Dynamics of interaction of RNA polymerase II with nucleosomes. I. Effect of salts

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Abstract

Mononucleosomes were labeled with the sulfhydryl-specific fluorescence probe 1,5-IAEDANS (5-(2-((iodoacetyl)amino)ethyl)amino-naphthalene-1-sulfonic acid) by attaching the dye to the single cysteine of H3 through a covalent linkage. The enzyme RNA polymerase II (pol II) utilized the native and the reconstituted fluorescent nucleosomes as templates with greatest efficiency when 0.2 M potassium acetate (AcOK) was used as the supporting salt; 0.2 M NaCl was found to be very much inhibitory. Measurement of polarity of the microenvironment of the dye at its binding site in the nucleosome showed the conformation to be more open in the presence of AcOK, compared to that in 0.1 or 0.2 M NaCl. The binding of pol II to the nucleosome resulted in a relatively more compact structure when measured in terms of the polarity of the microenvironment of the dye in various salt-dependent conformations of the nucleosomes. Time-resolved fluorescence spectroscopy showed that the probe molecule at its binding site undergoes certain excited-state processes, and the presence/absence or rate of these excited-state processes depends on the conformation of nucleosomes, which in turn depends on the type and concentration of the ion present in the medium. Time-resolved emission spectra showed that binding of nucleosomes by pol II established some new contacts that resulted in inaccessibility of the dye to the bulk solvent, reflecting a more hydrophobic environment for the dye in the steady-state spectra. Thus, binding or transcription of nucleosomes by pol II did not break open their structure. Rather, some transient internal adjustments within the histone octamer may take place to accommodate the bulky pol II molecule.

Keywords: 1,5-IAEDANS; Lippert plot; mononucleosomes; time-resolved fluorescence; transcription elongation; yeast RNA polymerase II

The regulation of gene expression at the molecular level is governed directly or indirectly by DNA–protein interactions. Many of these interactions can be described in terms of acid–base pairs, where a negatively charged phosphate backbone of DNA interacts with domains of a protein having either overall basic characteristics or positively charged residues at precise positions. Such electrostatic interactions are, as expected, sensitive to ionic environments. A unique example is the interaction of basic histones with DNA to form nucleosomes. The nucleo-

somes are proposed to be not only the structural elements of chromatin but also the “regulators of gene expression” (Grunstein, 1990a). How the presence of nucleosomes on chromatin allows its replication and transcription to take place is an important and interesting question. Although both the status of nucleosomes and the role of histones in transcription initiation are becoming more and more clear (Grunstein, 1990b; Laybourn & Kadonaga, 1991; Workman et al., 1991), the status of nucleosomes during transcription elongation remains controversial (Lorch et al., 1988; Grunstein, 1990b; Clark & Felsenfeld, 1991; Kornberg & Lorch, 1991; Lee & Garrard, 1991; Thoma, 1991; Felsenfeld, 1992). The answer to this question spans two extremes: nucleosomes either dismantle or remain intact. In an analogous way, with varying salt concentration, association of the histone octamer with DNA varies between these two extremes. Various salt-dependent conformations identified by *in vitro* studies (van Holde, 1988) may represent, on the one hand, conformational variations of

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Abbreviations: AcOK, potassium acetate; DTT, dithiothreitol; dye/probe, 1,5-IAEDANS; H1, H3, subtypes of histones; H3.3, variant of H3; 1,5-IAEDANS, 5-(2-((iodoacetyl)amino)ethyl)amino-naphthalene-1-sulfonic acid; 1,5-MeAEDANS, adduct of β -mercaptoethanol and 1,5-IAEDANS; nTPs, nucleoside triphosphates; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; pol II, RNA polymerase II; SDS, sodium dodecyl sulfate; TE, 10 mM Tris-HCl-1 mM EDTA (pH 7.5); TRES, time-resolved emission spectrum.

the nucleosome in the highly packed form of the inactive chromatin or, on the other, an approachable state of the DNA in the active chromatin. Thus, if RNA polymerase induces an opening of the nucleosome structure, it can probably be measured in terms of salt-induced opening.

Fluorescence spectroscopic methods have been employed in a fair number of studies in the past to determine the conformation, shape, and size of nucleosome core particles under varying physiological or nonphysiological salt concentrations. In such studies, either the intrinsic tyrosine fluorescence of histone protein, or signals from fluorescence probes attached to the DNA or protein part of the core particle, were followed (Daban & Cantor, 1982; Libertini & Small, 1982; Chung & Lewis, 1985). Out of all core histones, only H3 has a single cysteine conserved at position 110 (the cysteine at 96 being variable) (van Holde, 1988). If a sulfhydryl-specific fluorescence probe is attached to this cysteine, its fluorescence properties reflect the properties of its immediate environment, which in turn is controlled by the overall conformation of the core particle (Daban & Cantor, 1989). Using different sulfhydryl-specific fluorescence probes, previous studies have indicated that cysteines of H3 are somewhat buried and lie on the dyad axis of the nucleosome (Dieterich et al., 1979). Thus, even a slight opening of the nucleosome can bring about a substantial change in the microenvironment of this cysteine.

The conformation of nucleosomes is known to be sensitive to the ionic concentration of the medium (Ausio et al., 1984; Yager & van Holde, 1984), and ionic concentration is one of the decisive factors in the polarity of the medium. Therefore, for this study, a sulfhydryl-specific fluorescence probe, 1,5-IAEDANS, was selected as the polarity probe (Hudson & Weber, 1973). Steady-state emission properties of a macromolecule-bound fluorophore are determined by a number of parameters such as excited-state interactions, solvent relaxation, polarity of the binding site, and specific interactions with amino acid residues near it. All these interactions collectively prepare the excited-state system on a nanosecond time scale, so time-resolved fluorescence studies were also conducted to complement the steady-state fluorescence analysis. In the presence of potassium acetate, the efficiency of transcription by RNA polymerase II over nucleosomes as templates was found to be better than that in the presence of NaCl. A comparison of the effect of these salts on nucleosomal conformation revealed that, in AcOK, nucleosomes are maintained in a conformation that is probably better poised for transcription, compared to their conformation in NaCl of the same concentration.

Results

Depending upon the salt concentration in the medium, various degrees of opening of the compact core nucleosome structure have been reported in the literature

(Dieterich et al., 1979; Ausio et al., 1984; Yager & van Holde, 1984; Yager et al., 1989). At intermediate ionic strengths, salts do not produce any drastic change in conformation of nucleosomes, and the extent of dissociation is governed by particle concentration to a fair degree (Ausio et al., 1984). In the present study, nucleosomes were used at 10–20 $\mu\text{g/mL}$, and total salt concentration used in any experiment was not above 250 mM. Therefore, they fell in region 1 and partly on the line dividing regions 1 and 2 of the stability diagram of Yager et al. (1989) for core particles. For the time-resolved studies, nucleosome concentration was still higher. Thus, under the experimental conditions used in the present study, mononucleosomes were assumed to remain undissociated as far as the effect of salt or particle concentration was concerned.

In the native gel, a small amount of free DNA was detected in the final preparation (Fig. 1A, lane 1); this was probably generated during storage (Yager & van Holde, 1984). Shortwave UV exposure of the gel showed that the fluorescence probe (tagged to H3) comigrated with the DNA in the nucleosome band (not shown), indicating histones were bound to DNA. The preparation was found to contain DNA of ~150–180 bp (Fig. 1A, lane 2) and stoichiometric amounts of all four core histones. The preparation was also checked to determine if the fluorescence of the probe showed any response to the interaction of pol II with the nucleosome. Figure 1B shows the steady-state fluorescence emission spectrum of the 1,5-IAEDANS-labeled nucleosome. At 1 mM NaCl and 0.4×10^{-7} M particle concentration, an emission maximum at 483 nm was observed that shifted to 489 nm when 80 mM AcOK was added (Fig. 1B). A similar red shift with other AcOK concentrations was observed (not shown). The small quenching (Fig. 1B, curve B) may be due to the lower quantum yield associated with the red shift (Hudson & Weber, 1973). Addition of pol II at 80 mM AcOK resulted in a shift of the maximum back to 486 nm, which means that binding of pol II to nucleosomes makes the dye surroundings somewhat hydrophobic. The fine structures in the peak region (Fig. 1B) indicate a possible prevalence of excited state processes for the dye in nucleosomes, and excited state–solvent interactions have been proposed as key mechanisms underlying the sensitivity of a probe molecule to the solvent (de Toma et al., 1976). Therefore, the quenching and blue shift associated with pol II binding (Fig. 1B, curve C) may be the results of changes in these excited state interactions. To determine if the fluorescence changes due to the addition of pol II are actually due to its binding, nucleosome–pol II complex was allowed to form and was resolved on a glycerol gradient following the protocol of Baer and Rhodes (1983). A peak fraction containing a nucleosome–pol II complex was seen in the lower half of the gradient. There was no peak at the corresponding position in a control gradient loaded with only the reconstitute. The presence of the complex in the peak fraction was confirmed by SDS-PAGE analysis, wherein

Fig. 1. A: Size analysis of reconstituted nucleosomes over a 4.5% native polyacrylamide mini-gel run in 40 mM Tris-acetate, 2 mM EDTA (pH 7.5). Lane 1, reconstituted, fluorescent nucleosome; lane M, Hinf I-digested pBR322; lane 2, free DNA isolated from reconstituted preparation of lane 1. Sizes of fragments in bp are shown. **B:** Effect of AcOK on steady-state fluorescence of 1,5-IAEDANS-labeled nucleosomes (excitation at 340 nm). Spectra were obtained as given in the text, corrected for dilution effects, and replotted. Curve A, mononucleosome in the presence of 1 mM NaCl; curve B, addition of 80 mM potassium acetate brings a red shift in the emission maximum; curve C, addition of pol II quenches the fluorescence.

pol II subunits and all core histones could be seen in the same fraction (results not shown).

Mononucleosomes as templates for pol II in vitro

To study the interaction of yeast pol II with rat liver nucleosomes in different conformational states, it was necessary first to check the efficiency of the enzyme and template activity of the nucleosomes within different ionic environments, especially because of the nonhomologous nature of the system. The results of an assay of the en-

zyme activity are presented in Table 1. Gel electrophoretic analysis of the transcription product (Bhargava, 1993) showed that a transcript of a size equal to that of the DNA in the template is synthesized during transcription. This suggests an efficient utilization of the full length of the template, indicating that pol II enters from one end of the template.

Yeast pol II is known to prefer denatured over native DNA template for transcription in vitro (Frederick et al., 1969). Therefore, activity of the enzyme over denatured calf thymus DNA in a nonspecific transcription assay

Table 1. Use of rat liver nucleosomes as template by the yeast RNA polymerase II^a

Transcription condition (template/salt)	cpm incorporated/mL	% Relative activity
Denatured calf thymus DNA		
0.1 M (NH ₄) ₂ SO ₄	182,115 ± 29,900	100.0
0.2 M NaCl	52,810 ± 6,800	29.0
0.2 M AcOK	69,010 ± 11,000	37.9
Reconstituted nucleosomes		
0.1 M (NH ₄) ₂ SO ₄	136,000 ± 16,600	74.7
0.1 M NaCl	112,940 ± 12,230	62.0
0.1 M (NH ₄) ₂ SO ₄ + 0.1 M NaCl	66,590 ± 7,690	36.6
0.2 M NaCl	56,470 ± 12,420	31.0
0.1 M NaCl + 0.1 M AcOK	73,180 ± 5,790	40.2
0.1 M (NH ₄) ₂ SO ₄ + 0.1 M AcOK	77,180 ± 8,740	42.4
0.2 M AcOK	168,945 ± 27,030	92.8

^a The assay was carried out by counting DE81 paper-bound radioactivity as described in Materials and methods in 100 µL volume having 24 µg/mL yeast RNA polymerase II and 100 µg/mL denatured calf thymus DNA or 10 µg/mL rat liver nucleosomes as templates, in the presence of the indicated salt concentrations. Values given in the middle column were normalized for equal template amounts and represent the mean of values from three independent experiments.

(Bhargava & Chatterji, 1988) was used as a positive control. The enzyme was found to transcribe nucleosomes with a comparatively lower efficiency and a dependence on the nature of the salts (Table 1). Whereas 0.1 M $(\text{NH}_4)_2\text{SO}_4$ was appropriate for routine assay over denatured calf thymus DNA templates (because activity in the presence of other salts was comparatively lower), 0.2 M AcOK was the salt of choice when nucleosomes were used as the template. Taking the highest activity of the enzyme over calf thymus DNA as 100%, activity of the enzyme over both the templates was reduced to the level of 30% only in the presence of 0.2 M NaCl. Ammonium sulfate (at 0.1 M) was a better salt than 0.1 M NaCl for transcription over nucleosomes, and about 93% of the activity was recovered in the presence of 0.2 M AcOK. On the other hand, except for AcOK at 0.2 M salt concentration, any other combination giving a total salt concentration of

more than 0.1 M was found to be inhibitory for transcription over nucleosomes (Table 1). The reason for such a differential effect of salts on enzyme activity *in vitro* is not clear, but it may be related to the salt-induced conformational changes, at least in the case of nucleosomal templates.

Binding of pol II with the nucleosomes

For further studies on the interaction of pol II and nucleosomes, it was thought necessary to learn the binding characteristics of the two. A gel mobility shift assay was used to obtain evidence for the binding and nondissociation of nucleosomes (Fig. 2A, inset), and a fluorescence titration study (Fig. 2) was used to determine the stoichiometry of binding in the presence of the two salts. Although DNA did not move as sharp bands in the gel

Fig. 2. Binding of 1,5-IAEDANS-labeled nucleosomes with varying concentrations of pol II in the presence or absence of transcription cocktail and 0.2 M salts. Intensities were corrected for dilution effects. **A:** Titration of fluorescence emission intensity at 485 nm. The nucleosome concentration was 38 nM. The inset shows the gel mobility shift assay for the nucleosome-pol II complex. The complex was assembled in the presence of 0.2 M AcOK as described in the Materials and methods and loaded on the gel without adding tracking dye. Free DNA was isolated from the nucleosome after deproteinization. Lane F gives the mobility of free DNA and its complex with pol II at a 1:6 molar ratio for comparison. Lane 1 is the nucleosome control. Pol II was present at 0.5-, 1.0-, 2.0-, 4.0-, 6.0-, 8.0-, and 10.0-fold molar excess over nucleosomes in lanes 2-8, respectively. **B:** Titration of fluorescence intensity at 485 nm in the presence of 0.2 M NaCl with 58.2 nM nucleosomes and transcription cocktail (open circles) or without transcription cocktail and 50 nM nucleosomes (filled circles). The inset shows SDS-PAGE analysis of the nucleosome-pol II complex resolved on a native gel as described in the Materials and methods. Lanes 1 and 4 were loaded with the gel pieces carrying control unbound nucleosomes, while lanes 3 and 5 were loaded with those having the bands showing the mobility of the nucleosome-pol II complex in the gel mobility shift assay. Lane 2 shows the mobility of the various subunits of the yeast pol II, and "H" marks the position of the histones on this gel. Lanes 1 and 5 were loaded with twice the amounts loaded in the lanes 4 and 3, respectively.

(probably due to the presence of 0.2 M salt), bands due to bound or unbound DNA could be distinguished. Short-wave UV exposure also could not detect free histones in the gel (not shown), suggesting that nucleosomes do not dissociate due to pol II binding. Although an increase in intensity of the complex was observed with increasing pol II concentration, along with a decrease in intensity of the unbound DNA (Fig. 2A, inset), the gel mobility shift assay was not used to calculate the stoichiometry of the binding. The major reason for this was the simultaneous presence of two types of receptors before pol II binding, i.e., the nucleosomes and the small but unavoidable population of free DNA. Besides, even after binding, the large size of the nucleosome-pol II complex may cause its dissociation during the electrophoretic run; thus, a binding with 1:1 molar stoichiometry may also show saturation only at a higher molar ratio of the ligand. The gel mobility shift assay (Fig. 2A, inset) for the complex formation under the binding conditions of Baer and Rhodes (1983) showed the presence of retarded bands at as low as twofold molar excess of pol II, and all the nucleosomes were complexed with pol II at its eightfold molar excess. However, to make sure that the retarded bands are due to the complexation of the pol II with the nucleosome, the gel pieces corresponding to the control and retarded nucleosomal bands were cut and loaded onto a gel for SDS-PAGE analysis. The inset of Figure 2B shows the presence of the bands corresponding to the subunits of pol II only in lanes 3 and 5. Dark bands in the middle portions of lanes 1, 3, 4, and 5 were due to the DNA in the nucleosomes. Core histone bands could be seen in all four lanes, although with very low intensity in lanes 3 and 4, probably due to insufficient loading. Comparison of lanes 1 and 5 suggested that, although all the core histones are present in the unbound nucleosome, the pol II-bound nucleosomes seem to be deficient in histones H2A and H2B (cf. lanes 1 and 5), as reported previously by Baer and Rhodes (1983). However, because resolution of individual histones was unclear and the aim was only to establish their presence, bands were not quantitated to check these points.

Because a distinct response of nucleosomes to binding of pol II was observed (Fig. 1B), a fluorescence titration to determine the stoichiometry of binding was carried out wherein quenching of the fluorescence emission intensity was followed. In the presence of either AcOK or NaCl, a saturation effect was observed at 1:1 molar ratio (Fig. 2). Further addition of pol II did not result in any more quenching, implying a molar stoichiometry of 1:1 for nucleosome-pol II binding. Pol II was found to have only slightly different binding constants in the presence of either of the salts under the transcribing conditions (K_d of 13 nM with AcOK and 17 nM with NaCl). At the saturation level, only 13% or 17% of the total fluorescence was quenched, and titrations in the absence of transcription cocktail gave saturation at 15% quenching in the presence of both the salts.

Baer and Rhodes (1983) reported a molar stoichiometry of 1:1 for binding of calf thymus pol II to mouse myeloma mononucleosomes in the presence of a 10-fold molar excess of pol II. However, under the binding conditions used by them, only 15% of the total nucleosome population was found to enter the complex at the saturation level. In the present study, fluorescence titration showed that 13% of the nucleosomes enter the complex in the presence of an equimolar amount of pol II under transcribing conditions, indicating that only one-eighth of the total population of nucleosomes at a time was transcribed by pol II. Because quenching of the probe fluorescence is associated with binding of pol II, it is possible that pol II binds to this 13% of the nucleosomes in close proximity to the probe molecule. Thus, these nucleosomes may also represent the 15% fraction of nucleosomes with actively transcribed sequences described previously by Baer and Rhodes (1983). However, binding of the remaining nucleosomes to pol II at its higher molarities, observed in the gel mobility shift assay (Fig. 2A, inset), could not be seen as quenching in the fluorescence titrations. This observation suggested that the latter binding takes place in a region of nucleosome that does not affect the fluorescence properties of the probe molecule. Thus, while fluorescence could not detect the low-affinity binding of the pol II with the bulk nucleosomes, the high-affinity binding of the pol II to a selected fraction of nucleosomes detected by fluorescence at an equimolar ratio could not be differentiated by the gel mobility shift assay. Under nontranscribing conditions, pol II binds with the same affinity to this 15% fraction of nucleosomes in the presence of both salts. However, under transcribing conditions, due to the higher transcription in the presence of AcOK and therefore higher turnover, only 13% of the nucleosomes were finally bound at the saturation level in the presence of AcOK, whereas 17% were bound in the presence of NaCl.

Making a calibration curve for the measurement of polarity of the medium for the dye 1,5-IAEDANS

Changes in fluorescence properties of naphthylamine sulfonic acid derivatives when attached to a macromolecule have been used as a measure of the polarity of the binding site, which is generally more hydrophobic than the aqueous buffer in which the macromolecule is dissolved (Turner & Brand, 1968; Brand & Gohlke, 1972). Steady-state fluorescence studies were conducted to examine environmental changes for the cysteines at the dyad axis under different conformational states of nucleosomes. As described in the Materials and methods, absorption and steady-state fluorescence emission spectra of the free dye in media of varying polarity were obtained (Table 2), and the Lippert equation was used to generate a calibration curve using these data (Fig. 3A). Because spectral properties of the dye when attached to free cysteine or reacted

Table 2. Steady-state fluorescence properties of 1,5-MeAEDANS in response to solvent polarity^a

Solvent	Absorption maximum (nm)	$\bar{\nu}_a$ (cm ⁻¹)	Fluorescence emission maximum (nm)	$\bar{\nu}_f$ (cm ⁻¹)	$\bar{\nu}_a - \bar{\nu}_f$ (cm ⁻¹)	Δf
TE (pH 7.5)	336 ± 1.5	29,761	508.1 ± 1.9	19,681	10,080	0.3196
20% ethanol	337 ± 1.1	29,673	497.9 ± 0.8	20,084	9,589	0.3108
40% ethanol	337 ± 2.0	29,673	491.6 ± 2.7	20,341	9,332	0.3021
60% ethanol	337 ± 1.1	29,673	485.6 ± 0.9	20,590	9,083	0.2957
80% ethanol	338 ± 2.0	29,585	482.0 ± 1.2	20,746	8,839	0.2911
100% ethanol	340 ± 3.3	29,411	474.8 ± 3.1	21,048	8,363	0.2887

^a 1,5-MeAEDANS from a stock solution was added to various ethanol-buffer mixtures and spectra were recorded. Percent values in the first column represent the proportion of ethanol in the given ethanol-buffer mixture. Δf (orientation polarizability) was calculated by obtaining reported values of $\epsilon_{25^\circ\text{C}}$ and $n_{D,25^\circ\text{C}}$ for these mixtures from literature using the formula given in the text. $\bar{\nu}_a$ and $\bar{\nu}_f$ were calculated for the maxima obtained from the absorption and corrected emission spectra, respectively.

with β -mercaptoethanol are similar (Hudson & Weber, 1973), the 1,5-MeAEDANS derivative of the dye was used as representative of the free dye. The Δf values for ethanol-TE buffer mixtures of various polarities were calculated by obtaining the values of the refractive index ($n_{D,25^\circ\text{C}}$) and static dielectric constant ($\epsilon_{25^\circ\text{C}}$) for these mixtures from the literature (Timmermans, 1960). A straight line was obtained in the Lippert plot (Fig. 3A) for polarities up to 80% ethanol-buffer mixture, which signifies that specific solvent effects are saturated in these mixtures and that general solvent effects predominate (Lakowicz, 1983). The point for 100% ethanol was very much off the line (Fig. 3A), indicating a prevalence of specific solvent effects in this case, in accordance with the reported heterogeneity associated with emission in the more blue regions for this dye (Hudson & Weber, 1973). Because orientation polarizability did not show a perfect linear change with change in polarity of the medium, a second calibration curve (Fig. 3B) was prepared to double check the polarity measurement of the dye's environment.

Measurement of the polarity of the dye environment in nucleosomes using the Lippert plot

Because NaCl and AcOK were found to have almost opposite effects on transcription of nucleosomal templates, and $(\text{NH}_4)_2\text{SO}_4$ remained intermediary (having effects more like NaCl at equal concentrations; Table 1), only NaCl and AcOK were used for further fluorescence studies. When nucleosomes were shifted to various salt concentrations by mixing an aliquot of the stock directly into a salt-containing buffer in the fluorescence cuvette, rapid shifts in emission maxima could be seen.

Results summarized in Table 3 are from the steady-state fluorescence measurements for nucleosomes in various

Fig. 3. Lippert plots for 1,5-MeAEDANS in ethanol-TE buffer (pH 7.5) mixtures of varying polarities. Data in Table 2 were used to generate both plots.

salt concentrations. The $(\bar{\nu}_a - \bar{\nu}_f)$ values in each case were used to obtain the corresponding ethanol-buffer environment from the calibration curves given in Figure 3A and B. Nucleosomes in 0.1 M NaCl were found to have a conformation in which the dye was in the highest alcoholic concentration or the maximum hydrophobic environment. Therefore, taking these nucleosomes as the ones with maximum compactness, and assigning a value of 100, the relative compactness of nucleosomes in each case

Table 3. Effect of salt on steady state fluorescence of nucleosome labeled with 1,5-IAEDANS^a

Condition	Fluorescence emission maximum (nm)	$\bar{\nu}_f$ (cm ⁻¹)	$\bar{\nu}_a - \bar{\nu}_f$ (cm ⁻¹)	Δf (Fig. 3A)	% Alcohol equivalent (Fig. 3B)	% Relative compactness
Histone octamer	491	20,366	9,395	0.3050	33	30
Nucleosomes						
No salt	488	20,491	9,270	0.3018	41	37
10 mM NaCl	486	20,576	9,185	0.2995	46	42
30 mM NaCl	484	20,661	9,100	0.2972	55	50
100 mM NaCl	476	231,008	8,753	0.2882	110	100
200 mM NaCl	478	20,920	8,841	0.2905	86	79
200 mM NaCl + pol II	476	21,008	8,753	0.2882	110	100
780 mM NaCl	487	20,533	9,228	0.3005	44	40
200 mM AcOK	488	20,504	9,257	0.3015	41	37
200 mM AcOK + pol II	486	20,576	9,185	0.2996	46	42
200 mM Ac OK + pol II + nTPs	485	20,618	9,143	0.2985	50	45

^a An aliquot of nucleosomes stored with 40 mM NaCl was salt jumped to a given final salt concentration in the cuvette. The absorption maximum in each case was taken as 336 nm. Corrected emission spectra were recorded (excitation at 336 nm) after 1 min of mixing and were found to remain the same after 10 min of mixing. Given values of emission maxima are the averages ± 0.5 nm of three independent measurements, rounded off to the nearest whole numbers. Fluorescence of dye in the octamer did not change in this range of salt concentration, therefore no salt concentration for the octamer is mentioned. The alcohol equivalent of different salt conditions was obtained from Figure 3B by using the Δf values, which were in turn obtained from the Lippert plot (Fig. 3A).

was calculated. Although the emission maximum showed only small variations with changing ionic conditions (Table 3), the effect was seen better when the relative compactness of the nucleosomes in each case (Table 3) was calculated using the Lippert plots given in Figure 3. Changing the NaCl concentration from 0.1 M to higher or lower values reduced the measured compactness of the nucleosomes. In comparison, the histone octamer, irrespective of the salt concentration present, showed a relatively less compact structure. Compactness of nucleosomes in 0.2 M NaCl was found to be almost twice that of nucleosomes in 0.2 M AcOK, which is also close to that in 0–10 mM NaCl.

The change in fluorescence emission maximum of the dye in nucleosomes with NaCl concentration in this study followed a pattern reported previously by Dieterich et al. (1979). The present study, however, is restricted to a narrower salt concentration range, normally used for in vitro DNA–protein interaction studies and aimed at finding if interaction of nucleosomes with pol II can be described in terms of ionic interactions modeled by salts. Thus, in the presence of 0.2 M AcOK, a complex with a relatively more open conformation forms between pol II and the nucleosome, whereas in the presence of 0.2 M NaCl this complex is comparatively compact. This may be the reason the transcription efficiency of pol II over nucleosomes is so different for the two salts (Table 1). Measurement of polarity using the Lippert plot thus shows that binding of pol II results in a conformation of the nucleosome that is more compact than that of the histone octamer, and probably resembles the nucleosomes in 10–100 mM

NaCl. Thus, the results also demonstrate that a more compact nucleosomal structure is transcribed by pol II with a lower efficiency, and this efficiency can probably be measured by using the Lippert plot if required.

Time-resolved fluorescence measurements for the fluorophore attached to the nucleosomes

Time-resolved studies can demonstrate the effect of solvent and excited state reactions of a probe. Therefore, to gain a better understanding of the salt-induced changes in the probe environment and thus in nucleosome conformation under different conditions, fluorescence lifetimes and time-resolved emission spectra for the reconstituted nucleosomes were obtained under various conditions. Different values of lifetimes in the presence of NaCl or AcOK (not shown) indicated that different rates of excited state processes and therefore a different disposition of the probe molecule inside the nucleosome may exist in both cases.

The TRES in the presence of 0.1 M NaCl (the most compact organization; Table 3) showed that several changes occur around 40 ns (Fig. 4A). A peak at 466 nm was found to rise slowly in intensity until 40 ns with a blue shift, suggesting formation of an intramolecular charge-transfer complex. In contrast, the major peak, initially at 484.5 nm, overlapped a newly developed peak at 500 nm at 40 ns, after which it showed a blue shift to 481 nm. As the peak at 481 nm decayed, the peak at 500 nm persisted. This observation suggested intermolecular charge-transfer complex formation, probably as a result of interaction of

Fig. 4. TRES recordings of mononucleosomes obtained in stroboscopy mode at 25 °C (excitation wavelength 337 nm). **A:** In the presence of 0.1 M NaCl in TE (pH 7.5) buffer, plotted in 10-ns increments and at a 35° angle. **B:** In the presence of 0.2 M AcOK in TE (pH 7.5) buffer, plotted in 20-ns increments and at a 25° angle.

the two probe molecules on the two cysteines sitting close to each other (Dieterich et al., 1979).

The TRES in the presence of 0.2 M AcOK (Fig. 4B) was found to be different from that in the presence of 100 mM NaCl, and instead resembled the TRES for free dye in 20% ethanol (not shown). However, unlike the free dye, the peak at 471.5 nm showed a small blue shift of 1.5 nm to 470 nm after 80 ns. The decay of intensity of the 470-nm peak was accompanied by disappearance of a small and transient peak at 491.5 nm, after 80 ns, such that a peak at 511 nm (toward the red end of the spectrum) developed around 80 ns; in contrast, a 510-nm peak developed at 40 ns for the free dye in 20% ethanol. This peak at 510 nm may be due to the formation of an intramolecular charge-transfer complex in the excited state, in contrast to a peak at 500 nm in the presence of 0.1 mM NaCl (cf. Fig. 4A,B), which may be due to an intermolecular charge-transfer complex.

The TRES for the binding of pol II to the nucleosome in the presence of 0.2 M AcOK described some changes in the excited state solvent interactions (Fig. 5). The ma-

jor TRES peak, still at 471.5 nm, showed the same blue shift to 470 nm, and a short-lived intermolecular complex developing within 20 ns was observed as a small peak at 502 nm. These observations suggest that binding of pol II to nucleosomes results in a conformational change whereby the dye gains a more compact environment compared to that before binding. Due to the inherent complexity of the system, it was difficult to correctly assign different TRES peaks to identifiable emitting species, and therefore no quantitative analysis was attempted on TRES data in this study.

Discussion

The presence of nucleosomes on the transcribed portions of a gene may be inhibitory to an elongating RNA polymerase, even after productive initiation (Kornberg & Lorch, 1991). This interaction may also depend on the conformation of the nucleosome the RNA polymerase encounters. In this study, *in vitro* interaction of yeast pol II with various salt-dependent conformations of a mono-

Fig. 5. TRES of mononucleosomes under binding conditions for pol II (molar ratio 1:1 in TE [pH 7.5] buffer and 0.2 M AcOK), recorded at 25 °C in stroboscopy mode of the instrument, exciting at 337 nm. Plots are in 10-ns increments with a 20° angle.

nucleosome was followed. A fluorescence spectroscopic method was used to monitor the changes in the protein core rather than the status of the DNA in the nucleosome. As yeast H3 is known to be devoid of cysteine (Smith, 1984), rat liver rather than yeast nucleosomes were used as the template. The aim was to follow the changes in a single nucleosome following interaction with pol II, so mononucleosomes, rather than di- or oligonucleosomes (which probably resemble the natural chromatin template better), were used.

Binding and transcription of nucleosomes by pol II is facilitated by AcOK

Fluorescence titration data demonstrated a molar stoichiometry of 1:1 for the binding of nucleosomes and pol II, and an *in vitro* transcript size equal to the size of the template DNA (Bhargava, 1993) suggested read through from one end of the template to the other. Therefore, it was concluded that nucleosome and pol II probably form a high-affinity complex with 1:1 molar stoichiometry. Such binding may be possible at the ends of the nucleosomal

DNA, as pol II is known to have high affinity for DNA ends. The pol II probably binds at only one end of the DNA in the nucleosome, since the two DNA ends are close to each other in space and the presence of one pol II molecule at one of the ends will cause steric hindrance of the binding of another pol II molecule.

The gel mobility shift assay and fluorescence titration also suggested a better interaction between nucleosomes and pol II in the presence of AcOK (not shown); activity of the pol II over the nucleosome was found to be highest in the presence of 0.2 M AcOK. Compared to AcOK or glutamate, chloride is known to be an ion that inhibits favorable DNA-protein interactions (Leirmo et al., 1987; Lue & Kornberg, 1987). The reason for this differential effect is unknown, but it may be one explanation for why activity of RNA polymerase over nucleosomes is higher in the presence of AcOK compared to NaCl or other salt combinations. Another explanation could be that the conformation of nucleosomes in the presence of AcOK probably allows greater accessibility for the enzyme than in the presence of NaCl. Close resemblance of nucleosomes in 0.2 M AcOK to ones in "no salt" suggested an open conformation, described for nucleosomes in very low ionic strength buffers by Eshaghpour et al. (1980).

Conformation of nucleosomes differs under various salt conditions

Steady-state fluorescence studies showed changes in compactness of nucleosomes with varying salt concentrations. Differences in observed lifetime values and types and rates of excited state processes for nucleosomes in the presence of 0.1 M NaCl or 0.2 M AcOK resulted also in several observed differences in TRES obtained under the two conditions. For example, the TRES peak with lowest energy is seen at 500 nm in 0.1 M NaCl as compared to 511 nm in 0.2 M AcOK, suggesting a relatively more polar environment for the dye in the latter case. Similarly, a higher red shift of 20 nm in 0.2 M AcOK compared to 15 nm in 0.1 M NaCl for initial single peaks and a peak in the blue region at 460 nm in 0.1 M NaCl may explain why emission is seen at a lower energy level in the presence of 0.2 M AcOK in steady-state spectra. The TRES also showed that the dye was inaccessible to the bulk solvent in the presence of 0.1 or 0.2 M NaCl, and therefore nucleosomes in these salts are probably more compact than in the absence of NaCl (not shown) or in the presence of 0.2 M AcOK. An excited state complex, probably due to intermolecular interaction of the probe molecules sitting on the cysteines of the two H3 molecules, could be generated in the presence of 100 mM NaCl, where due to a more compact conformation, cysteines were probably closer in space. Thus, TRES analysis could confirm a more open conformation of nucleosomes in the presence of AcOK.

Binding of pol II to nucleosomes results in a more compact structure

Pol II binding did not produce a further opening of the nucleosome structure in the presence of NaCl and AcOK. In fact, a very compact structure was obtained from this binding in the presence of 0.2 M NaCl. Due to limitations in its application, the Lippert equation could be used in the present study only to make the comparative measurements of the polarity of the dye's environment under various ionic conditions. Thus, using the Lippert plot it could be estimated that binding of pol II to nucleosomes resulted in about a 25% increase in compactness of the nucleosomes in the presence of 0.2 M NaCl, whereas this increase was only 13% in the presence of 0.2 M AcOK.

TRES recordings of nucleosomes showed that the intermolecular excited state reactions of the probe molecule depended on the distance between the two cysteines, which was decided by the salt-dependent conformation of the nucleosome (Dieterich et al., 1979). Thus, whereas the steady-state spectra described the conformation in terms of polarity of the probe surroundings, time-resolved emission spectra described it in terms of molecular crowding around the cysteines. Both studies demonstrated that polarity of the dye's microenvironment was inversely correlated with the compactness of the nucleosome structure; therefore, lower polarity of the dye's surroundings, as measured by using the Lippert plot, was taken as indicative of a more compact nucleosomal conformation. The absence of solvent relaxation effects on the behavior of TRES peaks was taken as suggestive of exclusion of solvent molecules around the probe molecule as a result of a more hydrophobic environment. In the presence of 0.2 M AcOK, binding of pol II to nucleosomes resulted in changes in the excited state solvent interactions in the TRES and a blue shift of the steady state as well as the 502-nm TRES peak, all suggesting a more hydrophobic surrounding for the dye. In addition, the lack of mobility of solvent molecules in the probe's surroundings indicated blocking of the open solvent channels of the nucleosomes (Burlingame et al., 1985; Arents et al., 1991), which were probably available in all other cases.

Figure 6 is an attempt to collectively describe all the conformational changes of nucleosomes found in this study through a diagrammatic representation of the histone octamer in the nucleosome (based on the structure given originally by Burlingame et al. [1985]). Although these authors have revised their structure, the description of the solvent channels, which forms the basis of the model given in Figure 6, remains unchanged (Arents et al., 1991). When a probe is attached to the cysteines lying at the dyad axis (Fig. 6A), three types of excited state interactions may predominate: solvent relaxation and intra- and intermolecular interactions, such as proton abstraction and charge-transfer complex formation. In the presence of 100 mM NaCl, where nucleosomes maintain a more

Fig. 6. Schematic diagram of nucleosomes in various conformational states. The drawing is based on the crystal structure of a core particle given by Arents et al. (1991). For clarity, DNA strands are omitted and only outlines of the histone octamer are given to highlight the positions of the solvent channels. The double-headed arrow shows interacting species. **A:** In the presence of 100 mM NaCl. **B:** In the presence of 200 mM AcOK. **C:** In the presence of 200 mM AcOK and pol II.

compact organization, the probe molecules sitting in close proximity to each other can interact. Because solvent channels are probably far off from the fluorophores in space, the probe surroundings are less polar in nature. Thus, a low rate of excited state processes, such as delayed solvent relaxation, is observed. The position of the probe molecule allows it to interact with other amino acid residues also in its vicinity. In lower ionic environments, the nucleosome is more expanded and cysteines are comparatively more distant (Dieterich et al., 1979). In the presence of 0.2 M AcOK, cysteines are probably away from each other (Fig. 6B), and the probe has free access to the bulk solvent through the solvent channels (Fig. 6B). Interaction of the probe molecules with neighboring molecules may be possible, but interaction of probe molecules with each other is not, and the rate of excited state reaction remains low. When pol II binds, several internal adjustments take place within the histone octamer to accommodate it (Fig. 6C). Cysteines remain at the same distance as in Figure 6B, but due to the new spatial adjustments, the probe molecule now has a more compact environment around it (as seen by steady-state analysis;

Table 3). However, this new conformation may be energetically unfavorable, reverting back to the original state the moment pol II leaves the nucleosome. This hypothesis is further substantiated by results reported in the accompanying paper (Bhargava, 1993).

The opening of the structure is thought to facilitate transcription of nucleosomes. Thus, RNA polymerase can probably transcribe nucleosomes with greater efficiency in the presence of AcOK than in the presence of NaCl. It will be worth analyzing the exact mechanisms leading these salts to show such different effects on DNA-protein interactions, considering that both constitute monovalent ions. One of the reasons could be that NaCl is a salt of a strong acid and a strong base. Therefore, in aqueous solution it is 100% dissociated and the ionic concentration is the same as the molar concentration. As AcOK is a salt of a weak acid with a strong base, its dissociation may not be 100%; thus, the ionic concentration is less than its molar concentration in aqueous solutions. Due to this property, for certain DNA-protein interactions that are basically acid-base interactions (as in the case of nucleosomes), at low salt concentrations NaCl has a stabilizing effect, whereas AcOK either tends to break the interactions or its ionic concentration does not reach the required level to stabilize them.

Materials and methods

All common chemicals were of the highest purity available. Ethanol was distilled three times to attain 100%. The fluorescence probe 1,5-IAEDANS was purchased from Molecular Probes (Eugene, Oregon).

Purification of pol II and transcription assay

Pol II was purified from a protease-negative strain, pep 4/3, of *Saccharomyces cerevisiae* as described by Bhargava and Chatterji (1989). Its activity was checked and assayed over denatured calf thymus DNA (or nucleosomal templates) measuring DE81 paper-bound radioactivity following a nonspecific transcription protocol described previously (Bhargava & Chatterji, 1987). The transcription cocktail used in the fluorescence spectroscopic studies was a 10× concentrate of a mixture of all four ribonucleoside triphosphates in a pH 7.5 buffer, which gave 0.5 mM concentration of each along with 1.6 mM MnCl_2 and 10 mM Tris-HCl at 1× concentration.

Purification of core histones and mononucleosomes and fluorescence labeling of H3

Rat liver nuclei, prepared according to Jones et al. (1989), were used for purifying the core histones (Stein & Bina, 1984) and mononucleosomes (Lutter, 1978). Ultrafiltered histones were stored frozen at -20°C at 7–8 mg/mL concentration in a buffer containing 10 mM Tris-HCl (pH 8),

1 mM EDTA, 1 mM PMSF, 0.5 mM DTT, and 2.5 M NaCl. They were stable and remained unproteolyzed in this form (checked by SDS-PAGE) even after 1 year of storage. H3 of these histones was labeled with 1,5-IAEDANS according to the procedure of Murphy et al. (1982) and stored similarly. Purified mononucleosomes were concentrated by ultrafiltration and dialyzed against a storage buffer having 50% glycerol, 0.1 M NaCl, 5 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 0.5 mM PMSF, and 2 mM β -mercaptoethanol. Stored nucleosomes were kept in a sterile, siliconized vial at -20°C , at 4–5 mg/mL DNA concentration, and were found to be stable for long periods under these storage conditions.

Histone H3 of nucleosomes was labeled according to Dieterich et al. (1977) under careful pH control to avoid nonspecific labeling of other amino acids such as tyrosine and lysine. Reconstitution was carried out by the descending step-gradient salt-dialysis method. Fluorescent reconstituted mononucleosomes were purified over a 5–30% linear sucrose density gradient in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 0.2 mM EDTA, 0.25 mM PMSF, and no NaCl. The mononucleosomes concentrated near the middle of this gradient and could be identified by (1) comigration of peaks of A_{260} and A_{280} , and fluorescence emission intensity at 476 nm (excitation 340 nm) and (2) native gel electrophoresis and localization of labeled H3 in the same position as the DNA band, by shortwave UV illumination before ethidium bromide staining. A very high degree of fluorescence labeling of nucleosomes could be achieved. Fractions containing mononucleosomes from a number of gradients were pooled and dialyzed against the storage buffer containing 40 mM NaCl, 0.5 mM PMSF, 2 mM β -mercaptoethanol, 50% glycerol, and 10 mM Tris-HCl (pH 7.5). Mononucleosomes stored in this way at -20°C were found to be stable for 6–8 weeks without change in their fluorescence properties. For certain experiments, aliquots of these nucleosomes were dialyzed against the same storage buffer without NaCl and used as “no salt” nucleosomes.

Formation of the pol II-nucleosome complex

Interaction of pol II with mononucleosomes was checked at different molar ratios by a gel mobility shift assay. Complex formation was allowed under binding conditions described by Baer and Rhodes (1983), and the reaction mix was loaded directly onto a 4.0% polyacrylamide native mini-gel run at 3 V/cm in TE (pH 7.5) buffer. The position of histones was visualized by UV exposure before staining, and DNA was seen after ethidium bromide staining. For further analysis of the proteins in the complex, the retarded as well as the unbound nucleosomal bands were cut, boiled with the loading buffer, and put into the wells of an SDS-polyacrylamide gel. The gel had 11% running gel, 5% stacking gel, and a short plug of 18% running gel at the bottom. Gels were silver stained.

Steady-state fluorescence studies

All steady-state fluorescence measurements were carried out at 25 °C on a Hitachi F4000 spectrofluorimeter, and corrected spectra were obtained in the appropriate buffers. The excitation wavelength for labeled nucleosomes was 340 nm, and a concentration range of $0.5\text{--}1.0 \times 10^{-7}$ M was used to avoid the inner filter effect (Daban & Cantor, 1989). Standard free dye was prepared by reacting the dye with excess β -mercaptoethanol in TE buffer, according to Hudson and Weber (1973).

Lifetime measurements and time-resolved emission spectroscopic analysis

All lifetime measurements and recordings of time-resolved spectra were carried out on a Photon Technology International (Canada) LS-100 luminescence spectrometer by the pulse method, in the stroboscopic mode. This instrument model employs a thyatron-gated, nanosecond, nitrogen flash lamp, giving a pulse width of less than 1.6 ns. A nonlinear least-squares fitting program is supplied by the manufacturer for single or multiexponential decay analysis, and gives output as a time-resolved fluorescence decay curve plot along with the decay parameters. The program involves deconvolution of the data by iterative reconvolution (O'Connor & Phillips, 1984) to obtain the best fit for the decay curve in the calculations used in statistical and plotting subroutine packages. A check for the best fit is based upon the test values for chi-square, Durbin-Watson parameter, covariance matrix, autocorrelation function, and a runs test Z for the residuals.

The instrument could also be used to obtain the reconstructed time-resolved emission spectra for the decay data but not for the steady-state or fluorescence anisotropy measurements. The TRES was obtained by accumulating data for decay over a range of individual emission wavelengths and reconstructing the emission spectra from the deconvolved decay curves at each time point, obtained as described above, for the complete decay period. A three-dimensional TRES plot was thus obtained that gave the change in fluorescence intensity with time over a range of wavelengths, following an excitation pulse at the nanosecond level. With such a plot, the prevalence of excited state interactions of a fluorophore can be envisaged. Rates of these processes on a nanosecond time scale describe the solvent or environmental effects on the fluorescence of the probe under investigation. When solvent effects are observed in TRES analysis, they indicate the accessibility of the probe molecule to the bulk solvent (Lakowicz, 1983).

Measuring polarity of the probe environment in fluorescent mononucleosomes

Fluorescence properties of the probe used in this study, 1,5-IAEDANS, are sensitive to the medium polarity in

which it is kept (Hudson & Weber, 1973). When a probe molecule is attached to a macromolecule like a protein, its immediate microenvironment is confined to a pocket generated by the folding of the macromolecule in which the probe may sit. Unfolding of the macromolecular structure may bring about a change in the size of this pocket or in the polarity of the microenvironment of the probe by exposing it more to the bulk solvent. Under conditions where specific solvent effects are saturable, the general solvent effects on steady-state fluorescence of a dye or probe molecule are described by the Lippert equation (Lippert, 1957; Lakowicz, 1983):

$$\bar{\nu}_a - \bar{\nu}_f = \frac{2(\mu^* - \mu)^2}{h \cdot c \cdot a^3} \left(\frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \right) + \text{constant},$$

where $\bar{\nu}_a$ and $\bar{\nu}_f$ are wave numbers in cm^{-1} of absorption and emission maxima, respectively; n is refractive index of the solvent; ϵ is the static dielectric constant of the solvent; h is Planck's constant; c is the speed of light; μ^* and μ the dipole moments of the fluorescence probe in excited and ground states, respectively; and a describes the radius of the Onsager cavity in which the probe may reside. For a given probe, a and $(\mu^* - \mu)$ are constant values. The term "constant" in the Lippert equation takes care of the vibrational energy loss. The terms inside the parentheses comprising the solvent properties contribute together to the "orientation polarizability," Δf , of the solvent molecule and thus give a measure of the probe environment. By keeping the probe in solvents of known Δf values and measuring its fluorescence in these solvents, a calibration curve can be prepared by plotting Δf versus $(\bar{\nu}_a - \bar{\nu}_f)$, which gives a straight line if specific solvent effects are not there and solvent relaxation is complete before emission. Mixtures of ethanol and water (or a buffer) in different ratios have different general properties, but they may have the same specific solvent effects due to the same type of hydrogen bonding present in all such mixtures. Fluorescence properties of a probe kept in a series of such mixtures are used to generate a Lippert plot to be used as a calibration curve for measuring properties of an unknown environment in which the probe's fluorescence is measured. The validity of the Lippert equation, mentioned above, is based on certain assumptions, which include a solvent continuum around a free probe molecule and completion of solvent relaxation prior to emission (Lakowicz, 1983). In the case of a probe bound to a macromolecule, emission is largely from a not-fully-relaxed state, and the dye may undergo specific interactions with certain amino acid residues at its binding site (Gafni et al., 1977). Structural studies of the histone core (Burlingame et al., 1985; Arents et al., 1991) have revealed the presence of two large solvent channels approaching the dyad axis of the nucleosome. Thus, the probe molecules attached to the cysteines sitting at this position may become freely accessible to the bulk solvent.

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