bp homology with a 3–7 fragment and <5 bp homology with a 3–6 fragment and so would probably wash off the paper. The SA probe would have 50 bp homology with these fragments.

(Table II; Lohr, 1983) of this region of chromatin originates from the presence of the nuclease-resistant region, flanked on both sides by strong cleavage sites.

One could try the same calculation for a naked DNA digest, but the calculation would be more difficult and the results less credible because of the larger number of cleavage sites (Figure 3). Also, although many of the same individual staphylococcal nuclease cleavage sites are present in both chromatin and naked DNA, the naked DNA profiles comparable to those in Table II have significantly higher background and more diffuse peaks, perhaps attributable to some nonsequence-specific cleavage in addition to the sequence-specific cleavage (Lohr, 1983).

Because of the presence of strong staphylococcal nuclease cleavage sites on opposite sides of the *Eco*RI site, peaks like 220 and 246 bp (Table II), which contain large contributions from bands with cleavage sites 6 and 7 as the downstream terminus (Table III), should be decreased in intensity in a pattern obtained with the RI–*Bgl*II hybridization probe compared to patterns obtained by using probes upstream of the RI site. However, bands with sites 8–10 as the terminus should be less affected. This is the case (Figure 6A). Some of the larger peaks do not behave quite as expected, but this may result from the presence of more nucleosomal length DNA in the RI–*Bgl*II profile. In Figure 6B, the patterns obtained with the *Sma*I–*Alu*I (SA) hybridization probe are compared to those obtained with the *Rsa*I–RI (RR) probe. The 246 and 220 bp bands are both reduced in the RR-probed profile, while the 285 bp band is less affected, consistent with the importance of site 6 for the 220 bp band, site 7 for the 246 bp band, and site 8 for the 285 bp band (Table III).

Chromatin Organization around the Transcription Initiation Site Depends on the Growth State. In the double cleavage site mapping (Figure 5), the relative intensities of the 125 bp band (frame from +30 bp) and the 90 bp band (frame from +5 bp) are quite different, depending on whether the core particle DNA was isolated from growing phase or stationary chromatin digests (Figure 5A, tracks 2 and 3). When DNA from growing cells is used, ~55% of the intensity within the profile is in the band arising from the nucleosome locus at +30 bp while ~10% is in the band from the locus at +5 bp. When core DNA from stationary cells is used, ~60% of the intensity is in the band corresponding to the nucleosome locus at +5 bp while ~15% is in the band from the locus at +30 bp. No attempt was made to correct for the hybridization efficiency decrease with size, so these are minimum estimates for the 90 and 125 bp bands.

The single cleavage site mapping data also show growth-state differences. The band which corresponds to the cleavage site at +5 bp is strongest in the chromatin from stationary cells (Figure 5B, track 4; brace vs. asterisk), whereas the +30 bp band is strongest in chromatin digests from growing cells (Figure 5B, track 3; open star). In fact, in stationary chromatin digests, there is not a well-defined band at the position corresponding to the +30 bp site. In contrast, the band cor-

responding to the +75 bp site appears strong in both chromatin digests.

Since the stationary phase is a quiescent phase, it should show low levels of transcription. Unfortunately, the transcription rate of rDNA in stationary cells is not known and cannot readily be ascertained. However, elongation of polymerase I transcripts is ~10–20-fold reduced in nuclei from stationary cells compared to those from log-phase cells (Lohr & Ide, 1979). It is likely that at least this magnitude of difference exists in vivo. Thus, these differences in chromatin organization correlate with changes in expression level and may be associated with the process of expression in these genes.

Discussion

The nuclease cleavage site data obtained here describe the chromatin structure around the transcription initiation site of the 35S genes in yeast. From +5 to about −220 bp, the chromatin is resistant to cleavage by staphylococcal nuclease, even though the deproteinized DNA itself contains many cleavage sites. The resistant chromatin region is flanked on both sides by strong staphylococcal nuclease cleavage sites. These features are determined by aspects of chromatin structure [see also Bloom & Carbon (1982)].

There is a transition from nonnucleosomal to nucleosomal chromatin structure (Lohr, 1983), the boundary of which is located by the cleavage sites immediately downstream of the resistant domain (+5 and +30 bp). While there are no chromatin cleavage sites for some distance upstream from this boundary, there are numerous cleavage sites in the downstream direction, within the coding sequences.

There is decreased recognition of naked DNA sites in chromatin digests of the 5′ flanking sequences, resulting mainly from the presence of a protected stretch of DNA in the region. In prokaryotes, many regulatory proteins bind to DNA by covering, and thus changing the accessibility of, stretches of DNA. If the protected region upstream of the 35S gene also results from proteins covering the DNA, then the nonnucleosome to nucleosome structural transition region in the 35S chromatin also marks a transition from protein-covered DNA upstream to DNA-covered protein (nucleosomes) downstream.

The strong chromatin cleavage site at ~+30 bp appears to be the major upstream locus of the coding sequence nucleosomal domain in growing cells. First, there are chromatin cleavage sites at the expected intervals downstream from this locus. Second, restriction endonuclease digestion of isolated core particle DNA gives a major band consistent with this locus. Third, DNase I cleavage site mapping in this region shows a ladder of DNA fragments, whose sizes and relative band intensities correlate with a locus at ~+30 bp. There is also some evidence for a nucleosome locus from +5 bp.

Some aspects of the chromatin structure change with growth state. The most likely explanation of the changes is a shift in the predominant nucleosome frame on these genes, from an origin at +30 bp in the growing phase but from +5 bp in the stationary phase. In possible agreement with this explanation, there is a cleavage site at −145 bp (approximately one core particle length from the +5 bp site) which is prominent only in stationary chromatin (Figure 5B). However, downstream in the coding sequences there is less corroboration for the shift in frame. Thus, it is possible that the changes noted arise from an alteration in the structure of the upstream end of the first core particle only, affecting the nuclease sensitivity of this DNA. In either case, the result is an increase (active state) or decrease (inactive state) in the cleavage frequency of the first ~30 bp below the transcription initiation site. In *Xenopus* rDNA, promoter sequences include the first several nucleotides (to +13) of the coding sequence (Reeder et al., 1982). If this is true in yeast, these chromatin changes could affect promoter recognition.

The presence of the strong chromatin cleavage sites at +75, +100, and +130 bp is puzzling. Since there is little corroborative evidence that they are nucleosomal loci, beyond their existence as cleavage sites, they may exist because they are strong naked DNA sites which continue to be recognized in chromatin. In general, since DNA wrapped around the histone octamer in the core particle is quite exposed, unless histones block a specific nuclease recognition feature, nucleases might still be able to recognize and cleave core DNA at strongly preferred, sequence-specific sites. In agreement with this idea, various physical and chemical studies (McGhee & Felsenfeld, 1980) show that association with histones causes surprisingly few restrictions for core DNA. This combined with an exposed structure on the upstream side of the first core particle (see above) could produce the chromatin cleavages noted, including the prominent one at +75 bp.

Techniques which involve digestion with staphylococcal nuclease are subject to two known types of related artifacts: cleavage at specific DNA sequences (Horz & Altenburger, 1981; Dingwall et al., 1981); preference to digest in A-T-rich regions of DNA (Von Hippel & Felsenfeld, 1964). The former can presumably be estimated by sufficiently careful comparison of chromatin and deproteinized DNA digestion profiles, both of which should be digested to similar extents because naked DNA profiles can vary with the extent of digestion (cf. Figure 2B). The former artifact should not compromise this work, since such naked DNA controls were done.

McGhee & Felsenfeld (1983) have recently described a more serious artifact resulting from the preference to cleave A-T-rich DNA. In an extensive digest, this preference can produce a population of core particle DNA depleted in A-T-rich components, which would then give biased double cleavage site mapping results. The results presented here seem not to be compromised by this artifact. First, the core DNA used in these experiments was isolated from pools combining DNA from various extents of digestion, including some quite moderate ones and excluding very extensive ones. Second, if an A-T selection process was producing selective spacer or subcore particle cleavage in the single cleavage site mapping, different sets of sites should be utilized in brief vs. more extensive digests, perhaps differing in increments of 10 bp with increasing digestion (McGhee & Felsenfeld, 1983). This behavior could be recognized in a time course analysis but was seen only occasionally in the profiles, which are precise enough to pick up 10 bp shifts. Third, DNase I, which shows no tendency for the artifacts associated with A-T selection (McGhee & Felsenfeld, 1983), gives nucleosome location results consistent with the data from staphylococcal nuclease digestion. Lastly, the changes in predominant cleavage sites between growing and stationary chromatin, noted in both single and double cleavage site mapping analyses, argue that chromatin and not DNA sequence features are being detected. Thus, although one cannot unequivocally rule out artifacts, the consistency and diversity of the data suggest that nucleosomes can take up particular positions on DNA and that the locations might vary in response to environmental stimuli.

The term phasing has been used to describe the specific location of histones on DNA. However, the word connotes long-range regularity and thus may not be appropriate. The crucial issue is whether any single nucleosome can be precisely positioned on any DNA sequence because there is evidence that nucleosomes take up restricted locations with respect to one another (Lohr et al., 1977b; Lohr & Van Holde, 1979; McGhee & Felsenfeld, 1980). Specific positioning of histones

on any particular stretch of DNA is entropically expensive and thus statistically unlikely, unless there is a compensating stabilization resulting from the binding of a histone octamer to this particular DNA sequence. Cooperation from other favorable contributions could aid positioning by lowering the specific histone–DNA binding energy requirement. For example, as may occur on the 35S gene, a stretch of inaccessible DNA, covered by specific (regulatory?) proteins, could effectively direct nucleosomes to the first available DNA, immediately adjacent to the protected domain. From a first nucleosome, regularity could be maintained by the forces which determine internucleosomal locations, forces which seem able to compensate for the unfavorable entropy of specific locations. However, since these forces probably do not specify unique internucleosomal locations (Lohr & Van Holde, 1979), the initial precise positioning could degenerate downstream.

Registry No. Staphylococcal nuclease, 9013-53-0; DNase I, 9003-98-9.

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Resonance Raman Detection of Fe–CO Stretching and Fe–C–O Bending Vibrations in Sterically Hindered Carbonmonoxy "Strapped Hemes". A Structural Probe of Fe–C–O Distortion†

Nai-Teng Yu,* Ellen A. Kerr, Brian Ward, and C. K. Chang*

ABSTRACT: We report resonance Raman studies of the Fe–C–O distortion in sterically hindered heme–CO complexes. The steric hindrance is provided by a hydrocarbon chain strapped across one face of the heme. Increasing the steric hindrance (by decreasing the chain length), which reduces the CO binding affinity, is found to increase the Fe–CO stretching frequencies: heme 5 (unstrapped), 495 cm^{-1}; FeSP-15, 509 cm^{-1}; FeSP-14, 512 cm^{-1}; FeSP-13, 514 cm^{-1}. This is interpreted in terms of a decrease in the CO effective mass and increased interactions between the C atom of CO and the N atom(s) of the pyrrole ring(s). Resonance Raman enhancement of the Fe–C–O bending mode upon Soret excitation may be correlated with the overlap between the porphyrin (π^*) and CO (π^*) orbitals when the CO ligand is tilted. Its intensity relative to that of the Fe–CO stretching mode increases with increasing steric hindrance in these "strapped hemes". In addition, we have estimated the Fe–C–O angles from isotope data in various heme–CO complexes. It is inferred that the angles are 167 ± 5° (FeSP-15) and 175 ± 5° (FeSP-14, FeSP-13, Mb·CO, and Hb·CO).

The steric hindrance by distal residues in hemoproteins plays an important role in the regulation of heme reactivity toward different ligands (Moffat et al., 1970, and references cited therein; Szabo, 1978). The heme pockets of biological oxygen carriers such as hemoglobins (Hb) and myoglobins (Mb) have a geometry that fits dioxygen (O_2) in its natural bent, end-on configuration (Collman et al., 1974; Phillips, 1978, 1980; Shaanan, 1982) but not a carbon monoxide (CO) molecule, which preferentially binds to the iron in a linear and perpendicular fashion (Hoard, 1975; Peng & Ibers, 1976). It has been proposed that the distal steric effect lowers the affinity

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332 (N.-T.Y. and E.A.K.), and the Department of Chemistry, Michigan State University, East Lansing, Michigan 48824 (B.W. and C.K.C.). *Received January 4, 1983.* This work was supported by National Institutes of Health Grant GM 18894 and National Science Foundation Grant CHE-8210200. C.K.C. is an Alfred P. Sloan Fellow (1980–1984) and a Camile Henry Dreyfus Teacher–Scholar (1980–1985).

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