Heparin increases chromatin accessibility by binding the trypsin-sensitive basic residues in histones

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Recent evidence indicates that chromatin accessibility to transcription factors is of regulatory significance. The polyanion heparin is known to increase chromatin accessibility to DNAase I and to stimulate both RNA and DNA synthesis. In the present study, chromatin structure and its modification by polyanions were examined by using trypsin and micrococcal nuclease as probes. Both heparin and poly(glutamic acid) were found to be equivalent to trypsin digestion of histones in their ability to increase nuclease accessibility in chromatin. However, no increase in nuclease accessibility was observed when trypsin-digested chromatin was further treated with heparin, indicating that polyanions and trypsin are not additive in their effects on chromatin accessibility. Moreover, sucrose-gradient analysis demonstrated that heparin binds tightly to intact nucleosomes but not to trypsin-digested nucleosomes. These data suggest that polyanions interact predominantly with the trypsin-sensitive lysine and arginine residues in histone H1 and the N-terminal segments of the core histones. The possible relevance of these results to the chromatin structure of actively transcribed regions is discussed.

INTRODUCTION

Eukaryotic DNA is condensed to fit inside the nucleus by the histones, which compact DNA into the nucleosome and the 30 nm chromatin fibre (Reeves, 1984; van Holde, 1988). Previous studies have shown that the chromatin structure around active genes is perturbed in that actively transcribed chromatin is preferentially digested by either DNAase I or micrococcal nuclease (MNase), suggesting that chromatin packaging must be altered in order for transcription to occur (Weintraub & Groudine, 1976; Bloom & Anderson, 1978; Reeves, 1984). Supporting this notion, recent data strongly indicate that the DNA packaged within chromatin is often not accessible to transcription factors and that histones can hinder both polymerase initiation and elongation (Elgin, 1990; Grunstein, 1990; Felsenfeld, 1992; van Holde et al., 1992). Thus a basic unanswered question is how chromatin is modified in order for transcription to occur.

The acidic polyanions heparin and poly(glutamic acid) have been reported to alter chromatin thermodenaturation and c.d. and to stimulate DNAase I sensitivity, RNA transcription and DNA replication in vitro (Smith & Cook, 1977; Brotherton et al., 1989). These changes in the chromatin template were not accompanied by major disruptions of chromatin structure. In the present study I have attempted to elucidate the specific mechanism by which these polyanions act by focusing on the role of the histone trypsin-sensitive domains (Bohn & Crane-Robinson, 1984).

To examine changes in chromatin structure on treatment with heparin, MNase was used as a probe for nuclease accessibility, whereas trypsin was used to determine the role of the trypsin-sensitive histone domains. MNase was chosen as a probe of chromatin structure because a characteristic limit digest of 50% DNA solubilization is reached on prolonged digestion of nuclei or chromatin (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976). This limit digest apparently reflects the nature of the histone-DNA interactions within the nucleosome (Camerini-Otero et al., 1976). If chromatin is first digested with trypsin to preferentially remove histone H1 and the N-terminal ‘tails’ of histones H2A, H2B, H3 and H4, there is a significant increase in the susceptibility to MNase attack (Weintraub & Lente, 1974; Lilley & Tatchell, 1977). Since my initial experiments indicated that polyanion treatment of chromatin leads to a similar increase in the susceptibility to MNase digestion, I have directly compared the effects of polyanion treatments of chromatin with the effects of trypsin digestion. The data indicate that polyanions interact preferentially with the trypsin-sensitive regions of the histones, suggesting that the polyanion-induced stimulation of endogenous RNA and DNA synthesis (Smith & Cook, 1977; Brotherton et al., 1989) is best accounted for by electrostatic masking of the trypsin-sensitive lysine and arginine residues in the histones.

MATERIALS AND METHODS

Isolation of nuclei, chromatin and nucleosomes

Nuclei were isolated from calf thymus as previously described (Weintraub & Groudine, 1976). To prepare chromatin, nuclei were resuspended in 0.35 M-sucrose/10 mm-Tris·HCl/1 mm-phenylmethylsulfonyl fluoride/2 mm-CaCl₂, pH 7.9, followed by centrifugation of the homogenate at 13000 g for 10 min. The pellet was washed twice with the same buffer containing 0.5% Triton X-100, and then once with 0.25 M-NaCl/25 mm-EDTA, pH 6.0. The purified nuclei were then washed twice in 10 mm-Tris·HCl, pH 8.0, lysed with a glass/Teflon homogenizer, and washed twice more with 10 mm-Tris·HCl, pH 8.0. Salt-washed nucleosomes lacking H1 were prepared from calf-thymus nuclei as described previously (Martinson et al., 1979).

Treatment of chromatin or nucleosomes with polyanions and MNase

A sample of chromatin or nucleosomes (125 μg/ml; determined by A₂₆₀ in 5 mm-NaCl/1 mm-NaOH) was suspended in 10 mm-Tris·HCl (pH 7.9)/25 mm-CaCl₂, and treated with various amounts of heparin (porcine intestinal mucosa, grade II, Sigma) or poly-L-glutamate (15–50 kDa, Sigma). The chromatin was typically digested with MNase (5 μg/ml; Sigma) for 30 min at 37 °C. The reaction was stopped by adding NaCl and HClO₄ to

Abbreviation used: MNase, micrococcal nuclease.
final concentrations of 0.5 M and 5% respectively. The acid precipitate was centrifuged for 10 min at 12000 g and the percentage of HClO₄-soluble DNA was calculated from the DNA remaining in the supernatant.

Trypsin digestion of chromatin and nucleosomes
Chromatin or nucleosomes were digested with 4-36 μg of trypsin (Worthington Biochem. Co.)/ml for 20 min at 37 °C as described previously (Weintraub & van Lente, 1974). The trypsin digestions were stopped by incubating the samples in soybean trypsin inhibitor (500 μg/ml; type II-S, Sigma) for 20 min at 37 °C. The trypsin-digested samples were then digested with MNase as described above or treated with heparin before MNase digestion.

Histone analysis
Chromatin [125 μg/ml in 10 mM-Tris/HCl (pH 7.9)/25 mM-CaCl₂] was treated with various concentrations of heparin or poly(glutamic acid). Polyanion-treated and untreated samples were then centrifuged at 40000 rev./min (4 °C) for 2.5 h in an SW41 rotor. The pellets were dissolved directly in electrophoresis loading buffer [0.125 M-Tris/HCl (pH 7.0)/2% SDS/0.5% β-mercaptoethanol/3 M-urea]. Aliquots of the supernatants were made 25%, in trichloroacetic acid, incubated on ice for 30 min to precipitate the histones, and then pelleted by centrifugation. The pellets were washed once with acetone, vacuum-dried and then resuspended in electrophoresis loading buffer. Histones from whole or trypsin-digested chromatin were analysed on 18% polyacrylamide gels as described previously (Villeponteau et al., 1978).

Sedimentation of nucleosomes
Treated or untreated nucleosomes (6 A₂₆₀ units) were layered on a linear 5-25% sucrose gradients containing 10 mM-Tris/HCl (pH 7.9)/25 mM-CaCl₂, and centrifuged at 41000 rev./min (4 °C) for 16 h in a Beckman SW41 rotor. The histones from each fraction were collected by trichloroacetic acid precipitation for analysis by PAGE as described above.

RESULTS
Chromatin treated with polyanions or trypsin has nearly identical MNase limit digests
About one-half of the DNA within chromatin is normally resistant to MNase attack (Clark & Felsenfeld, 1974). Fig. 1 shows that treatment of chromatin with the polyanion heparin increases the accessibility of chromatin to MNase. To determine the dose response for this effect of polyanions on chromatin, various concentrations of heparin or poly(glutamic acid) were added to a fixed amount of chromatin followed by digestion with MNase (5 μg/ml). The maximum susceptibility of chromatin to MNase was achieved at a polyanion/DNA ratio of 0.2-0.4 (Fig. 2a). Above this ratio poly(glutamic acid) has little further effect, whereas heparin exhibits a strong inhibition of the nuclease (see below). The addition of a neutral protein such as BSA has no effect on the accessibility of chromatin to nuclease attack (results not shown).

Digestion of chromatin with trypsin generates the well-characterized limit histone fragments P1-P5, which contain the globular C-terminal domains of the core histones (see Bohm & Crane-Robinson, 1984; Weintraub & van Lente, 1974; and the inset to panel of Fig. 2a). Trypsin-digested chromatin has an increased susceptibility of the chromatin to MNase attack (Weintraub & van Lente, 1974; Lilley & Tatchell, 1977). Fig. 2 shows that the increased availability of trypsin-digested chromatin.
Heparin binds histone basic residues to unfold chromatin.

tin to nuclease attack closely parallels that found for polyanion-treated chromatin. Moreover, the size distributions of the MNase-resistant limit-digest fragments from heparin-treated and trypsin-digested chromatin are also similar (results not shown), further supporting the link between trypsin digestion and heparin treatment.

To ascertain whether heparin or poly(glutamic acid) has a direct effect on MNase activity, naked calf thymus DNA was digested with MNase (5 μg/ml) in the presence of various concentrations of polyanion. Fig. 2(b) shows that heparin inhibits MNase activity, whereas polyglutamic acid has no effect. The same result was obtained when the rate of MNase digestion of naked DNA was monitored by hyperchromicity using a recording spectrometer (results not shown). Thus the most likely explanation for the biphasic heparin curve in Fig. 2(b) is that heparin at low concentrations binds histones and increases the availability of the DNA for digestion, but at high concentrations excess free heparin becomes available for direct inhibition of the MNase. These data suggest that heparin and polyglutamic acid act similarly in altering the structure of chromatin.

Polyanion-induced extraction of histones from chromatin is not the primary cause of increased MNase susceptibility

To determine the extent to which polyanions extract histones from chromatin, calf thymus chromatin was incubated with various concentrations of heparin or poly(glutamic acid) and then pelleted in the ultracentrifuge. Analysis of the histones in the pellet and supernatant fractions was then carried out using PAGE. Fig. 3 shows that some extraction of H2A and H2B at moderate concentrations of heparin. Densitometric scans of these gels indicate that there was less than a 15% loss of H2A and H2B below the 0.3 level of heparin. Moreover, even at high concentrations, poly(glutamic acid) removes negligible amounts of core histone from calf thymus chromatin, although H1 is removed at the highest concentrations tested.

Comparing the MNase digestion data on heparin-treated chromatin in Fig. 2 with the heparin-induced-histone-extraction data shown in Fig. 3, it is apparent that near maximum susceptibility to MNase digestion is achieved at a heparin/DNA weight ratio of 0.2 below that which leads to substantial H2A and H2B extraction. For poly(glutamic acid), hardly any of the core histones appear to be extracted at polyglutamic acid/DNA ratios substantially above those that give rise to maximal susceptibility of chromatin to MNase digestion. Thus the increased susceptibility of polyanion-treated chromatin to MNase attack appears not to correlate with histone extraction.

The effects of trypsin and heparin are not additive

The similarity in MNase digestion limits with trypsin digestion or heparin treatment suggests that heparin interacts with the same regions of the histones in chromatin as does trypsin. To compare trypsin- and heparin-treated chromatin directly, trypsin-digested chromatin was further treated with heparin before MNase digestion. Table 1 demonstrates that subjecting chromatin to both trypsin digestion and heparin treatment has the same effect on chromatin accessibility to MNase as does trypsin or heparin treatment alone. This complete lack of additivity with trypsin and heparin supports the notion that heparin interacts with the same histone domains as does trypsin, namely H1 and the N-terminal tails of the core histones.

Sucreose-gradient analysis of heparin-treated nucleosomes

Because heparin increases chromatin accessibility to MNase

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Fig. 4. Sucrose-gradient centrifugation of heparin-treated nucleosomes

Nucleosomes at 600 µg/ml were incubated for 20 min with 0 (a; control), 120 (b and c; 0.2 µg of heparin/µg DNA), or 300 µg/ml (d; 0.5 µg heparin/µg DNA) of unlabelled heparin mixed with a negligible constant amount of tritiated heparin tracer. A 0.5 ml portion of the treated and untreated nucleosomes were then layered on a 5–25% sucrose gradient containing 10 mM-Tris/25 mM-CaCl₂, pH 7.9, and centrifuged. In (c), 100 µg of unlabelled heparin/ml was included in the gradient buffer. Fractions were collected from the bottom of the tube. The A₂₆₀ (●) and tritium activity (○) were then determined for each fraction.

shown) demonstrate that these sedimented nucleosomes contain normal amounts of the four core histones, bind heparin, and exhibit maximal susceptibility to MNase digestion. Taken together, these data indicate that heparin binds tightly to nucleosomes and partially unfolds them.

Heparin does not bind trypsin-digested nucleosomes

Table 1 shows that heparin has no effect on MNase chromatin accessibility of trypsin-digested chromat, indicating that heparin binds to the trypsin-sensitive domains of the histones. To test for the binding of heparin to the trypsin sensitive regions of the core histones definitively, trypsin-digested nucleosomes were treated with tritiated heparin and then centrifuged in a 5–25% sucrose gradient as before. Fig. 6 clearly demonstrates that heparin does not bind to trypsin-digested nucleosomes. PAGE analysis of the peak fractions in the gradient revealed the typical trypsin-limit-digest pattern of the histones (see the inset in Fig. 6). These results strongly indicate that heparin binds to the

Fig. 5. Histone content and MNase accessibility of isolated heparin-treated nucleosomes

Each of the peak fractions from the second gradient in Fig. 4 was divided into two aliquots. Histones were isolated from one portion and aliquots were electrophoresed on 18% PAGE gels containing 0.1% SDS. MNase was added to the other aliquot (0.04 µg of MNase/µg of DNA) and the nucleosomes were exhaustively digested as before. The percentage of acid-soluble DNA is shown below the histone profile for each fraction. Control nucleosomes treated (0.2 µg of heparin/µg of DNA) or untreated with heparin yield 85 or 65%, acid-soluble DNA respectively. Note that MNase-sensitivity of nucleosomes is increased by H1 removal.

Fig. 6. Sucrose-gradient centrifugation of trypsin-digested heparin-treated nucleosomes

Nucleosomes (125 µg of DNA/ml) were digested with 30 µg/ml of trypsin for 20 min. The reaction was stopped with trypsin inhibitor as before. ³H-labelled and unlabelled carrier heparin were then added to the trypsin-treated nucleosomes and they were layered on a sucrose gradient and sedimented as described in the text (Fig. 4). Free DNA sediments at approximately fraction 13. PAGE analysis of the histones in the A₂₆₀ peak fractions revealed the expected quantity of histone-limit-digest products (see the inset).
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trypsin-sensitive regions of the histones in the nucleosome core.

DISCUSSION
Heparin unfolds chromatin and increases chromatin accessibility

Figs. 1 and 2 demonstrate that, when chromatin is treated with the polyamions heparin or poly(glutamic acid), the DNA contained within chromatin becomes markedly more accessible to MNase digestion. The MNase limit digest is known to depend on histone–DNA interactions (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976), so that heparin likely disrupts histone–DNA binding. Although heparin and poly(glutamic acid) will extract certain histone species from chromatin at sufficiently high concentrations, maximum accessibility of chromatin to MNase is observed at lower polyamion concentrations, where little histone is removed by heparin and none by poly(glutamic acid) (Fig. 3; Brotherton et al., 1989). Thus the altered MNase digestion characteristics of polyamion-treated chromatin is not due to extensive histone extraction, but rather to a perturbation in the histone–DNA binding interactions of otherwise intact nucleosomes.

In addition to increasing sensitivity to MNase, treatment of nucleosomes with heparin leads to slower sedimentation on sucrose gradients (Fig. 4), suggesting that nucleosomes are unfolded by heparin. This result is consistent with the reports that heparin-treated chromatin has enhanced sensitivity to DNase I, decreased thermal stability, and a c.d. spectrum more like that of free DNA (Smith & Cook, 1977; Brotherton et al., 1989). Taken together, these data indicate that heparin increases transcription and replication by unfolding chromatin and increasing chromatin accessibility.

Heparin interacts with trypsin-sensitive histone H1 and the N-terminal tails of the core histones

Trypsin digestion of chromatin or nucleosomes generates a similar pattern of limit digest fragments, P1–P5, which mainly consist of the globular C-terminal domains of the core histones H2A, H2B, H3, and H4 (Weintraub & van Lente, 1974; Bohm & Crane-Robinson, 1984). In nuclei, the central globular core of H1 is also protected from trypsin (Bohm & Crane-Robinson, 1984). In chromatin, histone H1 and the basic N-terminal tails of 20–30 residues are thought to be preferentially degraded, owing to their accessibility to trypsin rather than to enzyme specificity (Bohm & Crane-Robinson, 1984), as chymotrypsin digestion of chromatin gives the same limit-digest pattern (Sollner-Webb et al., 1976). Moreover, the highly basic N-terminal tails removed by trypsin have been identified as lacking in secondary structure by n.m.r. (Cary et al., 1978) and are the known in vitro sites of histone acetylation and phosphorylation (van Holde, 1988). In contrast, the C-terminal globular domains of the core histones fail to bind anti-histone antibodies (Goldblatt & Bustin, 1975) and are responsible for the histone–histone interactions in the octamer core (van Holde, 1988).

Several lines of evidence indicate that heparin interacts mainly with the trypsin-sensitive domains of the histones. First, trypsin-treated chromatin has been reported to have enhanced DNAase I-sensitivity, reduced thermal stability and a shift in c.d. spectrum toward that of free DNA (Lilley & Tatchell, 1977; Whitlock & Simpson, 1977; Whitlock & Stein, 1978; Lundell & Martinson, 1989) that closely resembles similar changes seen with heparin-treated chromatin (Smith & Cook, 1977; Brotherton et al., 1989). Secondly, chromatin treated with heparin or trypsin has equivalent levels of sensitivity to MNase (Fig. 2). Thirdly, after chromatin is digested with trypsin, addition of polyamions has no further effect on the accessibility of chromosomal DNA to MNase attack (Table 1). Finally, trypsin-digested nucleosomes no longer bind heparin (Fig. 6). Taken together with the data showing that H1 and the N-terminal tails of the core histones are preferentially accessible to acetylases, phosphatases, anti-histone antibodies and proteinases, the results reported here provide compelling evidence that heparin interacts preferentially with H1 and the N-terminal tails of the core histones. Given the pattern of heparin binding to the histones, heparin can be postulated to increases chromatin accessibility by masking the electrostatic charges of the lysine and arginine residues in histone H1 and the N-terminal tails of the core histones.

Since histone H1 is the principal histone involved in the higher-order folding of chromatin, the interactions of heparin with H1 could account for all of the effects of heparin on nuclease accessibility in chromatin. However, the data in Fig. 5 argue against the possibility that the effects of heparin on chromatin depend solely on interactions with H1. Whereas the MNase-sensitivity of nucleosomes is markedly increased by H1 removal, H1-free nucleosomes become even more accessible to MNase digestion after heparin treatment. These results strongly suggest that the heparin-induced increases in chromatin accessibility involve polyamion interactions with both H1 and the core histones.

Does heparin-induced unfolding provide a model for the alterations in chromatin structure required for transcription?

Active genes are preferentially accessible to DNAase I (Weintraub & Groudine, 1976; Reeves, 1984), DNAase II (Gottesfeld & Partington, 1977), and MNase (Bloom & Anderson, 1978; Bloom & Anderson, 1979). Preferential DNAase I sensitivity of active genes is lessened by trypsin digestion (Lundell & Martinson, 1989), so that the histone domains available to trypsin and polyanions are likely to play a prominent role in active chromatin. The nuclear high-mobility-group proteins, which have a high content of acidic amino acid residues in one domain (Walker et al., 1976), represent a naturally occurring class of non-histones that may function in a manner mimicked by heparin or poly(glutamic acid). The high-mobility-group proteins have been localized to actively transcribed regions and have been reported to promote DNAase I sensitivity (Levy-Wilson & Dixon, 1979; Weisbrod et al., 1980; Stein & Townsend, 1983).

Recent genetic experiments have demonstrated that the core histones (Han & Grunstein, 1988; Grunstein, 1990) or the N-terminal region of histone H4 (Kayne et al., 1988) can repress the initiation of transcription of various genes. Conversely, many transcription factors can specifically counteract histone-mediated repression (Elgin, 1990; Grunstein, 1990; Laybourn & Kadonaga, 1991; Felsenfeld, 1992). The activation domains of many transcription factors contain acidic residues that may form an amphiathic a-helix with negatively charged residues lined up along one surface (Gill & Ptashne, 1988; Ma & Ptashne, 1988; Ptashne, 1988). Whereas transcription factors are thought to bind each other (Ptashne, 1988), acidic activation domains may also counteract histone-mediated repression (Han & Grunstein, 1988; Laybourn & Kadonaga, 1991) by binding the trypsin-sensitive arginine and lysine residues in H1 and the N-terminal tails of the core histones as postulated for heparin in the present paper.

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