Cations and the accessibility of chromatin to nucleases

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ABSTRACT

When rat liver nuclei prepared with polyamines as stabilising cations are digested with DNAase II, release of both inactive chromatin and Mg-soluble, active chromatin is greatly reduced, in comparison to digestion of liver nuclei prepared with Mg^{2+} as stabilising cation. Chromatin release from polyamine stabilised nuclei is also inhibited relative to Mg-stabilised nuclei following digestion with micrococcal nuclease under two very different cation conditions. Nuclei prepared with polyamines and monovalent ions as stabilising cations exhibit properties intermediate between these two extremes with both nucleases. These effects are due to residual binding of polyamines to chromatin, which is thus maintained in a condensed state, inaccessible to nucleases. Since polyamine binding is not easily reversed, concentrations of polyamines and other cations must be rigidly controlled in experiments on chromatin structure if artefacts are to be avoided. The significance of these findings to the nature and properties of active chromatin within the intact nucleus is considered.

INTRODUCTION

The nucleosome model of chromatin structure is now well established (1). Digestion of nuclei or chromatin with nucleases has contributed essential evidence for this model. Micrococcal nuclease and the endogenous Ca-Mg activated endogenous endonuclease, found in many nuclei, initially cleave DNA in the linker region between nucleosomes to yield a characteristic 200 base pair repeating unit (1-3). Digestion of nuclei with DNAase II at low temperatures, in the absence of divalent cations, also yields a 200 base pair repeat, but digestion at 37°C, or in the presence of divalent cations yields a 100 base pair repeat due to intranucleosomal cleavage by the DNAase II (4-5). Following DNAase II digestion, in the absence of divalent cations some of the chromatin released remains soluble when divalent cations such as Mg^{2+} are added (6). This Mg^{2+} -soluble chromatin is reported to be enriched in actively transcribed DNA sequences (7,8).

Marshall & Burgoyne (9) have emphasised the importance of preventing

digestion of DNA by endogenous endonuclease during isolation of nuclei, and recommend the use of buffers containing EGTA[†] and EDTA[†], with the polyamines spermine and spermidine as stabilising cations. During the course of our experiments on DNAase II fractionation of chromatin, we have therefore examined the effects of preparing nuclei with different stabilising cations, including polyamines. Our results indicate that polyamines can markedly reduce the accessibility of chromatin to nucleases, when monovalent cations are absent, and throw some doubt on the origin of Mg²⁺-soluble "active" chromatin.

METHODS

Isolation of nuclei. Rat liver nuclei were prepared by one of three methods. In all methods tissue was homogenised in solution 1, containing 0.32 M sucrose, nuclei were pelleted by centrifugation for 1 hour at 70,000 x g in solution 2, containing 2.4 M sucrose, and finally washed by resuspension in solution 3, containing 0.25 M sucrose. 1 mM PMSF[†] was present during homogenisation. In the three different methods solutions 1, 2 and 3 contained the following in addition to the concentration of sucrose indicated.

"Magnesium nuclei": solutions 1, 2 and 3 all contained 1.5 mM MgCl₂

"Polyamine nuclei": solution 1 contained 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA pH 7.4, solution 2 contained 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM EDTA, 0.1 mM EGTA pH 7.4, and solution 3 contained 0.5 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA pH 7.4.

"Salt polyamine nuclei": as in the preparation of polyamine nuclei but with the following additions to all solutions: 60 mM KCl, 15 mM NaCl, 15 mM β -mercaptoethanol, 15 mM Tris-HCl pH 7.4. This is essentially the method of Hewish and Burgoyne (3).

Hen erythrocyte nuclei were prepared by a modification of the procedure of Harlow and Wells (10) which involved osmotic lysis of cells, washing crude nuclei in 0.5% Triton X-100, homogenisation in a Virtis high speed homogeniser (10) and centrifugation through 2.25 M and 2.35 M sucrose solutions. Cation conditions were maintained throughout as in the preparation of magnesium nuclei or polyamine nuclei from rat liver.

Digestion of nuclei with DNAase II. Nuclei were pelleted from their appropriate washing solution 3 and lysed by resuspension in 0.2 mM EDTA pH 8 at a concentration of 0.5-2.0 mg DNA per ml. Sodium acetate was added to

a final concentration of 25 mM (pH 6.6) and the lysed nuclei incubated at 24°C with 100–200 units/ml DNAase II (E.C. 3.1.4.6. – enzyme from Worthington and Sigma had similar levels of ribonuclease and protease activity, and gave identical results). The reaction was terminated by adjusting the pH to 7.5 with 0.1 M Tris, and chromatin separated into a first supernatant and a nuclear residue (P1) by centrifugation (at 3,000 g for 15 min). The supernatant was further fractionated by addition of MgCl_2 to 3 mM, and after 15 min incubation at 4°C, centrifuging (3,000 g for 15 min) to yield a pellet of Mg^{2+} -insoluble chromatin (P2) and a Mg^{2+} -soluble supernatant fraction (S2) (6). Identical results were obtained when fractions were separated by centrifugation at 20,000 g for 20 min. Aliquots of fractions P1, P2 and S2 were taken, after resuspension in 0.2 mM EDTA pH 8 and sonication if necessary, for estimation of DNA content by absorption at 260 nm. An aliquot of S2 was also adjusted to 0.5 M perchloric acid, 1 M NaCl and centrifuged (3000 g for 15 min) for estimation of acid soluble nucleotides – a factor of 1.68 was used to correct for hyperchromicity (11). In some experiments fractions were analysed directly for DNA and RNA by the method of Munro & Fleck (12).

Digestion of nuclei with micrococcal nuclease. Nuclei were pelleted from their washing solution and resuspended at 3–5 mg DNA per ml in 10 mM TES^+ pH 7.4 (low cation conditions) or 0.34 M sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM β -mercaptoethanol, 15 mM Tris-HCl pH 7.4 (high cation conditions). CaCl_2 was added to 0.025 mM (low cation conditions) or 1 mM (high cation conditions) followed by 120 units/ml micrococcal nuclease (E.C. 3.1.4.7, Worthington) and the mixture incubated at 37°C. The reaction was terminated by addition of EDTA to a final concentration of 4 mM, and the mixture centrifuged (2,500 g for 10 min) to give a first supernatant and nuclear pellet. The nuclei were lysed by resuspension in 0.2 mM EDTA pH 8, and centrifuged again (2,500 g for 2 min) to produce a second supernatant and nuclear residue. The DNA contents of the two supernatants and of the nuclear residue were estimated by absorption at 260 nm.

Gel electrophoresis. DNA was purified from samples by adjusting to 1% SDS, 10 mM EDTA, 0.1 M Tris-HCl, pH 8.0, extracting twice with phenol, and precipitating the DNA with ethanol. DNA was analysed by electrophoresis on 3.5% polyacrylamide gels in 90 mM Tris-borate, 2.5 mM EDTA pH 8.3, using bromophenol blue and xylene cyanol FF as approximate molecular weight markers under the conditions described by Maniatis *et al.* (13). DNA from

undigested nuclei was analysed by electrophoresis on 1% agarose in Tris-borate, EDTA, using EndoR Eco RI digested λ DNA (Miles) for molecular weight calibration (14). Gels were stained with ethidium bromide and scanned for fluorescence with a Quick Scan R and D densitometer (Helena Laboratories).

Proteins were analysed after concentration of samples when necessary (15), by electrophoresis on SDS polyacrylamide slab gels according to Laemmli (16) with the following modifications: the separating gel contained 15% acrylamide and 0.3% bisacrylamide and the concentration of Tris was 0.75 M, the stacking gel contained 5% acrylamide and 0.13% bisacrylamide. Gels were stained in 0.1% Coomassie Blue, 50% methanol, 7% acetic acid, destained in 50% methanol, 7% acetic acid, and scanned with the Quick Scan R and D densitometer.

RESULTS

When rat liver nuclei, prepared with magnesium as stabilising cation (magnesium nuclei) are digested with DNAase II in 0.2 mM EDTA, 25 mM sodium acetate, pH 6.6, more than 70% of the chromatin DNA is released, with up to 56% in Mg^{2+} -insoluble form (fraction P2) and up to 15% in a Mg^{2+} -soluble fraction (S2) (Table 1). This fraction includes both acid-soluble material and the Mg^{2+} -soluble chromatin reported to be enriched in actively-transcribed DNA sequences (6-8). We obtained variable yields of fractions S2 and P2 when these fractions were separated by centrifugation in 2 mM $MgCl_2$ (6). In our hands, 3 mM $MgCl_2$ was required for maximal precipitation of Mg^{2+} -insoluble chromatin, with no further precipitation in 10 mM $MgCl_2$. We have therefore routinely used 3 mM $MgCl_2$ in our experiments.

When nuclei prepared in buffers containing spermine and spermidine (polyamine nuclei), or in buffers containing spermine, spermidine, NaCl, and KCl (salt polyamine nuclei) were digested with DNAase II and fractionated in a similar way to magnesium nuclei, striking differences were observed (Table 1). With polyamine nuclei, release of Mg^{2+} -soluble chromatin (S2) is reduced by 50%, compared to magnesium nuclei, and virtually no Mg^{2+} -insoluble chromatin (P2) is released. With salt polyamine nuclei, release of Mg^{2+} -soluble chromatin (S2) is similar to that with magnesium nuclei, while release of Mg^{2+} -insoluble chromatin (P2), although very variable, is intermediate between that with magnesium nuclei and polyamine nuclei. Similar results were obtained when nuclei were digested in the presence of 1 mM PMSF. Although under the conditions of DNAase II digestion used here,

Table 1. DNAase II fractionation of chromatin
Comparison of different nuclear preparations. S2, Mg²⁺-soluble fraction. P2, Mg²⁺-insoluble fraction

Nuclear preparation	Time of incubation (min)	% of input DNA (A ₂₆₀)		
		S2	P2	Acid soluble nucleotides
<u>Rat liver</u>				
Magnesium nuclei	2	7.5	27.0	2.3
" "	5	9.0	35.5	3.1
" "	10	11.2	45.7	3.6
" "	20	11.0	55.0	4.0
" "	30	15.1	56.0	4.7
<u>Polyamine nuclei</u>				
" "	10	7.1	1.2	2.1
" "	30	7.3	1.4	1.6
<u>Salt polyamine nuclei</u>				
" "	10	14.7	8.9	5.9
" "	30	14.7	19.9	6.5
<u>Hen erythrocyte</u>				
Magnesium nuclei	30	1.7	46.7	0.7
Polyamine nuclei	30	1.8	0	2.2

polyamines can condense free DNA, they have little effect on the rate of digestion of free DNA by DNAase II (data not shown). It is unlikely, therefore, that the differences observed between magnesium, polyamine, and salt polyamine nuclei can be explained by a direct effect of residual amounts of polyamines on DNAase II itself.

Since the buffers used to prepare polyamine and salt polyamine nuclei are designed to inhibit endogenous Ca-Mg activated endonuclease action during nuclear isolation (9), the larger release of Mg²⁺-insoluble chromatin from magnesium nuclei might be due to endonucleolytic cleavage of DNA during preparation of these nuclei, but not of polyamine or salt polyamine nuclei. Hen erythrocyte nuclei are reported to lack the endogenous endonuclease (17). Erythrocyte nuclei prepared under the conditions for magnesium nuclei or polyamine nuclei released little Mg²⁺-soluble chromatin, following DNAase II digestion, consistent with their transcriptional inactivity (Table 1). However, release of Mg²⁺-insoluble chromatin (P2) was again abolished in

polyamine nuclei compared to magnesium nuclei, indicating that the observed differences in release of this form of chromatin are independent of endogenous endonuclease activity.

These conclusions were confirmed by experiments in which rat liver nuclei were exposed to various cation solutions in an attempt to reverse the pattern of chromatin release following DNAase II digestion (Table 2). When magnesium nuclei are washed with polyamine solution 3 before DNAase II digestion, release of Mg^{2+} -soluble chromatin (S2) is reduced, and release of Mg^{2+} -insoluble chromatin (P2) almost completely abolished. A similar effect was observed when magnesium nuclei were washed with salt polyamine solution 3, or if residual Mg^{2+} ions were not removed from magnesium nuclei, by chelation with EDTA, before digestion. These results imply that binding of small amounts of cations (Mg^{2+} , Na^+ , K^+ or polyamines) to chromatin is sufficient to prevent release of Mg^{2+} -insoluble chromatin, and to reduce release of Mg^{2+} -soluble chromatin from nuclei, irrespective of nuclear isolation conditions.

However, when polyamine nuclei, salt polyamine nuclei, or magnesium

Table 2. The effect of washing nuclei with different cation solutions before digestion with DNAase II
Rat liver nuclei (1 mg DNA) were washed by resuspension and centrifugation in the solutions indicated, before resuspension in 0.2 mM EDTA, 25 mM sodium acetate, pH 6.6 and incubation with DNAase for 30 min (except magnesium nuclei (+ residual Mg^{2+}), which was resuspended directly in sodium acetate, pH 6.6, without EDTA). A3, solution 3 for preparation of magnesium nuclei. B3, solution 3 for preparation of polyamine nuclei. C3, solution 3 for preparation of salt polyamine nuclei. S2, Mg^{2+} -soluble fraction. P2, Mg^{2+} -insoluble fraction.

Nuclear preparation	Washing solution	% of input DNA (A_{260})		
		S2	P2	Acid soluble nucleotide
Magnesium nuclei	-	16.5	43.6	6.7
Magnesium nuclei (+ residual Mg^{2+})	-	4.6	3.4	1.9
Magnesium nuclei	3 ml B3	10.6	4.4	4.6
" "	3 ml C3	14.2	8.5	5.6
" "	3 ml B3 then 9 ml A3	11.4	2.1	4.9
Polyamine nuclei	-	9.2	0.8	-
" "	10 ml A3	8.8	0.6	-
Salt polyamine nuclei	-	18.9	0.7	-
" "	10 ml A3	12.5	1.0	-

nuclei which have been exposed to polyamine solution 3, are washed once or twice with an excess of magnesium solution 3 (0.25 M sucrose, 1.5 mM MgCl_2) prior to digestion, release of Mg^{2+} -insoluble chromatin is not increased (Table 2). Residual polyamines bound to chromatin are therefore not readily displaced by excess Mg^{2+} ions. Complete removal of polyamines bound to chromatin following preparation of polyamine and salt polyamine nuclei required exhaustive dialysis against 0.2 mM EDTA, 25 mM sodium acetate, pH 6.6. When this was performed (Table 3), subsequent digestion by DNAase II caused substantial release of Mg^{2+} -insoluble chromatin, and of Mg^{2+} -soluble chromatin, approaching the levels observed with magnesium nuclei.

Residual cation binding and consequent condensation of chromatin could result in either reduced accessibility to nucleases, or in reduced solubility of digested chromatin fragments, or both (18,19). To elucidate which of these factors is primarily responsible for the effects described, we analysed by gel electrophoresis the DNA fragment size in the various chromatin fractions following DNAase II digestion. DNA extracted from undigested nuclei was analysed on 1% agarose gels (data not shown). Up to 36% of the DNA from undigested magnesium nuclei was less than 10,000 base pairs in length, whereas all of the DNA from undigested polyamine and salt

Table 3. The effect of dialysing nuclei before digestion with DNAase II

Rat liver nuclei (1 mg DNA) were resuspended in 2 mls of 0.2 mM EDTA, 25 mM sodium acetate, pH 6.6, and dialysed twice against 500 mls of the same solution. The dialysed nuclear suspension was then split into two aliquots, and each digested with DNAase II for 30 min. The average of the results for the two aliquots is given. Undialysed nuclear suspensions were kept at 4°C for similar periods, before DNAase II digestion, for comparison. S2, Mg^{2+} -soluble fraction. P2, Mg^{2+} -insoluble fraction

Nuclear preparation	Dialysis	% of input DNA (A_{260})	
		S2	P2
Magnesium nuclei	-	15.8	38.3
" "	+	26.3	55.7
Polyamine nuclei	-	6.2	0.4
" "	+	13.8	27.7
Salt polyamine nuclei	-	12.5	0.9
" " "	+	23.8	48.5

polyamine nuclei was larger than 10,000 base pairs; this difference reflects the action of the endogenous endonuclease during isolation of magnesium nuclei, but not of polyamine or salt polyamine nuclei (9). DNA extracted from the different chromatin fractions following DNAase II digestion was analysed on 3.5% polyacrylamide gels (Fig. 1). If lack of release of Mg^{2+} -insoluble chromatin (P2) from polyamine and salt polyamine nuclei were due simply to reduced solubility of digested chromatin (in the presence of residual polyamines) one would expect the size distribution of DNA in the nuclear residue fraction (P1) from these nuclei to be similar to that of the DNA in Mg^{2+} -insoluble chromatin (P2) from magnesium nuclei. This is not the case; indeed DNA in P1 chromatin from polyamine and salt polyamine nuclei is much larger than most of the DNA in both P2 and P1 fractions from magnesium nuclei. (DNA from a P1 fraction from magnesium nuclei washed with polyamine solution 3, before DNAase II digestion, as in Table 2, was also larger than DNA from P2 and P1 fractions of unwashed magnesium nuclei; data not shown.) The small amounts of DNA recovered as Mg^{2+} -insoluble chromatin (P2) from polyamine and salt polyamine nuclei are again larger in size than much of the DNA in the nuclear residue (P1) from magnesium nuclei. Clearly lack of release of Mg^{2+} -insoluble chromatin from polyamine and salt polyamine nuclei is due to lack of digestion by nucleases, rather than simply to an effect on solubility of chromatin fragments.

The limited extent of digestion observed in Fig. 1 makes it impossible to decide whether chromatin condensed by polyamines is sensitive to intranucleosomal cleavage (generating a 100 b.p. repeat) as observed when Ca^{2+} or Na^{+} ions are bound to chromatin (4,5).

Is the reduced accessibility of chromatin with bound polyamines restricted to digestion by DNAase II or is it observed during digestion with other nucleases? We have also examined the digestion of magnesium, polyamine and salt polyamine nuclei from rat liver by micrococcal nuclease under two sets of conditions: (a) low cation conditions - 0.025 mM $CaCl_2$, 10 mM TES pH 7.4 - conditions insufficient, on their own, to cause chromatin condensation (19,20), and (b) high cation conditions - 1 mM $CaCl_2$, 0.34 M sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM β -mercaptoethanol, 15 mM Tris-HCl, pH 7.4 - conditions sufficient to cause chromatin condensation (19,20). Under low cation conditions (Fig. 2) micrococcal nuclease causes substantial release of chromatin into the first supernatant from magnesium nuclei, less from salt polyamine nuclei, and

Fig. 1. Size distribution of DNA fragments in chromatin fractions following DNAase II digestion. Magnesium, polyamine, or salt polyamine nuclei were prepared from rat liver, digested with DNAase II (120 units/mg DNA) for 30 min, and fractions isolated as described in Methods. Electrophoresis was on 3.5% acrylamide gels. AP1, AP2 and AS2, magnesium nuclei, BP1, BP2 and BS2, polyamine nuclei. CP1, CP2 and CS2, salt polyamine nuclei. AP1, BP1 and CP1, nuclear residue. AP2, BP2 and CP2, Mg^{2+} -insoluble fraction. AS2, BS2 and CS2, Mg^{2+} -soluble fraction. The migration of DNA from fraction BS2 was unaffected by prior treatment with RNAase.

much less again from polyamine nuclei. Following digestion under high cation conditions the majority of chromatin is released from magnesium nuclei into the second supernatant i.e. only after nuclear lysis, as reported by Noll *et al.* (21); release of chromatin into the second supernatant from salt polyamine nuclei is partly reduced in comparison, and release from polyamine nuclei virtually abolished. Therefore under two widely differing digestion conditions, polyamine nuclei appear much less susceptible than

Figure 2. Release of chromatin from different nuclear preparations during micrococcal nuclease digestion. Magnesium, polyamine, or salt polyamine nuclei from rat liver were digested with micrococcal nuclease (30 units/mg DNA) under low cation or high cation conditions. First supernatants (Δ) and second supernatants ($\circ$) were isolated and their DNA contents estimated. For further details, see Methods.

magnesium nuclei to digestion by micrococcal nuclease, as well as by DNAase II. This conclusion was confirmed when the size of DNA fragments in the total nuclear digests was analysed following incubation with micrococcal nuclease under high cation conditions (Fig. 3). After digestion with micrococcal nuclease (80 units/mg DNA) for 30 min, considerable amounts of DNA from magnesium and salt polyamine nuclei were reduced to sizes corresponding to nucleosome monomer and dimer, whereas only small amounts of DNA of this size were produced from polyamine nuclei. More prolonged

Fig. 3. Size distribution of DNA fragments following digestion of nuclei with micrococcal nuclease. Magnesium, polyamine, or salt polyamine nuclei from rat liver were digested with micrococcal nuclease (80 units/mg DNA) under high cation conditions for 30 min. DNA was extracted from total nuclear digests and analysed on 3.5% acrylamide gels. A, magnesium nuclei. B, polyamine nuclei, C, salt polyamine nuclei.

digestion of polyamine nuclei did produce significant amounts of mono-nucleosome and dinucleosome DNA fragments (data not shown). However, it is clear that the rate of digestion of DNA in polyamine nuclei is much slower than in magnesium or salt polyamine nuclei, again suggesting a reduced accessibility of chromatin to nuclease.

Does the binding of polyamines restrict the accessibility of all regions of chromatin in the nucleus to nuclease digestion? The results in Table 1 suggest that the release of "active" chromatin into the Mg^{2+} -soluble (S2) fraction during DNAase II digestion is only partially reduced from polyamine nuclei, but not significantly reduced from salt polyamine nuclei,

in comparison to magnesium nuclei. Since these results are expressed as % total A_{260} , and Gottesfeld and Butler (6) have reported that active chromatin (S2) is enriched in RNA, we have analysed directly the chromatin fractions obtained following DNAase II digestion of magnesium and polyamine nuclei, for both RNA and DNA. Table 4 shows that while the Mg^{2+} -soluble (S2) fraction from both magnesium and polyamine nuclei contains 30-35% of total nuclear RNA, the S2 fraction from polyamine nuclei contains 3 to 4 times less DNA (1.6%) than S2 from magnesium nuclei (5.6%). Therefore binding of polyamines (polyamine nuclei) in fact considerably reduces release of "active" Mg^{2+} -soluble chromatin DNA during DNAase II digestion as well as inactive Mg^{2+} -insoluble chromatin DNA (P2).

What then is the significance of the small amount of DNA (1.6%) that is released into the S2 fraction from polyamine nuclei? The size distribution of DNA fragments in this fraction is consistent with a 200 base pair repeat, although this is not as apparent as in the experiments of Gottesfeld and Butler (6) where digestion was more extensive. Indeed, much of the DNA is greater than 1000 base pairs in length, as in the S2 fractions from magnesium and salt polyamine nuclei: clearly the material in the S2 fractions is not merely partly degraded subnucleosomal particles (22) arising as an end product of chromatin digestion. Analysis of the proteins present in S2 fractions from magnesium and polyamine nuclei by SDS gel electrophoresis (Fig. 4) supports this view. The S2 fractions are greatly

Table 4. Analysis of chromatin fractions following DNAase II digestion
Fractions were analysed for DNA and RNA - figures are average of two fractionations using 4 mgs nuclear DNA. Digestion time: 30 mins.
S2, Mg^{2+} -soluble fraction. P2, Mg^{2+} -insoluble fraction. P1, nuclear residue

Nuclei	S2			P2		P1	
	DNA μ g	RNA μ g	Acid-soluble nucleotide	DNA μ g	RNA μ g	DNA μ g	RNA μ g
Magnesium nuclei	225	125	104	2218	57	1452	199
% of total DNA	(5.6%)		(2.6%)	(55.5%)		(36.3%)	
Polyamine nuclei	60	167	104	102	15	3734	297
% of total DNA	(1.5%)		(2.6%)	(2.6%)		(93.4%)

Fig. 4. SDS-gel electrophoresis of proteins in chromatin fractions following DNAase II digestion. Magnesium or polyamine nuclei from rat liver were digested with DNAase II and chromatin fractions separated as described in Methods. Proteins were analysed by electrophoresis on 15% acrylamide gels by a modification of the method of Laemmli (16) and the gels scanned at 605 nm. AP1, AP2 and AS2, magnesium nuclei. BP1, BP2 and BS2, polyamine nuclei. AP1 and BP1, nuclear residue. AP2 and BP2, Mg^{2+} -insoluble fraction. AS2 and BS2, Mg^{2+} -soluble fraction. H1, histone H1. hnRNP, proteins of heterogeneous nuclear ribonucleoprotein.

enriched in high molecular weight non-histone proteins, including 32-42,000 dalton molecular weight proteins (35) characteristic of heterogeneous nuclear ribonucleoprotein particles (labelled hnRNP in Fig. 4). Histone H1 and the core histones appear to be present in fraction S2 from magnesium

nuclei; although H1 is also present in S2 from polyamine nuclei, not all the nucleosome core histones can be unequivocally identified. Further work is required to establish the physical association of any or all of these protein species with DNA or RNA.

Of the other chromatin fractions obtained following DNAase II digestion (Fig. 4): fraction P1 from magnesium nuclei contains significant amounts of a wide range of different non-histone proteins, in addition to histones, whereas fraction P2 contains virtually no non-histone protein. Fraction P1 from polyamine nuclei is similar in composition to P1 from magnesium nuclei, whereas the small amount of chromatin released into fraction P2 has relative proportions of non-histone and histone protein intermediate between those of P1 and S2 from polyamine nuclei. More precise characterisation of the many protein species in these fractions will require two dimensional electrophoretic analysis (23-24).

DISCUSSION

We have shown that when rat liver nuclei are isolated with polyamines as sole stabilising cations, residual binding of polyamines maintains chromatin in a condensed state, thus restricting its accessibility to digestion by DNAase II and micrococcal nuclease. Additional, secondary effects on the solubility of chromatin fragments cannot be completely discounted. Nuclei stabilised during isolation by monovalent ions and polyamines appear much more accessible to both nucleases. The variable behaviour of such nuclei during DNAase II digestion may be related to their tendency to aggregate on resuspension in cation-free media. This problem might be avoided if the monovalent cation concentration is raised to 150 mM during nuclear isolation (25). Keichline *et al.* (26) and Schmidt *et al.* (27) have reported that polyamines can inhibit the digestion of chromatin by endogenous endonuclease, and DNAase I, respectively. Polyamines therefore have a general effect on the accessibility of chromatin to nucleases.

Spermine or spermidine can bind to free DNA molecules causing them to collapse into a highly compact state, which is extremely resistant to mechanical shear (28,29), but which can apparently still be digested by DNAase II (M.A.B. and T.J.H. unpublished observations). These polyamines also cause condensation of nuclei and isolated chromatin, binding to DNA-phosphate groups on chromatin with apparent stability constants ($\log K$ in mole λ^{-1}) of 4 to 4.5 (19,20). These stability constants are orders of magnitude greater than the apparent stability constants for binding of Ca^{2+}

and Mg^{2+} (3.1, refs. 19,30) and K^+ and Na^+ ions (1.1, ref. 19) to chromatin, which explains why polyamines are not readily displaced from chromatin by washing with Mg^{2+} solutions. However, it is surprising that nuclei exposed to saturating concentrations of polyamines in the presence of 75 mM monovalent cations (a concentration equivalent to half saturation of binding sites) are so much more accessible to nucleases than nuclei exposed to these concentrations of polyamines alone. Schmidt *et al.* (27) have observed that Na^+ ions compete effectively with Mg^{2+} ions for DNA phosphate groups on chromatin when both cations are in excess.

It is nevertheless clear that, depending on the other cations present, low polyamine concentrations can have profound effects on chromatin structure and conformation. Since these effects are completely reversed only with difficulty, they must be taken into consideration when polyamine containing solutions are used in experiments on chromatin or nuclei.

The release of Mg^{2+} -soluble (S2) chromatin from nuclei stabilised by different cations, during DNAase II digestion, deserves special consideration in view of its proposed correspondence with "active" chromatin (6-8). The S2 fraction from magnesium nuclei, and presumably from salt polyamine nuclei, is a complex mixture of DNA, histones, RNA, RNA packaging proteins (33) and other high-molecular weight non-histone proteins; others have shown this fraction to be enriched in acetylated histone H4 (34), high-mobility group proteins (32) and free ubiquitin (24), although the association of all of these components with DNA has not been proven.

Bulk chromatin becomes condensed and insoluble when Mg^{2+} or other cations bind to DNA phosphate groups, thus neutralising the net negative charge on chromatin (19,30,31). Histone H1 is required for this condensation to occur (18). Much of the DNA in the S2 fraction is greater than 1000 base pairs in length, and, at least in the case of magnesium nuclei, is associated with normal levels of histone H1 (32), indicating that the Mg^{2+} -solubility of this chromatin fraction is not due to excessive degradation during digestion. Instead, additional negatively charged groups, with a lower affinity for cations than DNA phosphate groups, must be associated with this chromatin so that, even in the presence of Mg^{2+} ions, it retains sufficient net charge to exist in an extended, uncondensed form (18,19,30). Although RNAase digestion of Mg^{2+} -soluble chromatin renders it Mg^{2+} -insoluble (6,34), these additional negatively charged groups are probably carboxyl or primary phosphate groups of non-histone proteins, which dissociate from chromatin during RNAase digestion, and not RNA

itself, which has a similar affinity for cations to DNA (36).

The greatly reduced release of Mg^{2+} -soluble, "active" chromatin when polyamines (polyamine nuclei) or Mg^{2+} ions (magnesium nuclei - Table 2) remain bound to chromatin in nuclei, suggests that either (a) the accessibility of "active" chromatin in the nucleus to nuclease is restricted by adjacent regions of inactive, condensed chromatin, or (b) that "active" chromatin is itself condensed in the nucleus by bound cations and therefore inaccessible to DNAase II. The latter situation would imply that the Mg^{2+} -soluble nature of "active" chromatin after release from nuclei is a consequence of the digestion process itself, due, for example, to the artefactual association of non-histone proteins. These possibilities require further investigation.

+ Abbreviations: EGTA, Ethylene glycol-bis-(2-amino ethyl ether)-N,N'-tetra acetic acid. EDTA, Ethylenediamine tetra acetic acid. PMSF, Phenylmethyl sulphonyl fluoride. TES, (N-tris[Hydroxymethyl]methyl-2-aminoethane sulphonic acid.

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