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Modulation of the Sensitivity of Chromatin to Exogenous Nucleases: Implications for the Apparent Increased Sensitivity of Transcriptionally Active Genes†

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ABSTRACT: We have examined the effects of changing the ionic composition of the buffers in which nuclei are isolated on the sensitivity of chromatin to micrococcal nuclease and deoxyribonuclease I. Unless nuclei are isolated in buffers containing physiological levels of monovalent (150 mM KCl) and divalent (2–5 mM $MgCl_2$) cations, there is a substantial loss of higher order structure. The ionic composition of the buffer in which the digestion is carried out also affects the amount of material digested both by modulating higher order structure and by determining the solubility of the released material. Magnesium ion concentrations greater than 2 mM and calcium ions at virtually any concentration precipitate substantial amounts of the released chromatin fragments. These observations can be interpreted in light of the known effects of the ions on 10- and 30-nm fiber structure and used as a basis for improvements in techniques for isolating chromatin and for studying its structure and function using exogenous nuclease probes. The apparent nuclease sensitivity of transcriptionally active chromatin was reexamined and shown to be more likely a reflection of differential solubility rather than an overall increase in nuclease sensitivity.

Chromatin is packaged in the nucleus of mammalian cells in a complex series of higher order forms that appear to relate to the functional state of each particular type of cell [for reviews, see Weisbrod (1982), Walker (1983), Butler (1983), and Tsanev (1983)]. Changes in gene expression during adaptation, proliferative activation, and differentiation are generally preceded by alterations in chromatin structure as genes are mobilized from a transcriptionally inactive state to a form that can be transcribed. Consequently, there is considerable interest in the regulatory mechanisms that govern these transitions. Furthermore, when the genetic material is replicated, there are also pronounced structural changes with chromatin becoming decondensed during the S phase of the cell cycle and subsequently becoming highly condensed during late G2 and mitosis.

Exogenous nucleases such as micrococcal nuclease (MNase) and deoxyribonuclease I (DNase I) have been widely used to probe the structure of chromatin in isolated nuclei. For example, they have played a central role in elucidating the structural organization of the 10-nm fiber and are currently being used to study the higher order 30-nm fiber [reviewed by Butler (1983)]. Furthermore, they have been used to detect and illustrate structural differences between transcriptionally inactive and transcriptionally active domains of chromatin (Weintraub & Groudine, 1976; Bloom & Anderson, 1978, 1979; Weisbrod, 1982). In addition, the "digestibility" of chromatin at various stages of the cell cycle has been examined (Prentice et al., 1985). However, in the latter study, reproducible results could only be obtained when the ionic composition of the isolation buffer was carefully controlled (Prentice & Gurley, 1983). Quite clearly, a prerequisite for all such studies is the development of techniques for isolating nuclei in which the native structure of chromatin is maintained.

Studies on the folding of the 10-nm polynucleosome chain into the 30-nm condensed fiber, and even higher order forms, have shown that monovalent and more particularly divalent cations have critical effects on fiber structure in vitro [see review by Butler (1983)]. With this information in mind, we have examined the effects of salt and divalent cations on chromatin integrity as measured by its sensitivity to exogenous nucleases. The data show that marked changes in chromatin structure can occur both during the isolation of nuclei and

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during the digestion reaction and these changes are directly related to the ionic composition of the isolation and incubation media. In general, the changes induced by these ions can be interpreted in relation to their above-mentioned effects on chromatin fiber structure, and this provides the basis for a rational approach to improvements in nuclear isolation technology. Furthermore, since the digestibility of chromatin by exogenous nucleases is shown to be so easily manipulated in vitro, the perceived enhanced sensitivity of transcriptionally active chromatin to exogenous nucleases is critically reexamined and shown to more likely reflect enhanced solubility of the fragments derived from active chromatin, rather than an intrinsic increased nuclease sensitivity.

Materials and Methods

Isolation and Digestion of Nuclei. Nuclei were isolated from livers of male (190–210 g) specific-pathogen-free Sprague-Dawley rats bred in this laboratory as described previously (Sikorska et al., 1980) following homogenization of the liver in 6 volumes of ice-cold buffer consisting of 0.25 M sucrose, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 150 mM KCl, and 5 mM $MgCl_2$. This same buffer was used throughout the nuclear isolation procedure, and the concentrations of monovalent and divalent cations were varied in some experiments as indicated in the figure legends.

Micrococcal nuclease (Sigma Chemical Co., St. Louis, MO) at a concentration of 50 units/mL (1 unit corresponds to a change of optical density at 260 nm of 1.0 at 37 °C, pH 8.0) and deoxyribonuclease I (DNase I, Pharmacia P-L Biochemicals, Montreal, PQ) at a concentration of 50 μg/mL were used to digest liver nuclei which had been resuspended at a concentration of 0.8–1.0 mg of DNA/mL in 10 mM Tris-HCl, pH 8.5, containing the concentrations of monovalent and divalent cations indicated in the figure legends. Digestions were carried out at 30 °C for the times indicated. The reaction was terminated by adding 0.2 M ethylenediaminetetraacetic acid (EDTA) [or in some cases 0.2 M ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)] to a final concentration of 10 mM followed by rapid cooling in ice–water. The suspension was then centrifuged at 25000*g* for 15 min in the 50 Ti rotor of a Beckman L8-70 ultracentrifuge at 4 °C to produce a supernatant (S1) containing released, soluble chromatin and a pellet (P1) of unreleased or insoluble material.

The concentration of DNA in these fractions was measured by the diphenylamine reaction as described previously (Walker et al., 1977).

Solubility of Chromatin Fragments in Solutions of Mono- and Divalent Cations. In these experiments, nuclei were resuspended in 10 mM Tris-HCl, pH 8.5, containing 0.5 mM $MgCl_2$ and digested for 5 min at 30 °C with 50 μg/mL DNase I. The reaction was stopped by rapid cooling, without the addition of chelating agent and a supernatant containing released material generated as described above. Samples (1.0 mL) of the supernatant were adjusted to approximately 1.0 mM DNA phosphate and different amounts of KCl, $MgCl_2$, and $CaCl_2$ added. After 10 min on ice, the samples were centrifuged, and the percentage of A_{260} material remaining in the supernatant (S2) was determined.

Gradient Centrifugation. The size distribution of released chromatin fragments was determined by layering 0.5 mL of supernatant onto a 10–35% (w/w) sucrose gradient prepared in 50 mM Tris-HCl, pH 7.6, containing 150 mM KCl followed by centrifugation in an SW40 rotor at 40000 rpm for 3.25 h at 4 °C in a Beckman L8-70 ultracentrifuge. The gradients were fractionated by using an ISCO Model 185 gradient frationator connected to an ISCO UA-5 absorbance monitor.

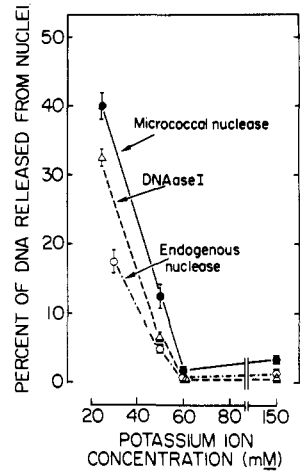

FIGURE 1: Effect of monovalent cation concentration in the isolation buffer on the sensitivity of chromatin to endogenous nuclease, DNase I, and micrococcal nuclease. Nuclei were isolated in buffer containing the indicated KCl concentration and then digested for 5 min in 10 mM Tris-HCl (+0.5 mM $MgCl_2$ in the case of DNase I) in the absence of added nuclease or in the presence of 10 units/mL MNase or 10 μg/mL DNase I. The data represent the mean ± SEM for four to six determinations.

Results

Effects of the Ionic Composition of the Isolation Buffer on Chromatin Digestibility. Nuclei isolated in the presence of different concentrations of monovalent cation (with the Mg^{2+} ion concentration being held constant at 5 mM) exhibited marked differences in digestibility (Figure 1). At low concentrations of K^+ in the isolation buffer, the nuclei that were produced were rapidly digested by both MNase and DNase I and even showed a considerable endogenous nuclease activity. In contrast, when the nuclei were prepared in the presence of 60 mM or greater K^+, there was little or no digestion by either endogenous nuclease or added DNase I or MNase at the enzyme concentrations used. The observation that nuclei prepared in buffers containing physiological levels of monovalent cation had little or no endogenous nuclease activity was particularly useful since it would permit studies using exogenous nucleases to be executed without interference.

A substantial increase in the nuclease:DNA ratio was required to digest chromatin in nuclei that were prepared in buffers containing high salt (Figure 2A). Thus, approximately 10 times more enzyme was required to release an amount of DNA similar to that released from nuclei prepared in 25 mM monovalent cation.

The concentration of divalent cation in the isolation buffer was also found to play an important role in determining the digestibility of chromatin (Figure 2B). Low concentrations of Mg^{2+} increased the sensitivity of the chromatin to MNase, but as the concentration of Mg^{2+} was increased from 0.5 to 2.0 mM, the chromatin became more resistant to nuclease attack. Since these effects of ions and the chromatin:enzyme ratio are markedly different from those observed by Prentice and Gurley (1983) in their work on CHO cells, it is clear that careful attention must be given to these experimental details.

Ionic Composition of the Digestion Buffer Also Affects Chromatin Digestibility. Since the ionic composition of the isolation buffer had such a marked effect on the sensitivity of chromatin to exogenous nucleases, we examined the effects of cations present during the digestion assay (Figures 3 and 4). In these experiments, all the nuclei were isolated in the

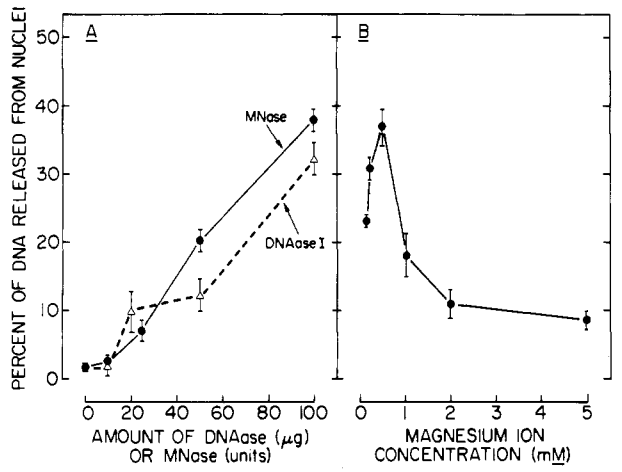

FIGURE 2: Effects of chromatin:enzyme ratio and divalent cation concentration of the isolation buffer on the digestion of chromatin. In (A), nuclei were isolated in the presence of 150 mM KCl + 5 mM $MgCl_2$ and digested for 5 min in 2 mL of 10 mM Tris-HCl (+0.5 mM $MgCl_2$ in the case of DNase I) in the presence of the indicated amounts of MNase or DNase I. In (B), nuclei were isolated in the presence of 150 mM KCl and the indicated concentrations of $MgCl_2$ and then digested for 5 min with 10 units/mL MNase in 10 mM Tris-HCl. In both experiments, the data represent the mean ± SEM of four to six determinations.

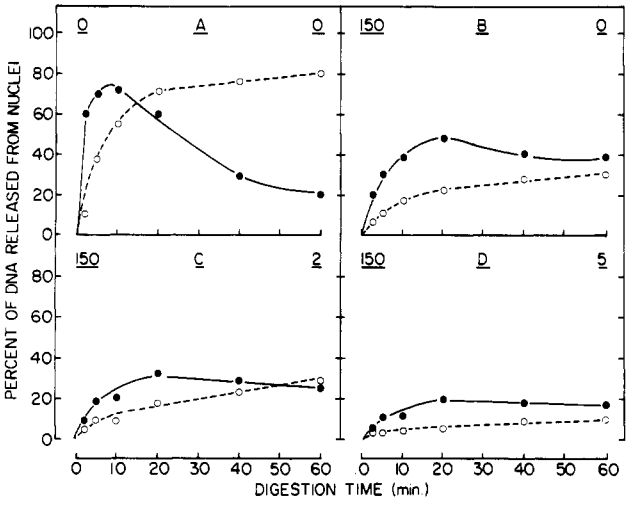

FIGURE 3: Effects of the concentration of mono- and divalent cations in the digestion buffer on the time course of digestion of chromatin by MNase. All the nuclei were isolated in the presence of 150 mM KCl and 5 mM $MgCl_2$ and digested with 50 units/mL MNase in 10 mM Tris-HCl containing the concentration (millimolar) of KCl indicated by the number in the top left-hand corner of each panel and the $MgCl_2$ concentration (millimolar) indicated in the top right-hand corner. Digestions were carried out in the absence (○) or presence (●) of 0.1 mM $CaCl_2$.

presence of 150 mM K^+ and 5 mM Mg^{2+} in order to maintain chromatin in a condensed state and to suppress endogenous nuclease activity.

Figure 3 shows the results of a series of time courses for the digestion of chromatin by MNase (50 units/mL) in the presence of various amounts of monovalent (K^+) and divalent cations (Mg^{2+}). All the digestions were carried out in the presence or absence of 0.1 mM Ca^{2+}. The nuclease rapidly digested chromatin when the nuclei were incubated in the absence of any added mono- or divalent cations with 70% of the nuclear DNA being released within 20 min (Figure 3A). The reaction reached a plateau shortly after this, however, and even after 60 min of digestion, 20% of the DNA remained in the nucleus. Micrococcal nuclease is a Ca^{2+}-requiring enzyme (Cuatrecasas et al., 1967), and its activity in the absence of

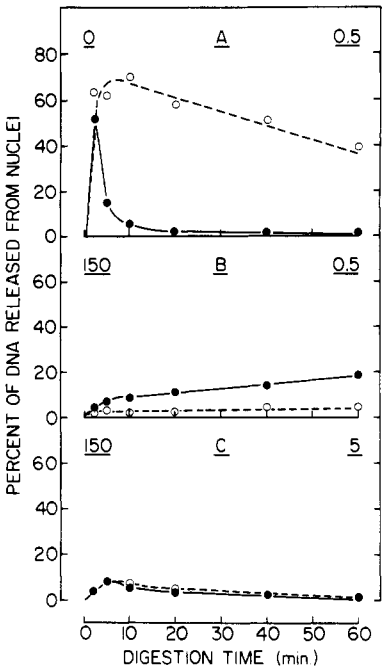

FIGURE 4: Effects of the concentration of mono- and divalent cations in the digestion buffer on the time course of digestion of chromatin by DNase I. Nuclei were isolated and digested with 50 μg/mL DNase I as described in the legend to Figure 3. (○) Digestions in the absence of $CaCl_2$; (●) digestions in the presence of 0.1 mM $CaCl_2$.

added Ca^{2+} ions observed above can be attributed to contamination of commercial preparations of the enzymes with low levels of Ca^{2+}. When 0.1 mM Ca^{2+} was added to the digestion mixture, there was a marked stimulation of the initial rate of digestion. However, during prolonged incubations, a substantial fraction of the released material was rendered insoluble, and by 60 min, only 20% of the total nuclear DNA remained in solution.

The inclusion of 150 mM K^+ in the digestion buffer substantially reduced the digestibility of chromatin (Figure 3B) with only 30% of the DNA being released in 60 min. Low concentrations of Ca^{2+} again stimulated the activity of the enzyme but did not restore the amount released to the level seen in the absence of cations. Most of the released material did, however, remain in solution. Magnesium ions at a concentration of 2 mM did not have any significant further effect on the amount of chromatin digested (Figure 3C), but at a concentration of 5 mM (Figure 3D), it rendered chromatin essentially resistant to nuclease attack in the absence of Ca^{2+}, and less than 20% of the DNA was released following 60 min of incubation in the presence of 0.1 mM Ca^{2+} (a Ca^{2+} concentration of 1.0 mM produced no further stimulation; data not shown).

Similar experiments were carried out with DNase I as the nuclease probe (Figure 4). Since DNase I has an absolute requirement for Mg^{2+} ions for enzymic activity, a minimum of 0.5 mM Mg^{2+} was present in all the incubation buffers. Furthermore, since calcium ions have been shown to activate DNase I in the presence of magnesium ions (Melgar & Goldthwait, 1968), digestions were also carried out in the presence and absence of 0.1 mM Ca^{2+} to further investigate the effects of this ion on the digestibility of chromatin and on the solubility of released material. Figure 4A shows that DNase I (50 μg/mL) also extensively digested chromatin in buffers of low ionic strength, with 70% of the nuclear DNA being released in 5–10 min, although some of this material appeared to precipitate at longer incubation times. The presence of 0.1 mM Ca^{2+} had a dramatic effect on this time

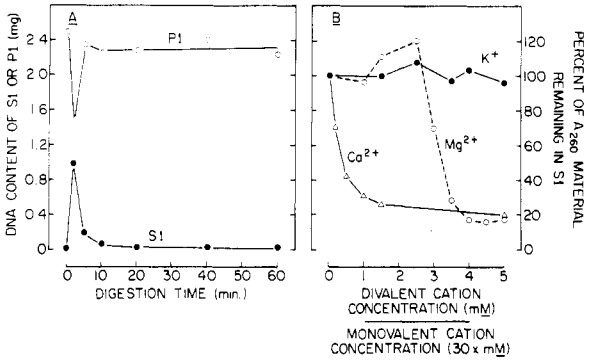

FIGURE 5: Solubility of chromatin fragments in buffers of various ionic composition. In (A), a time course of digestion was carried out on nuclei digested by 50 μg/mL DNase I in buffer containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The DNA contents of S1 and P1 were determined. In (B), nuclei were digested for 5 min by 50 μg/mL DNase I and an S1 generated without the addition of EDTA. Aliquots of this S1, at a concentration of 1 mM DNA phosphate, were then incubated on ice for 10 min in the presence of the indicated concentrations of KCl or $MgCl_2$ or $CaCl_2$, and the percentage of OD_{260} material remaining in solution was determined. In these experiments, all the nuclei were isolated in the presence of 150 mM KCl and 5 mM $MgCl_2$.

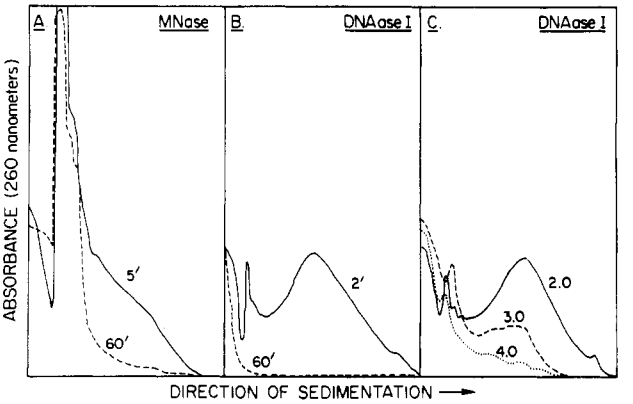

FIGURE 6: Density gradient sedimentation profiles of released chromatin fragments. Nuclei were isolated in the presence of 150 mM KCl + 5 mM $MgCl_2$ and then digested for the time indicated by (A) 50 units/mL MNase or (B and C) 50 μg/mL DNase I. In (A) and (B), 0.5-mL samples of S1 supernatants were loaded on gradients as described under Materials and Methods. In (C), a 2-min digestion was carried out and an S1 supernatant generated in the absence of EDTA. Aliquots of the supernatant were incubated for 10 min on ice in the presence of 2, 3, or 4 mM $MgCl_2$. 0.5 mL of S2 supernatant was loaded on the gradient.

course of digestion. For approximately 2 min, the digestion appeared normal with more than 50% of the nuclear DNA being released. However, all of this material apparently precipitated over the next 5–10 min, and there was no further release of material even when digestions were continued for 60 min.

When digestions were carried out in the presence of 150 mM K^+ (in addition to 0.5 mM Mg^{2+}, Figure 4B), there was essentially no release of DNA in the absence of Ca^{2+}. In the presence of 0.1 mM Ca^{2+}, 10–20% of the nuclear DNA was released. These effects may be due to a direct inhibition of DNase I by monovalent cations, an effect which can be partially overcome by Ca^{2+} ions (Melgar & Goldthwait, 1968). Moreover, when the Mg^{2+} concentration was further increased to 5 mM (Figure 4C), there was little or no digestion even in the presence of Ca^{2+} ions.

Effects of Cations on the Solubility of Released Material. The results presented in Figure 5A confirmed that in the presence of 0.1 mM Ca^{2+} the material that was initially released from nuclei by DNase I (cf. Figure 4A) was precipitated and reappeared in the pellet fraction within 2 min of its release (as opposed to being totally degraded to acid-soluble nucleotides). The nuclease was incapable of rendering this material soluble even after a further 50–60 min of incubation.

Since most nucleases require a divalent cation for optimal activity and this necessitates their inclusion in digestion buffers, we investigated the effects of these ions on the solubility of released material to assess what, if any, concentrations were permissible. Supernatants containing material released by DNase I were generated, and the effects of K^+, Mg^{2+}, and Ca^{2+} on the solubility of this material were tested directly (Figure 5B). Potassium ions up to 150 mM had no effect on the solubility of the chromatin fragments, indicating that digestions can be safely carried out in the presence of this ion. It is noteworthy that the solubility of chromatin fragments in salt may vary from tissue to tissue. Thus, Komaiko and Felsenfeld (1985) have shown that erythrocyte chromatin fragments are insoluble in 150 mM salt. This would be predicted from the "phase" map of Olins and Olins (1972) which shows a higher degree of compaction of erythrocyte chromatin by salt, presumably related to the presence of histone H5. Magnesium ions also had no effect on solubility below 2 mM, but concentrations greater than 2 mM caused the precipitation of substantial amounts of the material. In contrast, calcium ions were particularly effective precipitants, causing some material to be precipitated at the lowest concentration tested (0.1 mM) with virtually all of the material being removed from solution by 1.0 mM Ca^{2+}. The A_{260} material remaining in solution consists of nucleotides and short oligomers derived principally from RNA degradation. Direct measurements of DNA showed that no more than 2–3% of nuclear DNA remained in solution (cf. Figure 5A).

Characterization of the Chromatin Fragments Released by DNase I and MNase. The observation that there was a difference in the precipitability by Ca^{2+} of the material released by MNase and DNase I (cf. Figures 3A and 4A) suggested that there were differences in the nature of the material released by the two nucleases. To examine this in more detail, samples of S1 were analyzed on sucrose gradients (Figure 6). A 5-min digestion of chromatin by MNase, carried out in the presence of 0.1 mM Ca^{2+}, released approximately 80% of the chromatin from nuclei (cf. Figure 3A). This material sedimented in gradients with a profile corresponding to a large peak of 11S mononucleosomes together with short oligomers and a shoulder of larger fragments containing 10–20 nucleosomes per particle (Figure 6A). After 60 min of incubation, only 20% of this material remained in solution (cf. Figure 3A), and it sedimented almost entirely as a mixture of mononucleosomes and short oligomers.

The profile of material released by DNase I in the presence of 0.1 mM Ca^{2+} was substantially different from that of MNase (Figure 6B). The profile obtained following a 2-min digestion [the digestion was kept brief in order to minimize the amount precipitated (cf. Figure 5A)] showed that only a small proportion of mononucleosomes was released, whereas the bulk of the released material consisted of fragments containing 5–40 nucleosomes with a mean peak size of 10–12 nucleosomes. When the incubation time was extended, all of this material was precipitated (Figures 4A and 6B).

These results suggested that it was the higher molecular weight chromatin fragments that were insoluble in divalent cation containing solutions. This was examined by running gradient profiles on S2 samples derived from DNase I generated S1 supernatants that had been treated with Mg^{2+} di-

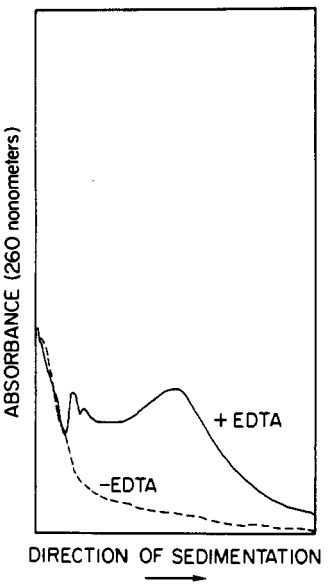

FIGURE 7: Effect of EDTA on the solubility of chromatin fragments. Nuclei were isolated in the presence of 150 mM KCl and 5 mM $MgCl_2$ and digested for 5 min with 50 μg/mL DNase I in the presence of 5 mM $MgCl_2$. The digestion was terminated in either the absence (-EDTA) or the presence (+EDTA) of 10 mM EDTA and 0.5 mL of S1 supernatant loaded on the gradient.

rectly (cf. Figure 5B). These profiles showed (Figure 6C) that as the Mg^{2+} concentration was increased from 2 mM, the largest polynucleosome fragments were precipitated first, followed by progressively smaller fragments. Magnesium ion concentrations of 2 mM or less had no effect on the solubility of the released material (Figures 5B and 6C compared to Figure 6B).

Effects of Chelating Agents on the Solubility of Cation-Precipitated Chromatin Fragments. Chelating agents, particularly EDTA, have been used routinely to stop nuclease digestion reactions and solubilize the released material although little attention has been paid to their effectiveness with regard to the ions present in the digestion buffer. Figure 7 illustrates that when a DNase I digestion is carried out in the presence of 5 mM $MgCl_2$ there is no release of material from the nuclei unless EDTA (10 mM) is added at the end of the digestion and prior to centrifugation. Therefore, this chelating agent must be used for all Mg^{2+} concentrations greater than 2 mM. When a similar experiment was carried out in the presence of 0.1 mM Ca^{2+} (cf. Figure 4A) and EGTA (or EDTA) at a final concentration of 10 mM was added to stop the reaction, there was no release of material (data not shown). It is clear, therefore, that when calcium ions are used, careful attention must be paid to the problems that may be encountered with the solubility of the products of digestion.

Discussion

These results demonstrate that, in isolated nuclei, the sensitivity of chromatin to exogenous nucleases (i.e., its digestibility) is affected by at least four factors. They are (i) the physiological state of chromatin inside the cell prior to isolation, (ii) the ionic environment to which the nuclei are exposed during isolation, (iii) the ionic environment in the digestibility assay, and (iv) the solubility of the released material. For an in vitro assay, which measures chromatin sensitivity to exogenous nucleases, to have any relevance to the physiological state of chromatin in vivo, then factors ii–iv must be carefully controlled, and this, in turn, requires an understanding of the effects of mono- and divalent cations on chromatin structure.

Numerous studies have shown that monovalent (K^+ and Na^+) and divalent cations (Mg^{2+} and Ca^{2+}) affect the condensation of chromatin. For example, Olins and Olins (1972) have produced phase maps for the effects of K^+ and Mg^{2+} (and also pH) on the degree of chromatin condensation in nuclei isolated from rat liver and chicken erythrocytes as measured by the degree of granularity of the nucleoplasm. For liver nuclei, chromatin is maximally condensed at concentrations of Mg^{2+} greater than 2 mM and of K^+ greater than 100 mM. Subsequent studies on isolated chromatin fibers have shown that monovalent cations in the 60–100 mM range and magnesium ions in the 0.5–1.0 mM range cause the 10-nm polynucleosome chain to condense to the 30-nm chromatin fiber [see Butler (1983) and Thomas (1984)]. Furthermore, Dixon and Burkholder (1985) have provided evidence that heterochromatin formation has an absolute requirement for Mg^{2+}. Heterochromatin appears to be generated by a higher order compaction of the 30-nm fiber which cannot be generated by monovalent cations. It is essential, therefore, to isolate nuclei in the presence of physiological concentrations of both monovalent and divalent cations (150 mM K^+ and 5 mM Mg^{2+}, respectively) in order to maintain chromatin in its native state. These conditions have the added advantage of suppressing endogenous nuclease activity in cells such as liver that have high levels of these enzymes. Isolation media which contain low levels of monovalent cations (10 or 25 mM are the concentrations used in virtually all conventional nuclear isolation buffers) and/or less than 2 mM Mg^{2+} will not maintain chromatin in a native state.

Chromatin that is maintained in a condensed state during the isolation is relatively resistant to exogenous nucleases when the digestion is also carried out at high ionic strength in the presence of Mg^{2+} ions (Figures 3C, 3D, and 4C). However, it rapidly disperses in buffers of low ionic strength or low divalent cation content and is then rapidly digested by both MNase and DNase I (Figures 3A and 4A). There is little doubt, therefore, that exogenous nucleases are sensitive indicators of the structural organization of chromatin inside the nucleus, but if the ionic environment has altered the native structure, then the results of digestibility assays become difficult to interpret.

The interpretation of experiments designed to measure the digestibility, or nuclease sensitivity, of chromatin is further complicated by the demonstration (Figure 7) that the fragments of chromatin released by exogenous nucleases are not soluble at Mg^{2+} concentrations greater than 2 mM or Ca^{2+} ions at virtually any concentration. Furthermore, solubility may be altered by the subsequent processing of released fragments during prolonged digestions (Figure 3A). Results based on the production of acid-soluble material by exogenous nucleases (Prentice & Gurley, 1983; Prentice et al., 1985) are also particularly difficult to interpret since the kinetics of the reaction reflect not only the action of the nuclease on the chromatin fiber but also the extensive processing of the released fragments that must occur to generate the small subnucleosomal species that are soluble in acid.

The observation (Figures 3A and 4A) that, under appropriate conditions, as much as 80% of the total nuclear DNA can be released in 2–5 min by either MNase or DNase I indicates that these nucleases are not just attacking a small fraction of "nuclease-sensitive or hypersensitive" transcriptionally active chromatin. Bulk, presumably transcriptionally inactive, chromatin also becomes nuclease sensitive under these conditions. It is clear, therefore, that there are two simultaneous events occurring during nuclease digestions of intact

nuclei. The nucleases (particularly DNase I) attack transcriptionally active (or potentially active) chromatin since genes in this configuration have a number of sites of enhanced sensitivity and a fraction of this active sequence material is liberated as monomers and short oligomers. Simultaneously, the nucleases attack bulk chromatin with the extent of digestion being dependent upon its degree of condensation, and this, in turn, is determined by the ionic environment in which the nuclei are digested (and what they were exposed to during isolation). If a high ionic strength and greater than 2 mM Mg^{2+} concentration is maintained throughout, then little or no bulk, inactive material is digested, whereas when low ionic strength conditions are used substantial amounts of this material are released. Finally, what is actually observed experimentally is determined by the relative solubilities of the released material. In general, the small amount of material released from active chromatin is highly soluble, even in the presence of divalent cation (Bloom & Anderson, 1979; Nicolas et al., 1983), whereas the fragments released from bulk, inactive material are relatively insoluble in buffers containing more than 2 mM Mg^{2+} or 0.05–0.1 mM Ca^{2+}. Chelating agents may, or may not, solubilize this material (Figure 7) which probably accounts for much of the variability that has been seen in these kinds of study.

We feel that it is mandatory to isolate nuclei using conditions which maintain chromatin in its native state and then choose a digestion strategy that will isolate and solubilize the desired fraction. For example, large amounts of bulk, predominantly transcriptionally inactive material can be conveniently prepared by brief digestions at low ionic strength (or at intermediate ionic strengths to generate material that is at definable levels of condensation/decondensation; P. R. Walker and M. Sikorska, unpublished observations). Micrococcal nuclease is the enzyme of choice for such studies since it cleaves almost exclusively at the linker region between nucleosomes, under these conditions, whereas DNase I introduces many nicks into the isolated fragments (P. R. Walker and M. Sikorska, unpublished observations). Micrococcal nuclease generated fragments have a full complement of core histones and are enriched in histone H1, making them suitable for studies on 30-nm fiber structure. This material can be prepared quickly without the need for lengthy dialysis steps if the concentration of divalent cations is kept to a minimum. The observation that MNase is active in the absence of added Ca^{2+} is particularly useful in this regard.

It is now apparent that techniques for the preparation of transcriptionally active chromatin must be reevaluated. Early work tried to exploit the apparent increased sensitivity of active chromatin to DNase II (Gottesfeld et al., 1974), MNase (Bloom & Anderson, 1979), and DNase I (Weintraub & Groudine, 1976) to release the active material. However, although enrichments of active fragments could be obtained, the fractionation was never complete. In retrospect, since both active chromatin and bulk chromatin were probably being digested in these studies, the enrichment obtained was more likely a reflection of the selective solubility of the fragments derived from transcriptionally active material in the presence of divalent cations. This approach has been further called into question by the results of Smith et al. (1984), who have shown that under low ionic strength conditions the transcriptionally inactive embryonic β-globin gene becomes just as sensitive as the expressed adult gene in murine erythroleukemia cells.

In addition, it has now been shown that transcriptionally active material is associated with the nuclear matrix (Abulafia et al., 1984; Barbashov et al., 1984; Hentzen et al., 1984) and that much of it remains in the pellet fraction, discarded by many workers, following nuclease digestions (Rose & Garrard, 1984; Cohen & Sheffery, 1985). Indeed, some transcriptionally active material may actually be protected by its association with the matrix and have a decreased sensitivity to MNase (Davis et al., 1983). We are currently examining which ionic environment is optimum for the isolation of this fraction of chromatin.

Finally, these observations, which show that the nuclease sensitivity of chromatin is essentially a function of ionic environment, do not necessarily preclude the existence in vivo of the "hypersensitive" sites that are usually, but not always, located immediately upstream from transcriptionally active (or potentially active) genes. The exquisite sensitivity of this small number of sites indicates that the structure of the chromatin fiber has been radically altered at strategic locations as a prerequisite for transcription (Weintraub, 1985). However, it is not clear whether the small amount of transcriptionally active chromatin that is released in digests of intact nuclei is derived solely from nuclease attack at these sites. Indeed, since Cohen and Sheffery (1985) showed that a probe to a 380 base pair sequence that is located entirely within the coding sequence of the globin gene hybridized to fragments released into S1, it appears that other sites are also involved in determining the release of material from transcriptionally active genes.

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Registry No. K, 7440-09-7; Mg, 7439-95-4; Ca, 7440-70-2; DNase I, 9003-98-9; micrococcal nuclease, 9013-53-0; nuclease, 9026-81-7.

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Dissociation of the Lactose Repressor–Operator DNA Complex: Effects of Size and Sequence Context of Operator-Containing DNA†

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ABSTRACT: The dissociation kinetics for repressor–^{32}P-labeled operator DNA have been examined by adding unlabeled operator DNA to trap released repressor or by adding a small volume of concentrated salt solution to shift the K_d of repressor–operator interaction. The dissociation rate constant for pLA 322-8, an operator-containing derivative of pBR 322, was 2.4×10^{-3} s^{-1} in 0.15 M KCl. The dissociation rate constant at 0.15 M KCl for both λp*lac* and pIQ, each of which contain two pseudooperator sequences, was $\sim 6 \times 10^{-4}$ s^{-1}. Elimination of flanking nonspecific DNA sequences by use of a 40 base pair operator-containing DNA fragment yielded a dissociation rate constant of 9.3×10^{-3} s^{-1}. The size and salt dependences of the rate constants suggest that dissociation occurs as a multistep process. The data for all the DNAs examined are consistent with a sliding mechanism of facilitated diffusion to/from the operator site. The ability to form a ternary complex of two operators per repressor, determined by stoichiometry measurements, and the diminished dissociation rates in the presence of intramolecular nonspecific and pseudooperator DNA sites suggest the formation of an intramolecular ternary complex. The salt dependence of the dissociation rate constant for pLA 322-8 at high salt concentrations converges with that for a 40 base pair operator. The similarity in dissociation rate constants for pLA 322-8 and a 40 base pair operator fragment under these conditions indicates a common dissociation mechanism from a primary operator site on the repressor.

Transcriptional control of protein synthesis, in which a group of functionally related structural genes is coordinately regulated, was first proposed by Jacob and Monod (1961). The lactose repressor serves as a negative control unit for the *lac* enzymes by binding to the lactose operator site of the *Escherichia coli* genome and thereby physically blocking RNA polymerase transcription of the lactose metabolizing enzymes (Miller & Reznikoff, 1980). In the presence of inducer sugars, the repressor undergoes a conformational change, which results in decreased affinity for operator DNA. The excess of nonspecific DNA binding sites in the genome can then compete effectively with the operator for repressor binding, and transcription of the *lac* enzymes can be initiated.

The specific, tight binding of the lactose repressor to the operator DNA was originally characterized by using the nitrocellulose filter binding technique (Riggs et al., 1970a,b). Studies of the repressor–operator DNA complex as a function of monovalent salt concentration have indicated participation of 6–7 ionic interactions, whereas ~11 ionic interactions appear to be involved in the repressor–nonoperator DNA complex (deHaseth et al., 1977; Record et al., 1977; Revzin & von Hippel, 1977; Barkley et al., 1981; Winter & von Hippel, 1981). Studies on the salt dependence of the *lac* repressor–operator DNA complex dissociation have been reviewed by Lohman (1985). In general, the binding of proteins to DNA varies with the type of monovalent or divalent salt; the salt dependence is specific for the particular counterion on the basis of the valence and relative affinity of the cation for the anionic DNA. In the absence of divalent cations, plots of log k_d vs. log [M$^+$] for the repressor–operator DNA complex exhibit curvature thought to reflect multiple dissociation steps or an alteration in the rate-limiting step (Barkley, 1981; Winter et al., 1981; Lohman, 1985). In addition, a decreased repressor affinity for operator observed with DNA fragments less than 170 base pairs (bp)[1] suggests "long-range" effects for operator binding (Winter & von Hippel, 1981; O'Gorman et al., 1980a). The stoichiometry of the repressor–operator DNA interaction has been investigated by using the nitrocellulose filter assay (O'Gorman et al., 1980a), gel filtration, and circular dichroism

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[1] Abbreviations: bp, base pair; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; ONPF, *o*-nitrophenyl β-D-fucoside; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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