THE EFFECT OF HEPARIN ON THE STRUCTURE AND TEMPLATE PROPERTIES OF CHROMATIN

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1. Introduction

The polyanion heparin, by virtue of its charge properties, is able to compete with nucleic acids in many protein–nucleic acid interactions. Thus heparin inhibits both ribonucleases [1–3] and initiation by bacterial [3,4] or eucaryotic DNA-dependent RNA polymerases. The presence of ribonuclease activity in chromatin preparations [3,5] has led to the suggestion that heparin be used to abolish this when studying RNA synthesis from such templates in vitro [3]. However, the present manuscript indicates that heparin also causes gross structural modifications to the chromatin template.

2. Materials and methods

[^{35}S] heparin (19.4 mCi/g) was purchased from the Radiochemical Centre, Amersham (Bucks, UK). Unlabelled heparin (Pularin Grade) was purchased from Evans Medical Co. (Speke, UK). All other chemicals were from sources previously given [5,6]. Unsheared chromatin was prepared as detailed previously [5,6] and was used immediately at a concentration of 5–600 μg DNA per ml. 0.25 ml assays for chromatin endogenous RNA polymerase activity were set up, incubated and processed as described previously [5,6]. Any modifications to this standard procedure are detailed in the legend to the figures. [^{3}H] UTP was present at a specific radioactivity of 324 counts/min^{-1}/pmoles^{-1} in all assays except those shown in figs. 1a and 2 where the specific radioactivities were 130 and 1625/min^{-1}/pmoles^{-1} respectively. Chromatin was present at 25–30 μg DNA per assay. Heparin or $(NH_4)_2SO_4$ were added to certain assays during the

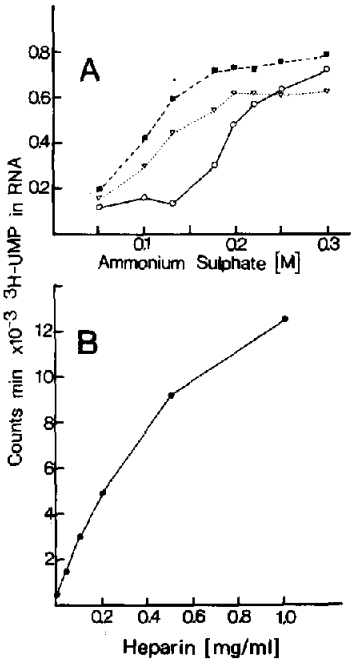

Fig. 1. The effect of increasing salt and heparin concentrations on chromatin endogenous RNA polymerase activity. A) Endogenous chromatin RNA polymerase activity was assayed at increasing concentrations of ammonium sulphate as described in Materials and Methods with no further additions, (o———o) with 120 μg/ml heparin added at each ionic strength (Δ.....Δ) and with 250 μg/ml rifampicin AF/0-13 added at each ionic strength (■– – – –■). B) Endogenous chromatin RNA polymerase activity was assayed at 0.13 M ammonium sulphate in the presence of increasing concentrations of heparin.

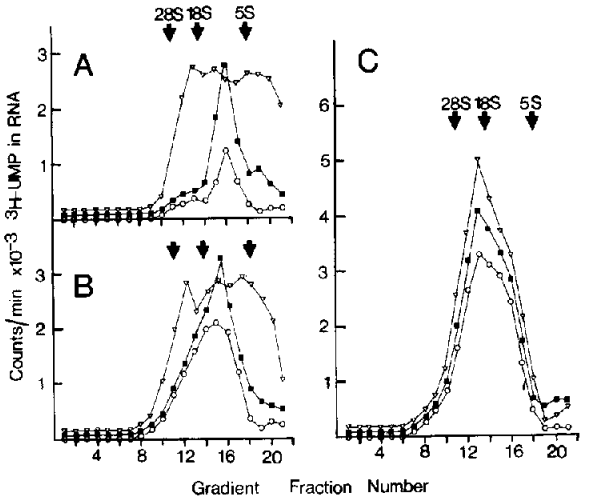

Fig. 2. The effect of ionic strength and heparin on the size range of RNA products synthesised by the endogenous chromatin RNA polymerase. Assays were carried out at A) 0.13 M ammonium sulphate, B) 0.25 M ammonium sulphate and C) 0.40 M ammonium sulphate in the absence of added heparin (○———○) with 200 μg/ml heparin, (■———■) or 1000 μg/ml heparin (△———△). RNA products were analysed on formamide gradients as described in Materials and Methods. Arrow heads mark the position of 28 S, 18 S and 5 S HeLa [^{3}H]rRNA run under the same conditions.

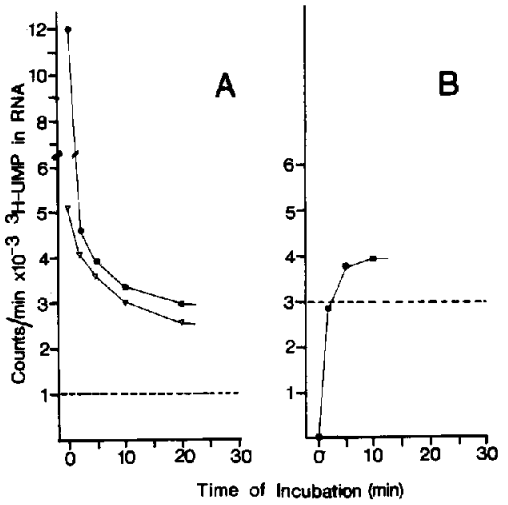

Fig. 3. The effect of addition of heparin or high salt concentrations before or after the onset of RNA synthesis as catalysed by the endogenous chromatin RNA polymerase or added from B RNA polymerase. A) Assays for chromatin endogenous RNA polymerase were begun at 37° C in the presence of 0.13 M ammonium sulphate. At the time points shown, heparin was added to 1 mg/ml (●———●) or ammonium sulphate to a final concentration of 0.40 M (△———△). The control with no additions is also shown (– – – – –). Time zero indicates addition immediately prior to incubation at 37° C. B) The template activity of chromatin was assayed at 0.13 M ammonium sulphate in the presence of 10 'units' purified form B RNA polymerase with the addition of heparin to 1 mg/ml at the times shown. Corresponding endogenous RNA polymerase activities (fig. 3A) have been deducted from all points so that the values shown represent only net added enzyme activity. (– – – – –) control level of net added enzyme activity with no additions; (■———■) with heparin (to 1 mg/ml) added at the times shown. All incubations were continued up to a total of 30 min at 37° C.

incubation but the additional volume was limited to 20 μl (see figs. 3 and 4). Assays for the experiment shown in fig. 4B also contained 10 units of form B rat liver RNA polymerase, prepared according to the method of Butterworth et al. [7].

Size ranges of in vitro synthesised RNA products (fig. 2) were estimated by a method described in detail elsewhere [5]. The assay mixtures, after incubation, were layered over 12 ml 2–10% linear sucrose gradients in an 85% formamide medium. These gradients were centrifuged for 18 hr at 284 000 *g* and 25° C. Gradient fractions were precipitated in the presence of 500 μg per fraction of carrier bovine serum albumin.

Possible binding of heparin to chromatin proteins was examined by comparing three samples respectively containing chromatin (800 μg DNA and 2000 μg protein per ml), [^{35}S] heparin (5×10^5 counts/min^{-1}/mg^{-1} and 1 mg/ml) and a mixture of these components, all in the absence of $(NH_4)_2SO_4$. Each of these samples (total vol. 4.0 ml) was layered over a 60 ml two-phase gradient from 5–60% sucrose in 10 mM Tris–HCl, pH 8.0 (the gradient profile is given in fig. 5). The gradients were centrifuged for 4 days at 100 000 *g* and 4° C, prior to fractionation into 42 1.5 ml fractions. The absorbance at 260 nm of the gradient material was continuously monitored during fractionation, and 100 μl samples were taken from each fraction of the labelled gradients and were mixed with 2 ml of ethanol and 10 ml butyl PBD/toluene scintillant [6] for counting. Selected, pooled fractions were assayed for protein content by the method

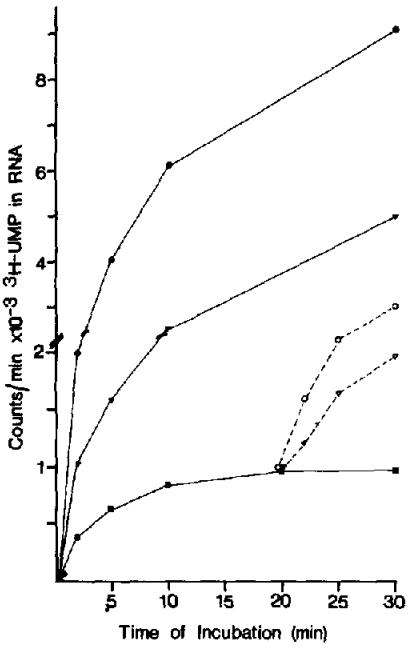

Fig. 4. Time course of chromatin endogenous RNA polymerase activity under various conditions. Normal assays were carried out as described in Materials and Methods at 0.13 M ammonium sulphate (■———■) 0.4 M ammonium sulphate (▲———▲) at 0.13 M ammonium sulphate in the presence of 1 mg/ml heparin from the outset (●———●) at 0.13 M ammonium sulphate but adding heparin to 1 mg/ml after 20 min (○– – – – –○) and at 0.13 M ammonium sulphate but adding ammonium sulphate to 0.40 M after 20 min (△– – – – –△).

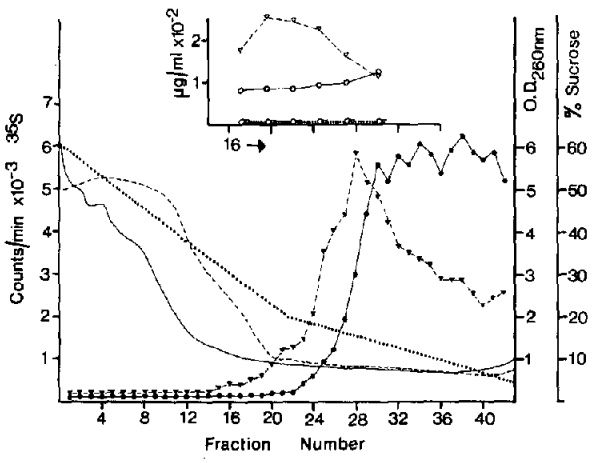

Fig. 5. The complexing of [^{35}S] heparin with chromatin associated proteins. Chromatin was treated with [^{35}S] heparin and sedimented in biphasic 5–60% sucrose gradients as described in Methods. Control gradients containing [^{35}S] heparin and chromatin alone were run in parallel. (▲– – – – –▲) ^{35}S radioactivity per 100 μl from each 1.5 ml fraction of the gradient loaded with chromatin-heparin mixture; (●———●) ^{35}S radioactivity per 100 μl from each 1.5 ml fraction of gradient loaded with [^{35}S] heparin alone; (– – – – –) absorbance 260 nm of gradient loaded with chromatin-heparin mixture; (———) absorbance 260 nm of gradient loaded with chromatin alone; (.....) profile of sucrose density gradient. The inset to this figure shows the protein content of fractions from the gradient loaded with chromatin–heparin mixture (△– – – – –△); protein content of fractions from gradient loaded with chromatin alone (○———○); the DNA content of fractions from the gradient loaded with the chromatin–heparin mixture (△.....△); the DNA content from gradient loaded with chromatin alone (○.....○).

of Lowry et al. [8] and for DNA by the diphenylamine method [9].

3. Results and discussion

A general parallelism between the effects on chromatin of high salt and heparin is apparent from figs. 1–3 and 4. Both agents are known to remove protein from chromatin [3,6] and it has been established that the net contribution by heparin to protein release decreases as the salt concentration is raised (see ref. [3] table 3, and from our own observations). Two primary effects may be distinguished: a massive activation of the endogenous RNA polymerase activity (figs. 1,3 and 4) and an increase in average and maximal RNA product size (fig. 2). The activation might be due to ribonuclease inhibition by heparin [1–3] or salt [5], an increased rate of transcription, the removal of proteins which otherwise slow down or halt transcription (whether specifically or not), the release of 'blocked' RNA polymerase molecules, or any combination of these possibilities. Inhibition of ribonuclease and/or protein release from chromatin might also explain the increase in product size induced by heparin or high salt concentrations.

Chromatin prepared by this method contains endogenous RNA polymerase activity which is totally sensitive to α-amanitin (form B) and is only evident at high ionic strengths [6]. In the presence of low concentrations of heparin (or even rifampicin AF/0-13), this activity becomes apparent at much lower ionic

strengths (fig. 1A). The effect of increasing concentrations of heparin at low ionic strength is shown in fig. 1B. Fig. 2 demonstrates a marked two-phase effect of heparin on chromatin endogenous RNA synthesis. In the presence of 200 μg/ml heparin a large stimulation is found at the lower ionic strengths, yet the product size is not increased. 1 mg/ml heparin however increases the product size up to a maximum close to 28 S under all ionic conditions. From this data ribonuclease inhibition seems unlikely, a conclusion reinforced by the absence of small RNA fragments at the top of the gradients from assays at lower ionic strengths to which no heparin had been added. A significant exonucleolytic activity has indeed been found in chromatin [5], but this would have relatively little effect on product size and its inhibition could not possibly account for the massive stimulation of endogenous activity with heparin at 0.13 M $(NH_4)_2SO_4$. It is unlikely that heparin or high ionic strength increase the rate of transcription; fig. 3B shows that most initiations by the added form B polymerase seem to occur within the first two minutes of the incubation (since heparin completely inhibits initiations when added at zero time) and addition of heparin at 5 or even 10 min results in little net increase in activity over the control level (no heparin added), implying that transcription rates must be similar both in the presence and absence of heparin.

The possibility that RNA synthesis might terminate more rapidly at 0.13 M $(NH_4)_2SO_4$ in the presence of heparin seems unlikely in view of the data given in fig. 4. A plateau of endogenous RNA synthetic activity is reached after 10–15 min at low salt in the absence of heparin but not at high salt concentrations or in the presence of heparin. Wholesale termination involving the dissociation of the polymerase from the template cannot explain this plateau phenomenon since RNA synthesis can be reactivated by the addition of high salt or heparin at 20 min, although both agents abolish initiation by free RNA polymerase. It seems most likely that the effect of high salt or heparin concentration is to remove proteins from the template which otherwise restrict transcription. Lower heparin concentrations (up to 200 μg/ml) might act via the release of 'blocked' polymerase molecules, there being no significant effect on product size.

One major anomaly remains in that the stimulation of RNA synthetic activity is drastically reduced when heparin is added 2 min after the start of incubation rather than at time zero (fig. 3A). This drop is far less marked with high salt (where it might be explicable in terms of true termination on a limited scale, consistent with the results in fig. 4). This alone should suggest caution in the interpretation of endogenous activities measured in the presence of high heparin concentrations ab initio.

Because of its similar charge properties to DNA, it might be expected that heparin would bind to some of the proteins it displaces. This is confirmed by fig. 5 which gives clear evidence of heparin–protein complex formation as judged by the sedimentation rate of the ^{35}S-labelled heparin in the presence and absense of chromatin. Protein contents, determined over the crucial range of fractions, show that at least 20% of the total protein loaded onto the chromatin–heparin gradient is associated with the leading edge of the heparin peak. This is over and above the protein levels found in parallel fractions from the gradient loaded with chromatin alone: no DNA is present in either case. Moreover, considerable dissociation of the chromatin DNA–protein complex in the presence of heparin is implied by the trailing out of the DNA as indicated by the 260 nm absorbance trace.

4. Conclusions

Although heparin may be a good ribonuclease inhibitor, its use in chromatin transcription studies must be approached with caution. Treatment with heparin results in gross modifications of the structure and template properties of chromatin. It must be concluded that heparin competes with DNA for chromatin-associated proteins, whether histones (which are known to be removed by high salt concentrations) or non-histone proteins has not been determined.

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