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Chromatin Structure Differs between Coding and Upstream Flanking Sequences of the Yeast 35S Ribosomal Genes†

D. Lohr

ABSTRACT: Staphylococcal nuclease (EC 3.1.4.7) and DNase I (EC 3.1.4.5) digestion analysis of the nuclear chromatin structure of the yeast 35S rDNA gene shows the presence of typical and homogeneous nucleosome patterns across the coding sequence. These nucleosomal patterns change abruptly, around the site of transcription initiation and upstream in the 5′-flanking sequences, to a unique pattern with both nucleosomal and nonnucleosomal character. The mix arises, at least partly, from heterogeneity within the population of upstream regions; some regions are nucleosomal, but the majority are nonnucleosomal. The nonnucleosomal set of upstream regions appears to be nucleoprotein associated and, in fact, may be an altered nucleosome structure rather than totally restructured. The abruptness of the transition from nucleosome to other structure suggests restricted nucleosome locations in the region around the transcription initiation site of this gene.

Transcription initiation is one facet of the control of eukaryotic gene expression. The specificity for the location and the frequency of transcription initiation resides in the upstream 5′ sequences for genes transcribed by RNA polymerase II (Breathnach & Chambon, 1981) and probably for RNA polymerase I (Grummt, 1981). There are indications that, in addition to the functional distinction between upstream (control) and coding sequences, there are chromatin structural differences between these regions. For example, DNase I and staphylococcal nuclease hypersensitive (cleaved very quickly) sites are found mainly in the upstream rather than coding sequences (cf. Elgin, 1981). Restriction endonucleases can also cleave preferentially in the control region, demarcating an accessible domain of ~400 base pairs (bp)[1] in SV40 which correlates with a nonbeaded region of the minichromosome (Varshavsky et al., 1979) and allowing complete excision of an ~115-bp fragment from the upstream region of a β-globin gene in chicks (McGhee et al., 1981). Distinctions between coding and noncoding regions may even be present in the DNA itself. Keene & Elgin (1981) have shown, by staphylococcal nuclease digestion of protein-free DNA, that noncoding sequences are cleaved preferentially at ~200-bp intervals on average, while adjacent coding regions are not so cleaved.

The SV40 data suggest another possible structural distinction between coding and control sequences, the presence or absence of nucleosomes. In SV40 the "open" region contains control elements (Varshavsky et al., 1979), while the nucleosome domain begins in the coding region. Other studies have detected nucleosomes preferentially located on the noncoding sequences adjacent to genes, cf. *Drosophila* histone genes (Samal et al., 1981) and *Drosophila* heat-shock genes (Wu, 1980). The interest in structural distinctions between control and coding sequences derives from the possibility that such structural distinctions could form some of the basis for the functional distinction, the specificity of transcription initiation.

To study the chromatin structure of individual genes, I developed a technique (Lohr, 1981) which permits analysis of the products of nuclease digestion of the chromatin on a single DNA sequence to the same level of detail and using the same spectrum of approaches as the original work which led to the elucidation of the bulk nucleosomal structure of chromatin. The technique utilizes electrophoretic transfer (Stellwag & Dahlberg, 1980) of DNA patterns to DBM paper (Alwine et al., 1980), which binds the DNA covalently. Thus one can transfer from high-resolution polyacrylamide gels, allowing the detailed analysis of staphylococcal nuclease and DNase I digestion products as double-strand patterns or as single-strand patterns on denaturing gels and permitting analysis of quite small sizes of DNA (down to 80 nucleotides). The extra effort involved in using this technique is justified because the method can produce "structural" information, in addition to "kinetic" information (hypersensitive sites etc.), which is useful but can tell little about why a sequence is preferentially cleaved and thus is difficult to interpret structurally. Application of this method to the region around the 35S rDNA transcription initiation site shows some unique chromatin structural features.

Materials and Methods

Cells were grown to early log (5×10^7/mL) or stationary [$(20–30) \times 10^7$/mL] in YEPD (1% yeast extract, 2% Bactopeptone, and 2% dextrose). Nuclei were isolated, digestions were performed with staphylococcal nuclease or DNase I, and DNA was extracted as described (Lohr et al., 1977b). Electrophoresis on composite nondenaturing or denaturing gels and DNA transfer to DBM paper was exactly as described in Lohr (1981) except the nondenaturing gels were treated in 0.25 M HCl for 15 min to depurinate the DNA as described in Alwine et al. (1980). All gels contained unlabeled PM2–*Hae*III restriction fragments which were visualized by including end-labeled PM2 fragments in the hybridization mix. Hybridizations were done according to Alwine et al. (1980) as described in Lohr (1981).

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[1] Abbreviations: bp, base pairs; b, nucleotide bases; DBM, diazobenzyloxymethyl.

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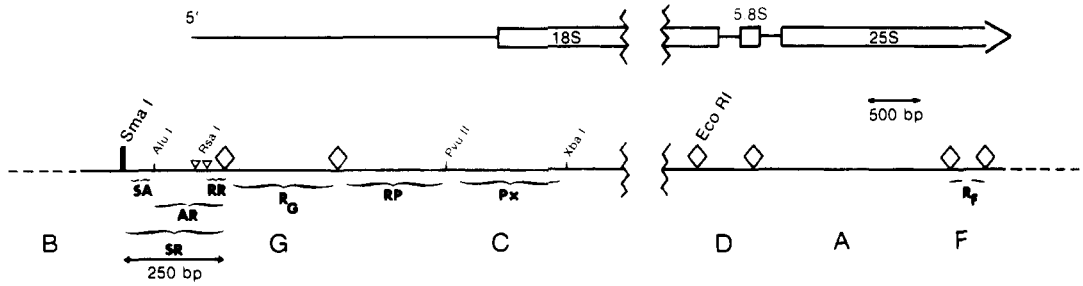

FIGURE 1: Map of the 35S region of the ribosomal DNA repeat in yeast. Locations within the repeat are described by *Eco*RI fragments A–G. *Eco*RI sites are denoted by (◊). Some other restriction sites of importance in this work are also shown. Much of the DNA in RI B, including the 5S gene, DNA between the 3′ ends of the 5S and 35S genes, and some of the DNA between the 5′ ends of the two genes, has not been shown in order to focus on the region studied in this work. The 35S transcript begins at 50 and/or at 80 bp upstream from *Eco*RI B/G. Transcribed spacer in the 35S RNA is shown as the solid line, while the mature RNAs, 18S/5.8S/25S, are shown as rectangles. Note that the scale changes in RI C, across the gap. The probes shown in this work are located in the map and are described in Table I.

Table I: Description of Hybridization Probes Used To Analyze Chromatin Structure on the 35S rDNA Gene and Flanking 5′ Region

RI fragment[a]	restriction sites[b]	abbreviation[c]	size (bp)
RI B	*Sma*I-*Eco*RI	SR	250
RI B	*Sma*I-*Alu*I	SA	50
RI B	*Rsa*I-*Eco*RI	RR	50
RI B	*Alu*I-*Eco*RI	AR	200
RI G	*Eco*RI-*Eco*RI	R_G	280
RI C	*Eco*RI-*Pvu*II	RP	260
RI C	*Pvu*II-*Xba*I	PX	260
RI F	*Eco*RI-*Eco*RI	R_F	370

[a] Denotes the RI fragment in which the probe is located. [b] Denotes the restriction enzyme sites which form the boundaries of the probe. [c] Denotes the abbreviation by which the probe will be known throughout the paper.

Escherichia coli carrying inserts of various parts of the yeast ribosomal genes in the plasmid pMB9 were grown in L broth plus tetracycline and chloramphenicol amplified. DNA was isolated by gentle lysis followed by CsCl/ethidium bromide equilibrium gradient centrifugation in a 50Ti rotor as described in Davis et al. (1980). The yeast insert was removed by *Eco*RI restriction, followed by isolation on a Savant preparative electrophoresis cell or according to Maxam & Gilbert (1980).

Hybridization probes were made from this DNA by (1) repairing 3′ ends of *Eco*RI restriction fragments using [^{32}P]dATP plus [^{32}P]dTTP as described in Goodman (1980) and (2) labeling 5′-OH ends of restriction fragments (treated with alkaline phosphatase) with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Single-strand probes were obtained by phenol extraction restriction enzyme redigestion, followed by isolation on a 5.5% polyacrylamide gel by the methods described in Maxam & Gilbert (1980). Double-strand probes were obtained by restriction enzyme digestion, then labeling, and gel isolation. The various probes used are described in Table I and locations shown in Figure 1. Throughout the paper they will be identified as named in Table I.

Results

rDNA in yeast consists of ~100 copies of a presumably homogeneous, ~9 kb, tandemly repeated unit which includes the divergently transcribed 5S and 35S genes, interspersed by ~1 kb of nontranscribed spacer DNA (Petes, 1980). This ~1-kb region should be rather rich in regulatory sequences since it contains sequences which allow two distinct RNA polymerases, I and III, to recognize and initiate RNA synthesis and perhaps an origin of replication (Szostak & Wu, 1979). Figure 1 shows a map of the 35S gene and adjacent, 5′ upstream ("control") sequences.

To analyze the chromatin structure on the gene, nuclei isolated from growing phase or stationary cells are subjected to nuclease digestion, the cleaved, purified DNA is electrophoresed, and the electrophoretograms are transferred to DBM paper and hybridized with various radioactive, cloned pieces of rDNA. In this work we use small hybridization probes, one to two nucleosome lengths of DNA or less, to focus on local features of the precise region being probed. Of course the smaller DNA in a digestion profile (e.g., monomer–trimer in a staphylococcal nuclease digest) will contain the highest proportion of signal from only the probed region since it is less than or equal to the probe size. Larger DNA sizes must necessarily contain some contribution from sequences beyond the probed region. With this in mind, we can describe chromatin structural results for various sections of the gene: "SMA", the region in RI B from the *Sma*I site to the RI site at the B/G border; "G", the region from the *Eco*RI site B/G to RI site G/C; "C", the region from RI B/C to the *Pvu*II site in RI C; "C′", the region from the *Pvu*II site in RI C to the *Xba*I site; "F", the region containing the *Eco*RI fragment F. Wherever these terms are used, they refer to the regions of chromatin containing these DNA sequences.

Fragment Patterns from Staphylococcal Nuclease Digestion. Results are shown in Figure 2A. A repeat pattern of protected DNA, differing little from the digestion pattern of bulk nucleosomal chromatin, is produced throughout the digestion course of F region chromatin, which is near the 3′ end of the gene (Figure 2A). Moving upstream, the C and C′ (not shown) chromatin regions, whose DNA sequences correspond to transcribed spacer and the 5′ end of the mature 18S rRNA, respectively, also show oligomeric repeat DNA patterns, while the pattern for G region chromatin differs slightly. However, for the SMA region chromatin, which contains the 5′-terminal 50–80 nucleotides of transcribed spacer plus ~170–200 bp of upstream, nontranscribed DNA (Klemenz & Geiduschek, 1980; Bayev et al., 1980), one obtains a quite unique and distinctive pattern which bears little resemblance to the repeat patterns for the coding sequences or for bulk chromatin.

Trivial explanations do not account for this altered pattern for the SMA region chromatin. First, all hybridizations were done on the same piece of DBM paper, so there can be no gel to gel variation. Second, the altered pattern was reproducible in subsequent hybridizations, whether performed before or after an entire series of hybridizations using the coding sequence probes. The altered pattern was obtained by using a hybridization probe labeled in both strands or a probe labeled only in the noncoding strand (i.e., complementary to the coding strand). The same pattern differences between the SMA region and coding sequence regions of chromatin were present

Table II: Nucleosomal DNA Length for Various Parts of the Coding Region of the 35S rDNA Gene in Yeast[a]

	IV			III			II			I		
	90 s	4 min	24 min	90 s	4 min	24 min	90 s	4 min	24 min	90 s	4 min	24 min
G	–	–	–	–	478	410	–	307	288	–	162	153/158
C	665	625	–	490	475	450	323	310	295	–	164	153
C′	650	617	–	–	478	445	–	310	298	–	165	154/161
F	660	600	–	495	470	440	334	315	288	–	163	154/163
total nuclear	665	615	–	470	470	433	322	308	291	–	163	151/162

[a] I, II, III, and IV refer to the monomeric, dimeric, trimeric, and tetrameric oligomers of the DNA repeat. Sizes were measured as the point of maximum intensity of the peak except for the few highly skewed peaks. In their case, some allowance was made for the effect of skewing on the intensity distribution. Where more than one size is shown, the size of the highest intensity peak is underlined. All measurements were made on Joyce-Loebel microdensitometer traces of autoradiographs taken from the same piece of DBM paper hybridized with various probes, with probe removal and hybridization as described in Lohr (1981). Sizes are expressed in base pairs determined against *Hae*III restricted PM2 phage DNA markers. Minus signs (–) denote the absence of peaks, due to the lack of small oligomers in a brief digestion or the depletion of large oligomers in an extensive one. In RI C, RP refers to the RI-*Pvu*II subfragment while PX refers to the *Pvu*II–*Xba*I subfragment.

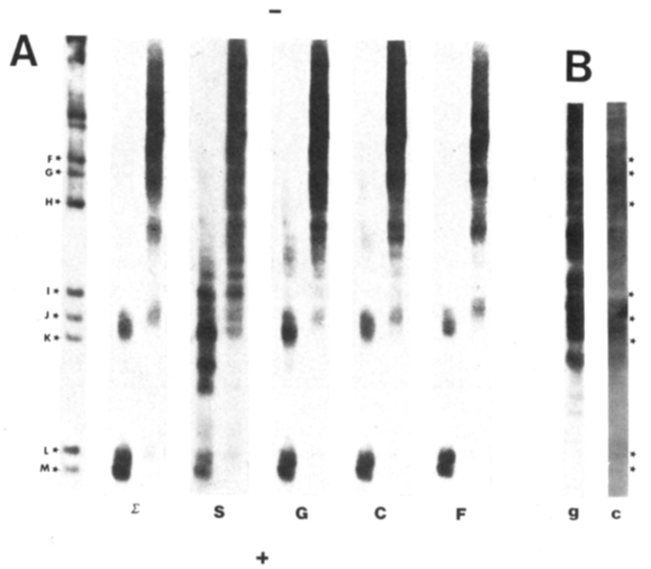

FIGURE 2: Staphylococcal nuclease digestion patterns of chromatin from upstream and coding regions of the 35S rDNA gene (nondenaturing). (A) DNA from two time points (4 and 24 min) of a staphylococcal nuclease digestion course of nuclear chromatin from rapidly growing cells was electrophoresed, transferred to DBM, and hybridized with various small pieces of cloned rDNA, labeled below each set of tracks: Σ, sonicated, ^{32}P-end-labeled total nuclear DNA; S, the 250-bp *Sma*I subfragment of RI B (SR); G, *Eco*RI G (R_G); C, the subfragment from *Eco*RI G/C to the *Pvu*II site in RI C (RP); F, *Eco*RI F (R_F). Direction of electrophoresis is from top to bottom. The PM2–*Hae*III markers from bottom to top are M = 152 bp, L = 168 bp, K = 272 bp, J = 301 bp, I = 336 bp, H = 525 bp, G = 635 bp, and F = 698 bp. (B) Time courses of staphylococcal nuclease digestion of protein-free nuclear DNA were performed as for nuclear chromatin except at decreased enzyme/substrate levels. Samples were electrophoresed, transferred to DBM, and hybridized with various small pieces of cloned rDNA: g, *Eco*RI G (R_G); c, the RI–*Pvu*II subfragment of RI C (RP). Only one time of digestion for each hybridization is shown here. The solid stars show the positions of the PM2–*Hae*III fragments for these gel tracks. The profile obtained by using the SR probe is shown in Figure 4.

in cells grown in another carbon source (galactose) or in a different strain of yeast (Y55, a diploid), even with respect to specific band sizes and relative band intensities in the SMA region (not shown). Thus, this altered pattern for the SMA region of chromatin reflects a conserved and presumably important aspect of chromatin structure in this transcription initiation region.

(*a*) *Nucleosome Structure on the Coding Sequences.* Throughout a time course of digestion, the DNA repeat patterns from chromatin on each of several regions of the coding sequence which were analyzed closely resemble bulk chromatin nucleosomal patterns both in repeat size (Table II) and in general shape and bandwidth, as determined by densitometric analysis of the autoradiograms (not shown). Since Keene & Elgin (1981) could obtain nucleosome-like periodic DNA fragment patterns from staphylococcal nuclease digestion of deproteinized DNA, such a control digest is needed in this work. Figure 2B shows typical results for such digests. There are discrete fragments but they are more uniformly spread throughout the gel (except for a lack of mononucleosome length DNA). Clearly such intensity profiles do not resemble the profiles from chromatin strongly enough to explain the results of Figure 2A.

Electrophoresis of DNA under denaturing conditions can detect single-strand nicking. Such a denaturing analysis of the same DNA as in Figure 2 again shows a nucleosomal pattern for chromatin from the C, C′, and G regions but a different pattern on the SMA region (Figure 3). Single-strand peak sizes are somewhat larger (3–8%) than the double-strand sizes. The difference in sizes is more pronounced for less extensive digestions and for smaller oligomers, which are relatively less extensively digested than larger oligomers within any given digest. These observations are consistent with some nicking during the staphylococcal nuclease digest. However, the single/double strand size differences are also present in bulk chromatin digestions and thus are not unique to the ribosomal genes. At any rate, as in the double-strand analysis, the single-strand patterns give the clear impression that, on the coding sequences, there is a repeating chromatin structure which strongly resembles nucleosomes. The structure is homogeneous and dominant through the coding region.

(*b*) *Unique Chromatin Structure in the SMA Region.* There is a disruption of this homogeneous coding sequence chromatin structure in the region around the transcription origin and upstream of the gene (Figures 2 and 3). To understand the chromatin structure in this latter region, it is useful to analyze separately monomer DNA (up to about 170 bp) and the rest of the DNA fragments larger than monomer in the profile. These larger fragments form a ladder pattern (Figures 2 and 3). The spacing increment is rather unique but nonuniform, varying between 20 and 40 nucleotides.

Is this region naked DNA? Comparison of staph nuclease digests of deproteinized DNA to digests of chromatin (Figure 4A) shows that while some of the sharp bands in the chromatin digest do coincide with bands in the deproteinized DNA digest, there are a large number of bands which have no complement. Furthermore, the bands in the chromatin profile appear quite distinctly above a low background and are regularly spaced in size, suggesting a periodic structure. During the course of

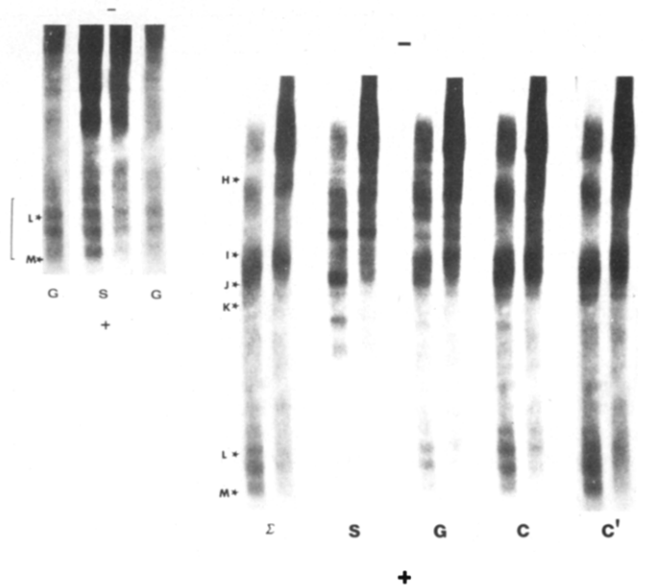

FIGURE 3: Staphylococcal nuclease digestion patterns of chromatin from upstream and coding regions of the 35S rDNA gene (denaturing). DNA from two time points (4 and 11 min) of a staphylococcal nuclease digestion course of nuclear chromatin from rapidly growing cells was electrophoresed, transferred to DBM paper, and hybridized with various small pieces of cloned rDNA: Σ, total genome sonicated, ^{32}P-end-labeled DNA; S, the *Sma*I–RI subfragment of RI B (SR); G, *Eco*RI G (R_G); C, the RI–*Pvu*II subfragment of RI C (RP); C′, the *Pvu*II–*Xba*I subfragment of RI C (PX). Electrophoresis is from top to bottom. The locations of PM2–*Hae*III fragments H–M are shown next to the Σ profile. The inset shows an overexposure around the monomer area of the profiles for two of the same autoradiograms as above, obtained with either the SR probe (S) or the R_G probe (G). Note the high intensity level of the larger bands in the SR probed track. The prominent, closely spaced bands referred to in the text are shown by the bracket.

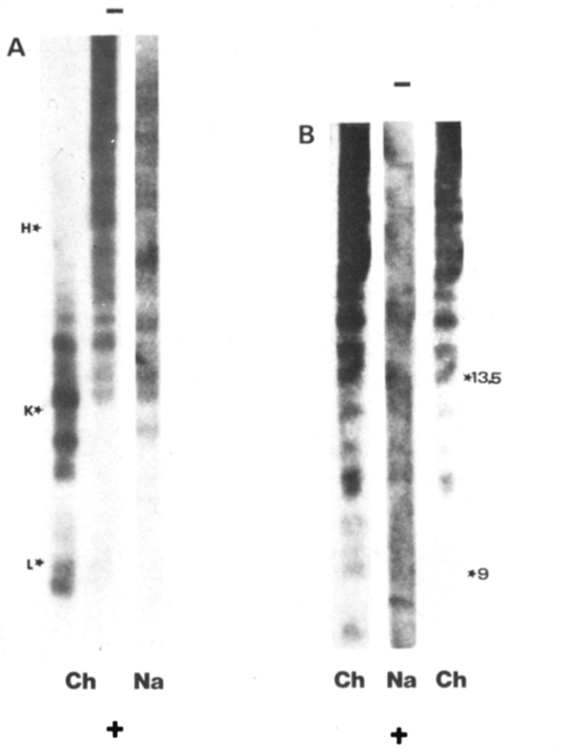

FIGURE 4: Staphylococcal nuclease and DNase I digestion patterns from naked DNA compared to chromatin. Time courses of staphylococcal nuclease or DNase I digestion of protein-free nuclear DNA were performed as for nuclear chromatin except at decreased enzyme/substrate levels. Samples were electrophoresed, transferred to DBM, and hybridized with various parts of the 35S gene. The *Sma*I–RI subfragment of RI B (probe SR) for the staphylococcal nuclease profiles and the *Alu*I–RI subfragment of RI B (probe AR) for the DNase I profiles are shown here. (A) Staphylococcal nuclease digest of nuclear chromatin, Ch, vs. protein-free DNA, Na. These samples are from different but comparable gels. The positions of a few PM2–*Hae*III fragments are shown for orientation. (B) DNase I digest of nuclear chromatin, Ch, vs. protein-free DNA, Na. These samples were run on the same gel. The nuclear chromatin sample happens to be from a stationary phase nuclear digest. Stationary phase chromatin shows slight differences in profile from growing phase chromatin digests, but the basic band pattern is present, and thus it serves as a valid comparison in this experiment. The different exposures of the chromatin track are shown to allow visualization of both the lower and extended ladder bands. Positions of band number 9 (9 × 10.5 b) ≃ 94 b and band number 13.5 ≃ 142 b are marked.

chromatin digestion, the relative intensities of some of the various bands change coordinately, suggesting the possibility of a precursor product relationship for these fragments and structural organization in the region. Such descriptions do not characterize the profiles in the digest of naked DNA. Rather this pattern consists of irregularly spaced, sharp bands superimposed on a broad smear of fragments, as if the staph nuclease preference for certain sites, which leads to the specific bands, is not strong enough to prevent cleavage at a large number of sites, which leads to the smear. All of these differences suggest that the SMA region is not present as naked DNA in nuclear chromatin. DNase I results (see below) also suggest nucleoprotein structure in this region.

There are no observable differences in the extent of digestion between this region and coding sequence regions, either in differences in mean DNA sizes between the two sets of profiles or in the relative concentration of SMA DNA sequences in the various parts of the digestion profiles, at any time of digestion. These kinetic characteristics cannot be interpreted to suggest protein–DNA complexes because chromatin and free DNA can digest at the same rate when present together in a digest (Sollner-Webb et al., 1976).

In contrast to the pattern at larger DNA sizes, the pattern in the monomer area in both the double-strand (Figure 2) and single-strand (Figure 3) gels resembles that for the coding sequences (which have nucleosomal structure). Although the information is limited, there is enough to suggest a strong resemblance, particularly on the denaturing gels where there is a distinctive pattern of three to five uniformly spaced bands in the monomer area (inset of Figure 3, bracket). On longer exposures of this area of the profile, the SMA chromatin digest shows a pattern which is identical in band sizes and relative intensities to that from the G (or C) regions of chromatin (inset of Figure 3). The close correspondences argue that the similarity to nucleosomal patterns does not arise by chance but because at least some part of the SMA region of chromatin is in nucleosome structure (in at least some of the genes). Of course, the complex pattern seen at DNA sizes larger than monomer requires that much of the SMA region is *not* in nucleosomal arrangement.

Such heterogeneity could arise from a nucleosomal to nonnucleosomal transition within the SMA region of chromatin in all or most of the rDNA gene repeats, or from heterogeneity within the population of rDNA genes, i.e., the SMA region being nucleosomal in some rDNA genes but not in others. In the former case, the two structural domains within the SMA region are responsible for the observed composite digestion pattern. In the latter, one might expect to see also oligomeric nucleosome length DNA in the profile, in addition to monomer-length DNA. However, recognition of a nucleosome profile depends on observation of a periodic pattern of intensity separated by null regions. If the nonnucleosomal SMA region exists in a majority of gene repeats,

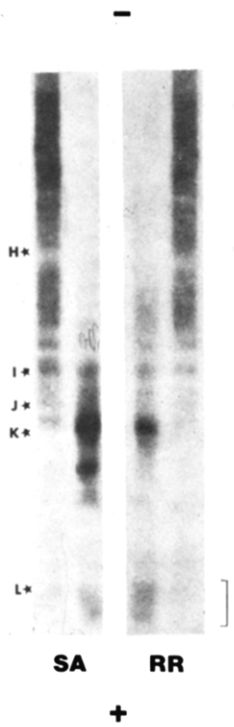

FIGURE 5: Detailed analysis of the staphylococcal nuclease digestion patterns from the chromatin region upstream of the 35S rDNA gene. The same DBM paper used in Figure 2 was hybridized with the *Sma*I–*Alu*I subfragment of RI B (SA) or the *Rsa*I–RI subfragment of RI B (RR), which come from opposite ends of the 250-bp *Sma*I–RI fragment. The pattern obtained by using the entire *Sma*I–RI subfragment (SR) can be seen in Figure 2 and is quite similar. The locations of some representative PM2–*Hae*III fragments are shown for orientation. The monomer region is designated with the bracket.

the fragments contributed by this majority effectively fill in much of the null region and would prevent definitive recognition of a nucleosome profile present as a minor component.

One should be able to distinguish these two alternatives by using very small hybridization probes. If the first explanation is correct, then the nucleosomal part of the profile (the monomer) should be present on some parts of the SMA region of chromatin but not on others. In the latter case, all parts of the region should show the mononucleosome since the heterogeneity lies in the population instead of within the region. When either SA (50 bp) or RR (52bp) DNA is used as probe, the profiles show a mononucleosome peak (Figure 5, bracket). The greater intensity on "RR" probably arises because this hybridization probe overlaps partially with the beginning of the coding sequence nucleosomal domain, which is about 20–45 bp upstream from RI B/G (D. Lohr, unpublished results). Thus, the partial nucleosomal character observed for the SMA region of chromatin is due both to repeat heterogeneity and to a nucleosomal to nonnucleosomal transition within the region, at the beginning of the coding sequence nucleosome domain. This explanation is consistent with the weaker monomer signal from the SMA region compared to G, C, or F regions, which are entirely nucleosomal (Figures 2 and 3).

Since the entire SMA region, each end of the region, and the region from the *Alu*I site to the *Eco*RI site B/G (not shown) all show a monomer pattern, it must be possible to find nucleosomes on all parts of the SMA region, albeit in a minor fraction of the rDNA gene repeats. Two nucleosomes are probably required because although a single 170-bp nucleosomal length of DNA, probably the largest possible in yeast, could span the 150 bp between the ends of the SA and RR probe fragments, there would only be about 10-bp homology left to recognize each probe, which seems insufficient to allow hybridization under the stringent conditions used. However, two explanations would both yield this result: *one* subset of SMA regions which is completely nucleosomal (i.e., two nucleosomes); *two* subsets of SMA regions, each of which contains one nucleosome but at different positions. Unfortunately, these experiments cannot distinguish between them. In the rest of the work we assume the former, that the subset of nucleosomal SMA regions are completely nucleosomal, though this cannot be proven. In summary, the observations suggest a minor subset(s) of nucleosomal and a major set of nonnucleosomal SMA regions of chromatin; this differs clearly from the coding sequence chromatin, which shows only a nucleosome pattern.

Fragment Patterns from DNase I Digestion. DNase I digestion of bulk chromatin produces two overlapping DNA repeat patterns in yeast (Lohr & Van Holde, 1979): the "lower ladder", arising from pairs of nicks within a core particle, with fragments up to 125 nucleotides, spaced at ~10.5-nucleotide intervals; the "extended ladder", arising from internucleosomal pairs of nicks, with fragments as small as 120 nucleotides, spaced at ~10.5-nucleotide intervals. Where the two ladders overlap, the extended ladder band sizes are out of phase with the sizes of the lower ladder bands by 5 nucleotides, presumably reflecting the presence of a 5 nucleotide increment in the spacer lengths (i.e., $10n + 5$ rather than $10n$ where $n = 0, 1, 2, \ldots$). The nicking sites which give rise to the extended ladder are thought to be the same sites within the core particle from which the lower ladder bands arise. Thus the extended ladder contains more information (intracore nicking sites and spacer DNA lengths) than the lower ladder.

From F (not shown), C, and G regions of chromatin (Figure 6) both ladder patterns can be obtained and are indistinguishable from the bulk chromatin patterns in either spacing or relative intensity of bands. The region of the DNase I digestion pattern where extended and lower ladders overlap contains detailed information about the structure within and between yeast nucleosomes; some aspects are peculiar to yeast (Lohr et al., 1977a; Lohr & Van Holde, 1979). The complete identity of the patterns from the 35S gene coding sequences to bulk chromatin strongly reinforces the conclusion (from staphylococcal nuclease digestion) that the coding sequences are nucleosomal.

DNase I ladder patterns reflect localized phenomenon because the size range analyzed is small, mainly 100–200 nucleotides; thus, the features which give rise to the entire pattern must be present in each set of neighboring nucleosomes. Computer simulation of the digestion process, based on the DNase I nicking frequencies in isolated rat liver core particles (Lutter, 1978), reproduces the observed pattern well (Lohr & Van Holde, 1979). On the basis of this calculation, the bands in the extended ladder share a common origin from the same subset of strong sites in the core particle. In fact, band intensity varies rather smoothly through the extended ladder region, suggesting such uniformity.

In contrast, the band intensity profile produced from the SMA region of chromatin is distinctly nonuniform, with regions of intense bands alternating with regions of much lower intensity (Figure 6, S). To rule out trivial explanations, I have repeated the experiment using either one or both strands of the probe labeled, looked at digests from cells grown in galactose, and looked at digests of another yeast strain. The alterations seen in Figure 6 occur at the same positions. There can be variation in the degree of alteration, particularly in stationary cells, which show the alterations less distinctively. Thus, these alterations must be another characteristic of the

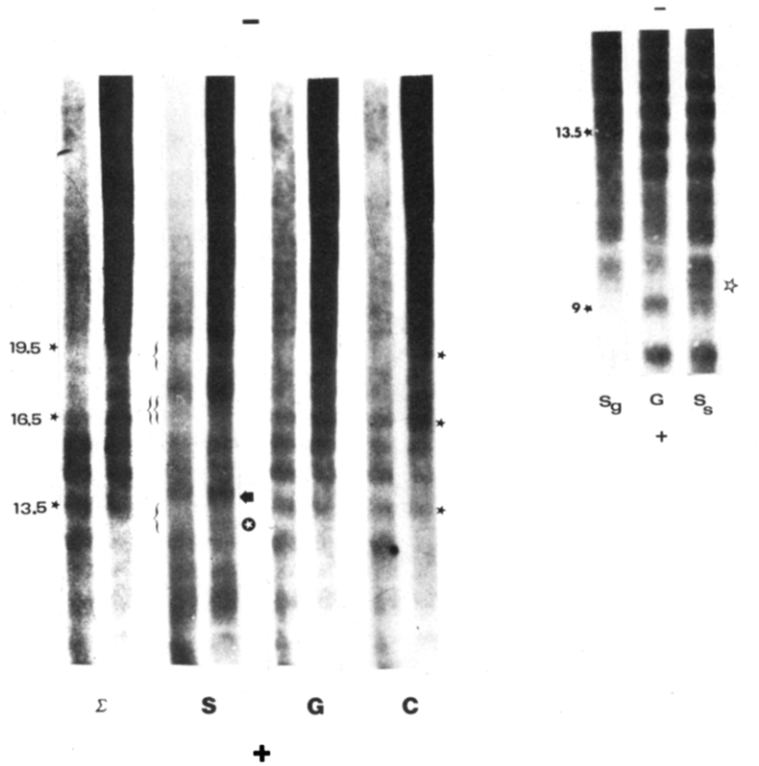

FIGURE 6: DNase I digestion patterns of chromatin from upstream and coding regions of the 35S rDNA gene. DNA from a time course of DNase I digestion of nuclear chromatin isolated from rapidly growing cells was electrophoresed, transferred to DBM paper, and hybridized with various small pieces of cloned rDNA, labeled below each set of tracks: S, the *Sma*I–RI subfragment of *Eco*RI B (SR); G, *Eco*RI G (R_G); C, the RI–*Pvu*II subfragment of *Eco*RI C (RP); $\sum$, sonicated, ^{32}P-end-labeled, total nuclear DNA. Electrophoresis is from top to bottom. The location of band number 13.5 (13.5 × 10.5 b ≃ 142 b), number 16.5 ≃ 173 b, and number 19.5 ≃ 205 b is indicated in the total genome DNA pattern. The sizes of these bands are taken from Lohr & Van Holde (1979). The inset shows results from another gel: S_g, chromatin from growing cells probed with SR; G, chromatin from stationary cells (growing gives the same pattern) probed with R_G; S_s, chromatin from stationary cells probed with SR. Bands number 9 ≃ 94 b and number 13.5 are marked near the S_g track for reference. Several features are labeled: band number 13, open star within circle; band number 14, reverse arrow; band number 9.5, open star; null regions around bands number 13.5 and number 19.5, brace; null region around band number 16.5, double brace.

general chromatin structural modification in the SMA region. DNase I digestion of protein-free DNA does not produce bands from these sequences (Figure 4B), confirming that the band pattern reflects *chromatin* structure.

Careful visual and densitometric analysis shows that several kinds of changes contribute to produce this altered pattern. DNase I bands can be described by band number rather than size in nucleotides to focus on the interrelationships of the bands. Band numbers correspond to the order of bands in a DNase I digest of core particles and run from 1 to 14, the full core particle length of 147 bp. The half-integral bands, e.g., number 16.5, refer to the extended ladder bands, which are incremented by five nucleotides from the lower series. First, a weak band at number 13 (Figure 6, open star within circle) and a strong band at about number 14 (Figure 6, reverse arrow) appear in the profile. I have never seen these bands in other autoradiograms (Figure 6, G; Lohr, 1981) or in ethidium bromide stained gels (Lohr et al., 1977a; Lohr & Ide, 1979; Lohr & Van Holde, 1979; D. Lohr, unpublished results). There is no intensity at the normally strong position of number 13.5, suggesting that the new band at about number 14 could correspond to the band usually found at number 13.5. The lack of intensity at number 13.5 creates the first null region (Figure 6, brace), most apparent in the late track where band number 13 is not present. Since band number 14.5 is also shifted slightly upward in the gel but number 15.5 occurs at the usual size, these three bands (number 14, number 14.5, and number 15.5) appear as a tight cluster, more closely spaced than the usual three bands (number 13.5, number 14.5, and number 15.5). There is a pronounced null region where band number 16.5 (Figure 6, double brace) should be; densitometry shows that band number 16.5 can sometimes be detected but in greatly diminished intensity. This null is accentuated by a slight upward shift of band number 17.5. There is another low intensity region around band number 19.5 (Figure 6, brace).

The gel inset in Figure 6 shows another general feature in digestion profiles from this SMA region of chromatin, a strong band at number 9.5 (open star). The profiles also show an apparent higher "background" in digests from growing cells compared to stationary (S_g vs. S_s). However, this may not reflect nonspecific digestion totally since there are many (new) discrete bands present in these patterns (number 13, number 14, several weak bands around number 11, number 16.5–17.5, and number 16.5–15.5). The presence of all these bands definitely complicates the appearance and interpretation of these profiles.

Results from staphylococcal nuclease digestion suggest that there is heterogeneity in the SMA region, a minor set of repeats in which the region is nucleosomal and a major set in which the region is nonnucleosomal. Digestion of the nucleosomal set of SMA regions should produce a normal DNase I ladder, which could account for the residual weak bands in the mainly null regions number 13.5, number 16.5, and number 19.5, but merely adds to the intensity already present at other band positions. The predominance of the altered DNase I motif and the anomalous staph nuclease pattern suggests that both originate from digestion of the major set (nonnucleosomal) of SMA regions. Although there may be some increase in background in the DNase I digestion profiles, it does not appear strong enough to represent digestion of the entire nonnucleosomal set. Thus it is likely that the greatly altered

staph nuclease pattern and the slightly altered DNase I pattern both reflect the same structural features.

Discussion

Staphylococcal nuclease digestion, in nuclei, of chromatin from coding sections of the 35S rDNA gene yields repeat patterns of DNA whose peak sizes remain constant across the transcription unit. The single- and double-strand peak sizes and peak shapes and widths closely resemble those obtained by digestion of bulk yeast (nucleosomal) chromatin. Nuclear DNase I digestion of chromatin from coding sequences of the rDNA gene gives the typical overlapping ladders of fragments, which are indistinguishable from bulk nucleosomal ladder patterns and also are invariant across the gene. Because there is extensive and explicit similarity of digestion profiles of 35S chromatin to profiles produced by digestion of bulk nucleosomes, for both nucleases, one must conclude that the coding regions of this gene are mainly nucleosomal. These results agree with earlier nuclease digestion studies of rDNA genes (cf. Mathis et al., 1980). The use of small hybridization probes, the increased level of detail in all analyses, and the addition of staph nuclease and DNase I denaturing gel analysis all tend to strengthen the conclusion, as well as allow the comparison of different regions of the transcription unit.

It seems most likely that nucleosomal coding regions would be present in the inactive 35S transcription units because so many electron microscopic studies have shown that active rDNA genes clearly appear to be nonnucleosomal. Nonnucleosomal genes would be expected to add nonnucleosomal lengths of DNA to the nuclease digestion profile. Since the nucleosomal patterns for the coding sequence chromatin seem to contain no more nonnucleosomal DNA than is present in bulk nucleosome patterns (Figures 2 and 3), there seems to be no evidence in this data for nonnucleosomal genes. One could offer trivial explanations for this lack. RNA polymerases alone could afford significant protection to rDNA, but, to explain my results, a polymerase–DNA structure would have to resemble histone–DNA structures (nucleosomes) in all details of staph nuclease and DNase I digestion. Nonnucleosomal regions could be quickly solubilized and thus removed from the digestion pattern. However, even naked DNA in the presence of chromatin is not necessarily preferentially digested (Sollner-Webb et al., 1976), and in my work, the nonnucleosomal SMA regions are not preferentially solubilized. If yeast rDNA transcription were very labile, the time necessarily spent in spheroplasting and nuclear isolation could allow runoff of the in vivo transcribing RNA polymerases and reversion of the active structure to an inactive state. However, Lohr & Ide (1979) showed that isolated yeast nuclei are exceptionally active in transcription ($\simeq 10\times$ rat liver nuclei). Furthermore, they have recently shown that isolated nuclei can elongate rRNA in vitro and even *initiate* transcription of 5S and 35S RNA at their in vivo sites (D. Lohr and G. Ide, unpublished results). These latter results in particular suggest that isolated nuclei maintain much of their in vivo character at this locus; therefore features observed in nuclei probably reflect the in vivo state.

However, there could be some yeast rDNA repeats in which the coding sequences are nonnucleosomal, despite the lack of direct evidence. Because nuclease digestion studies sample the entire population of rDNA genes while electron microscopy focuses on the active units, the two techniques could yield results in seeming conflict, when applied to a system with a mixed population where inactive repeats strongly predominate over active. This explanation would require that only a minor subset of the repeats be transcrib*ing* at any one time. Moreover, while the digestion profiles of naked DNA look much more uniform throughout than chromatin digestion profiles, the naked DNA profiles do show some accumulation of intensity around the sizes of di- and trinucleosomal DNA. This could cause an underestimation of the contribution of nonnucleosomal genes which digested like naked DNA, in a mix of nonnucleosomal and nucleosomal genes.

However, of utmost interest in this work are the comparisons of the chromatin structure on the upstream, putative control sequences to the chromatin structure observed on coding sequences. For the upstream regions, staphylococcal nuclease digestion produces a hybrid profile consisting of a regular pattern of approximately equally intense, closely spaced bands at larger DNA sizes and a mononucleosome peak which is typical in profile but of weak intensity. DNase I produces patterns which resemble nucleosomal patterns but are altered in size and in intensity at specific bands. Together these observations describe an alteration in structure which distinguishes this upstream region from the coding sequence (nucleosomal) domain. The use of small probes points to the vicinity of the RI B/G site as the region where the nucleosome to nonnucleosome transition takes place. This structural transition thus correlates roughly with the 35S transcription initiation sites, which lie 50 and 80 bp upstream from this RI site.

The digestion data suggest that these upstream sequences are nucleoprotein complexes rather than naked DNA. A DNase I ladder pattern, although altered, is still observed for this region; such ladders are diagnostic for DNA constrained to lie on a protein (Kirkegaard & Wang, 1981) or other surface (Rhodes & Klug, 1981). In contrast to digestion of chromatin, DNase I digestion of protein-free DNA yields a smeared pattern for this region. These results strongly suggest that the region is present as nucleoprotein in chromatin. Although the evidence is weaker, aspects of the staph nuclease pattern, as discussed under Results, support this conclusion. It is of course possible that only some fraction of the nonnucleosomal SMA regions is nucleoprotein and that fraction yields the behavior described above.

From staphylococcal nuclease digestion results, the majority of the upstream regions appear to be completely different from nucleosome structure, but the patterns are novel and do not suggest any particular interpretation. On the other hand, the DNase I patterns have more resemblance to the usual nucleosome ladder, suggesting perhaps merely an altered nucleosomal structure. There are two problems with such an interpretation. Computer simulation analysis suggests that the various ladder bands all arise from the same basic set of intracore particle cleavage sites, so that it should be hard to alter only some bands in a nucleosomal extended ladder pattern. However, the in vitro results may not adequately describe DNase I action on yeast chromatin in vivo since core particle cutting site frequencies could differ and the presence of spacer DNA have an effect. If an intracore site(s) sensitivity modification(s) can alter the intensity patterns specifically and selectively, to yield the types of profiles seen for the SMA region, then the DNase I patterns for this region become interpretable as a change in the usual DNA histone surface interaction or in the DNase I recognition of that interaction. This could result, for example, from a protein binding on the outside of the nucleosome, producing a nucleosomal ladder modified only at certain rungs.

The second problem is to explain why the staphylococcal nuclease pattern changes so much and the DNase I pattern so little. The two enzymes differ in digestion characteristics.

DNase I assesses the winding of DNA on a protein surface and thus is responsive to protein–DNA contacts, whereas staph nuclease responds mainly to the relative susceptibilities of compact core particle DNA compared to spacer. Thus, its digestion behavior toward nonnucleosomal nucleoprotein cannot be predicted. It is conceivable that even core particle conformational transitions, such as unfolding, could change the relative core–spacer susceptibilities and thus change the staphylococcal nuclease patterns without changing the histone–DNA contacts (DNase I patterns). An unfolding of the core, which maintains histone–DNA contacts, has been demonstrated in vitro (Zayetz et al., 1981). Furthermore, large changes in staphylococcal nuclease digestion patterns without changes in DNase I patterns occur on an active, single copy gene in yeast (D. Lohr, unpublished results). Thus the upstream region appears to be nonnucleosomal, but it may only be altered in conformation from the typical nucleosome.

To relate structural data to functional processes for a repeated gene like rDNA, one needs to know how many of the repeats display any given feature. In this work, evidence suggests a minor class with nucleosomal and a dominant class with "nonnucleosomal" upstream (control) regions. The most active genes may exist as a small, refractory class with nonnucleosomal *coding* regions. If the genes with nonnucleosomal coding regions are the transcrib*ing* repeats, as seems likely, the (dominant) class with nonnucleosomal upstream regions might well be the transcrib*able* ones. In these repeats, the control sequences differ from the coding sequences in chromatin structure. This seems an efficient way to demarcate regions for important control processes, aiding ready recognition by diffusible control molecules or enzymatic machinery. Such a region could also act as the nucleation site for the propagation of the structural alteration downstream, assuming that there is a coding sequence alteration during transcription of the gene, as suggested by electron microscopy. The structural discontinuity is also suggestive of some degree of nucleosome positioning on or around the site where the first nucleosome sits.

In this gene set, the primary distinction seems to be whether or not a region contains (classical) nucleosomes, not how they are spaced (repeat length etc.). Where they appear, nucleosomes are rather homogeneous, for example, across the entire ~7-kb 35S coding sequence. Furthermore, in most repeats, this distinction divides the two functional domains, coding vs. control sequences. For a repeated set of genes, whose number is probably much greater than the number needed to be expressing at one time but most or all of which may be capable of being expressed at some time, such structural distinctions could aid the necessary maintenance of the expression potential. The structural heterogeneity within each domain thus may have great utility in carrying out functional aspects of variations in expression.

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

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