Different Nucleosome Structures on Transcribing and Nontranscribing Ribosomal Gene Sequences

Abstract. *Monomeric DNA lengths from* Physarum *nuclear chromatin occur in two subunit forms which differ from each other and from higher oligomers of nucleosomes in content of transcribed ribosomal DNA sequences. Labeled DNA restriction fragments from ribosomal RNA coding regions reanneal most rapidly with DNA from a monomeric subunit fraction, A particles, isolated from growing plasmodia and containing 144 base pairs of DNA in an extended conformation. Higher oligomers of nucleosomes are depleted in sequences from transcribing gene regions but are enriched in sequences from the nontranscribed central spacer of the ribosomal DNA palindrome. Nucleosome configuration on two 26S gene intervening sequences resembles that on adjacent coding regions.*

Organization of the eukaryotic genome into DNA-histone subunits extends to those sequences that code for ribosomal RNA (rRNA) (*1–3*) or messenger RNA (*4*) products. Electron microscopic studies suggest that DNA in transcribing ribosomal gene chromatin of several organisms is in an unbeaded configuration when spread for visualization (*5–7*). The *Xenopus* ribosomal gene repeat, when incorporated into a circular plasmid and injected into oocytes, is transcribed in a fully extended configuration while adjacent inactive DNA sequences are contained in nucleosomes with a packing ratio of about 2:1 (*8*). The extrachromosomal rDNA of *Physarum polycephalum* is amplified (200 to 400 copies per nucleus) and can be purified in milligram quantities together with its associated nucleolar proteins (*9*), offering a system for biochemical studies of ribosomal gene subunits. At the end of G2 growth phase in plasmodial nuclei, nearly all ribosomal genes are in the process of transcription (*10*). Plasmodial ribosomal gene chromatin is digested by staphylococcal nuclease to release repeating chromatin subunits (*3*). However, several studies show that *Physarum* chromatin containing the rRNA coding regions is digested more rapidly than most chromatin to release subunits containing 144 base pairs (bp) of DNA (*3, 7, 11, 12*). These subunits include 11*S* monomers as well as 5*S* monomeric subunits (A particles) containing 144 bp of DNA in an extended configuration and an altered protein content (*11*). We find a different distribution of these chromatin subunits and higher oligomers on rDNA sequences comprising the ribosomal gene transcription unit and on associated nontranscribing rDNA sequences.

The *Physarum* rDNA molecule is a 61-kilobase (kb) palindrome containing two copies each of the 19*S*, 5.8*S*, and 26*S* rRNA coding regions (*9, 13*). The 26*S* gene contains two intervening sequences (*13*). Electron microsopic (*7*) and restriction mapping (*14*) procedures give approximate coordinates of the rDNA transcription unit. For this study rDNA restriction fragments were labeled with ^{32}P by nick-translation with DNA polymerase I and [α-^{32}P]deoxyribonucleoside triphosphates (*15*). These labeled fragments were annealed with DNA from chromatin subunits isolated from nucleoli after staphylococcal nuclease digestion (*11*). For our study we compared DNA from 11*S* nucleosomes, 5*S* A particles, and higher oligomers of nucleosomes (tetramers and larger, containing at least 690 bp of DNA). The DNA from each subunit fraction was reannealed in excess over each labeled restriction fragment DNA used as probe. Under these conditions, the rate of reannealing of the probe is proportional to the extent to which the labeled rDNA sequences are represented in the DNA from each chromatin subunit (*16*). Rates of reannealing of three representative rDNA restriction fragments with chromatin subunit DNA are plotted in Fig. 1. The Hind III c fragment is located entirely

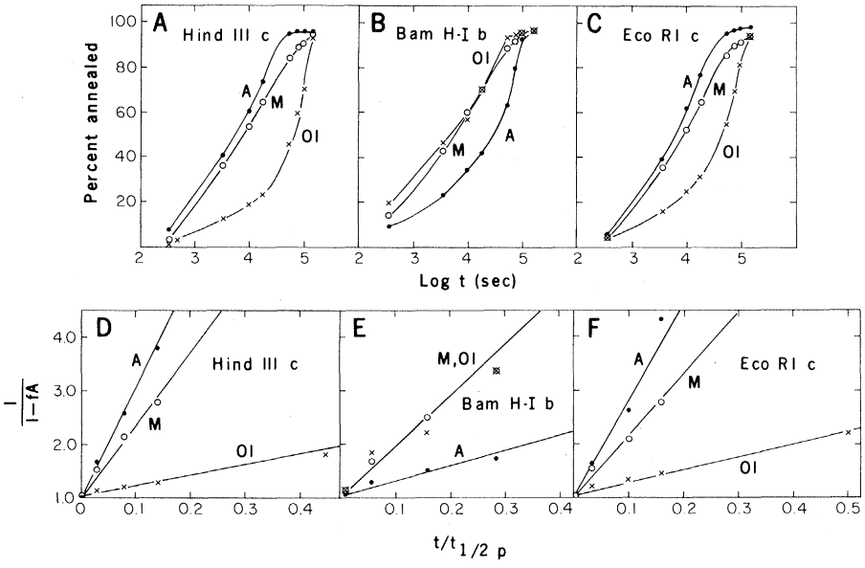

Fig. 1. Reannealing of ^{32}P-labeled rDNA restriction fragments with DNA from chromatin subunits. Isolated rDNA restriction fragments (*13*) were nick-translated with the use of [α-^{32}P]dATP and [α-^{32}P]dCTP (350 Ci/mmole) (*15*). To obtain DNA of separated chromatin subunits, nucleoli were treated with staphylococcal nuclease (15 percent digestion to trichloroacetic acid–soluble products), and resulting chromatin subunits were isolated by sucrose gradient centrifugation (*11*). Of the recovered DNA 28.7 percent was in A particles, 17.3 percent in monomers, and 34.4 percent in higher oligomers after DNA extraction (*11*). Prior to reannealing, all DNA fractions were boiled for 10 minutes in 0.3*N* NaOH to denature and partially degrade DNA. Average lengths of higher oligomer and labeled probe DNA strands were approximately 300 bp as measured by gel electrophoresis. Portions of each labeled rDNA fragment (5.0×10^{-4} μg; 7.2×10^{7} cpm/μg) were incubated with excess subunit DNA (0.5 μg) in 0.50 ml of solution containing 1.0*M* NaCl, 1.0 m*M* EDTA, and 0.14*M* sodium phosphate, *p*H 6.8. Reannealing was carried out at 68°C as indicated, and products were analyzed (*16*). (A to C) Kinetics of reannealing of DNA from A particles (*A*), monomer nucleosomes (*M*), and higher oligomers of nucleosomes (*Ol*) with labeled DNA of the Hind III c, Bam H-I b and Eco Rl c fragments, respectively. (D to F) Linearization of reannealing kinetics expressed as the reciprocal of 1 minus the fraction of labeled DNA annealed [$1/(1 - f_a)$] plotted against annealing time (t) divided by the half-time of reannealing of the labeled probe alone ($t_{1/2p}$). The probe was reannealed in the presence of excess calf thymus DNA, and the average value of the half-time for reannealing was calculated (*16*). Under the conditions used, values for $t_{1/2p}$ of the Hind III c, Bam H-I b, and Eco Rl c fragments were 35.0, 17.5, and 31.1 hours, respectively. Kinetics were plotted with all values of f_a from 0.03 to 0.80. Note differences among the chromatin subunits in content of sequences annealing to the Hind III c fragment (A and D; 8.0 to 13.0 kb, derived from a transcribing rDNA segment), to the Bam H-I b fragment (B and E; 23.9 to 37.3 kb, derived from the nontranscribed spacer) and to the Eco Rl c fragment (C and F; 5.3 to 7.6 kb, containing both 26*S* gene intervening sequences).

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within the rDNA transcription unit, while the Bam H-I b fragment is derived from the nontranscribed central spacer (*13, 14*). Annealing of the Hind III c fragment with DNA from A particles or monomers proceeds nearly ten times faster than with DNA from higher oligomers of nucleosomes (Fig. 1A). Of the three subunits, A particles contain the highest content of Hind III c sequences while these sequences, derived from the transcription unit, are only slightly represented in higher oligomers, as is indicated by slopes in the linearization of data shown in Fig. 1D. The Bam H-I b fragment reanneals nearly five times faster with DNA from 11*S* monomers or oligomers of nucleosomes than with DNA from A particles (Fig. 1, B and E). This fragment is represented to an equal extent in beaded monomers and oligomers. Reannealing of the Eco R1 c fragment proceeds most rapidly with DNA from A particles (Fig. 1, C and F), in a pattern similar to that seen with the Hind III c fragment. This fragment consists primarily of the two 26*S* gene intervening sequences (Fig. 2).

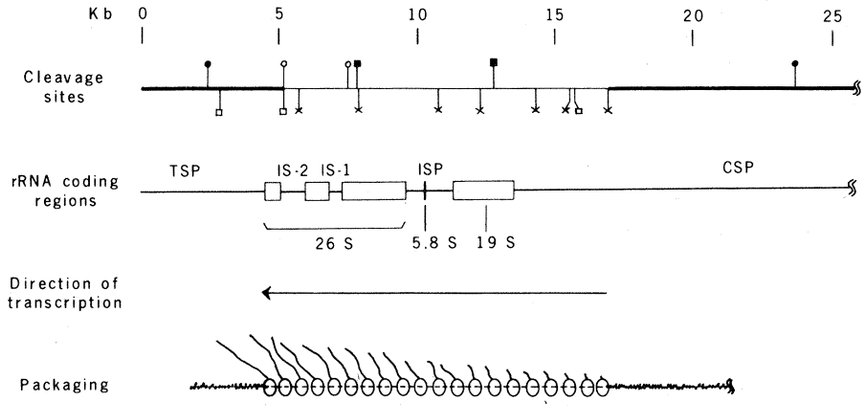

Fig. 2. Summary of reannealing of rDNA sequences with subunit DNA showing coordinates of rDNA restriction fragments as probes. One end of the 61-kb rDNA palindrome is shown. Restriction fragments produced by the five enzymes shown were reannealed with A, M, and O1 DNA (Fig. 1). The heavy solid line at top denotes those fragments containing rDNA sequences reannealing most rapidly with M or O1 DNA. The thin line denotes those fragments containing sequences reannealing most rapidly with A particle DNA. Pst I c and d, and Bam H-I c fragments at the rDNA termini deviate from second order kinetics upon reannealing with chromatin subunit DNA. In the text, restriction fragments for each enzyme are lettered alphabetically in order of decreasing size. Cleavage sites are for: Eco R1 (○), Hind III (■), Bam H-I (●), Pst I (□), and Xho I (x). Abbreviations are: *TSP*, terminal spacer; *IS-1* and *IS-2*, intervening sequences 1 and 2, respectively; *ISP*, internal spacer; and *CSP*, central spacer. The figure at bottom, drawn to the same scale, symbolizes extension of transcribing rDNA [dotted line (*7, 11*)] under RNA polymerases (ovals, not to scale) and shortening of inactive rDNA by nucleosomes with a repeat length of 170 bp (*3*) and a packing ratio of 2:1.

Our results indicate that subunits on the ribosomal gene transcription unit are in a configuration different from the oligomers of nucleosomes present on inactive rDNA regions. Peak A particles are derived primarily from the rDNA segment lying between the Pst I site at 5.3 kb and the Xho I site at 17.0 kb, this segment of 11.7 kb corresponding to most of the 12.3-kb transcription unit (Fig. 2). In contrast, oligomers of nucleosome beads are a distinct characteristic of that segment central to the Xho I site at 17.0 kb, which consists primarily of nontranscribed spacer sequences. (The Xho I a fragment reanneals most rapidly with oligomer DNA while the adjacent h fragment reanneals most rapidly with A particle DNA.) Our results are in agreement with earlier indications that all major DNA classes are found in monomers (*4*). However, we find that protected subunits derived from transcribing rDNA regions are not a homogeneous population of nucleosomes, but consist predominantly of the monomeric A form with altered physical parameters and protein content. Furthermore, oligomers of subunits are not derived significantly from the transcription unit. Either they are present to a very limited extent on transcribing sequences or they are more labile to nuclease than are oligomers on nontranscribing sequences. Previous studies on *Tetrahymena* (*2*) and mice (*17*) have shown rDNA sequences in repeating subunits, but the nature of these subunits has not been defined. In several cell types actively transcribed gene sequences are preferentially digested by deoxyribonuclease I (*18*), suggesting a stable modification in nucleoprotein structure. *Physarum* differs from certain other eukaryotes in that its ribosomal genes are segregated and that, in its less complex genome, a higher percentage of genes are active. Differences in histone structure also exist (*3*). The significant presence of a 5*S* monomer subunit in *Physarum* nuclease digests may be due to the high level of transcriptional activity of the organism, but may also be the result of specific characteristics of *Physarum* histones or their degree of modification. It is not known whether a conformational change of DNA in nucleosomes is required for transcription in vivo. Nucleosome beads have been visualized on nonribosomal genes sparsely populated with polymerases (*5, 19*). Evidence obtained with isolated RNA polymerases indicates that DNA in nucleosomes can be transcribed slowly, but without initiation (*20*). Increasing salt concentration enhances the rate of transcription severalfold (*20*). Extension of DNA in chromatin as seen by electron microscopy may be a function of the rate of transcription as indicated by the extent of polymerase binding (*6*), and thus particularly may be evident in ribosomal genes which are densely packed with polymerases. Alterations in the physical conformation of nucleosomes on functional genes may be transient (*21*) but also dependent on the more stable alterations in nucleoprotein structure responsible for conferring deoxyribonuclease I sensitivity (*18*). Our results are evidence that different chromatin subunit structures can be isolated from different functional regions of a single gene. The molecular transitions involved in these differences remain to be elucidated.

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is thus derived from structures localized throughout the genome. Peak A particles were not found in nuclei from the dormant microsclerotial stage of the life cycle.
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21. On ribosomal genes where RNA polymerases are not densely packed [Y. N. Osheim, K. Martin, O. L. Miller, Jr., *J. Cell Biol.* **79**, 126a (1978)] chromatin in a beaded morphology can be visualized between polymerase molecules by electron microscopy. Studies on replicating *Xenopus* ribosomal genes show that nucleosomes re-form rapidly on the DNA after passage of the replication fork [S. L. McKnight, M. Bustin, O. L. Miller, Jr., *Cold Spring Harbor Symp. Quant. Biol.* **42**, 741 (1978)].
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Time-Resolved Europium(III) Excitation Spectroscopy: A Luminescence Probe of Metal Ion Binding Sites

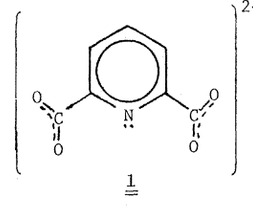
Abstract. *A laser-induced luminescence technique is introduced for probing the structure and equilibria of lanthanide complexes and lanthanide ion binding to macromolecules. The method involves the excitation of the* $^7F_0 \rightarrow {}^5D_0$ *transition between nondegenerate levels in the europium(III) ion by means of an intense pulsed dye laser source. Excitation profiles obtained by scanning the laser through the transition region reveal distinct peaks characteristic of individual europium(III) ion environments. The technique may be used to characterize the species present in complex equilibria in solution or to study europium(III) binding to macromolecules. Distinct europium(III) binding sites in thermolysin with long and short excited state lifetimes are observed.*

The ability of trivalent lanthanide ions, Ln(III), to serve as replacement probes for Ca(II) in calcium-binding proteins is well established (*1*-*6*). An especially promising property is the ability of several members of the series to luminesce in fluid solution at room temperature. We have shown (*7*) that useful luminescence emission can be observed at Ln(III) concentrations as low as 1 μM when an intense pulsed dye laser source is used for direct excitation of metal ion levels. Determinations of the reciprocal excited state lifetime, τ^{-1}, in both H_2O and D_2O solution provide a direct measure of the number of water molecules coordinated to a Ln(III) ion, which is itself bound to a macromolecule (*7*).

In this report we discuss a technique that is akin to absorption spectroscopy but that amplifies the sensitivity of the absorption experiment by several orders of magnitude. This is accomplished by monitoring absorption indirectly by means of emitted photons. Excitation spectroscopy with an intense, rapidly pulsed laser excitation source allows us to record absorption profiles of protein-bound Ln(III) ions with absorption coefficients, ε, as low as $0.01 M^{-1}$ cm^{-1} down to concentrations of 0.1 mM and below.

Our present concern is with the $^7F_0 \rightarrow {}^5D_0$ transition in Eu(III) at $\sim$ 17,250 cm^{-1} (580 nm), which has certain unique advantages. Since both the initial and final states are nondegenerate in the free ion, neither can be split by a ligand field, and thus a single sharp transition is to be expected for a particular Eu(III) ion. The energy of the $^7F_0 \rightarrow {}^5D_0$ transition (hereafter referred to as the 0-0 transition) is slightly dependent on the environment of the Eu(III) ion; and this transition is observed to range over slightly more than 1 nm for the systems we have investigated (1 nm $\simeq$ 30 cm^{-1} at 580 nm). The 0-0 transition is an extremely weak one ($\varepsilon \simeq 0.01 M^{-1}$ cm^{-1}) (*8*) and its study by absorption spectroscopy in dilute protein solutions is out of the question. Our experiment is performed, as described below, with much of the same apparatus employed for lifetime measurements (*7*). The Eu(III)-containing sample is irradiated with light (at or near 580 nm) from a pulsed (10 Hz) dye laser pumped by a nitrogen laser. The emission monochomator is set with wide slits to accept the intense $^5D_0 \rightarrow {}^7F_2$ emission at $\sim$ 612 nm. The excitation source, whose spectral bandwidth is approximately 0.01 nm, is then scanned through the 0-0 transition region. The intensity of the signal detected is proportional, among other factors, to the number of photons absorbed by this transition. For a single complex in solution, perfect Lorentzian peaks are observed with widths at half height of $\sim$ 8 cm^{-1}. They are thus sharp enough to be able to reflect fairly small changes in transition energy in going from one Eu(III) ion environment to another. Barring fortuitous coincidences, one observes as many 0-0 transitions as there are Eu(III) environments in the sample.

Our method may be applied to the study of complex equilibria in solution. Figure 1A shows the excitation profiles of the 0-0 transition during the course of the titration of Eu(III) (present as the chloride salt) with DPA^{2-} (dianion of dipicolinic acid, **1**, present as the sodium



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salt) in solutions with an adjusted pH of 6.0. As the DPA^{2-}/Eu(III) ratio is increased, marked changes occur in the spectra. Four distinct peaks are observed at various stages in the titration. On the basis of lifetime measurements, these correspond to the aqua ion: Eu_{aq}^{3+}, $[Eu(DPA)]^+$, $[Eu(DPA)_2]^-$, and $[Eu(DPA)_3]^{3-}$, which occur at progressively lower energies (Table 1). The energy differences are small, varying from 9 to 17 cm^{-1} between adjacent species, but with widths at half height of about 8 cm^{-1} the individual species are readily detectable.

The intensity of an individual peak in an excitation spectrum is determined by the product of the absorption coefficient for the band of the species in question and the quantum yield of luminescence of the emitting species. In the present experiment, signals of the Eu_{aq}^{3+} ion and the $[Eu(DPA)_3]^{3-}$ ion require much higher excitation intensities and signal ampli-

Table 1. Energies, excited-state lifetimes in H_2O and D_2O, and estimated numbers of Eu(III)-coordinated water molecules for the Eu(III)-dipicolinate system and Eu(III)-substituted thermolysin.

Species	Energy (cm^{-1})	τ_{H_2O} (μsec)	τ_{D_2O} (μsec)	q
Eu_{aq}^{3+}	17,272	104	2630	9.6
$[Eu(DPA)]^+$	17,263	169	2966	6.3
$[Eu(DPA)_2]^-$	17,248	304	3172	3.1
$[Eu(DPA)_3]^{3-}$	17,231	1640	3153	0.3
Thermolysin				
Site 1	17,253	555	1650	1.2
Sites 3 and 4	17,265	242	908	3.2

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