THE SUBUNIT STRUCTURE OF CHROMATIN: CHARACTERISTICS OF NUCLEOHISTONE AND NUCLEOPROTAMINE FROM DEVELOPING TROUT TESTIS

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1. Introduction

Recent work has demonstrated the existence of a subunit structure in chromatin.

Hewish and Burgoyne have reported [1] that a large proportion of DNA in isolated rat liver nuclei is digested by an endogenous Ca^{++} and Mg^{++} dependent endonuclease into fragments which are integral multiples of a unit length. Louie has found [2] that DNA from mouse liver nuclei or from the nucleoprotein complex of polyoma virus is also digested by this endonuclease into 200 base pair fragments. An important breakthrough has been provided by Noll [3] who has obtained similar DNA fragments from rat liver nuclei by digestion with commercially available DNase or micrococcal nuclease (E.C. 3.1.4.7). He has also succeeded in separating chromatin from nuclease-treated nuclei into 11.2 S 'subunit monomers' (containing chromosomal proteins on 200 base pairs of DNA) and dimers, trimers etc. on sucrose gradients.

Trout testis is a good tissue for the study of such chromatin subunits since: (i) large quantities of chromatin relatively free of cytoplasmic contamination are easily prepared; (ii) the subunits represent one approach to studying changes in chromatin structure when the histones are replaced by protamine during sperm development; and (iii) the subunits provide a model substrate for the study of the enzymatic modifications of histones in trout testis.

We wish to report that 11 S chromatin subunits can be isolated from trout testis nuclei treated with micrococcal nuclease at early stages (containing nucleohistone) of development, but that trout sperm nuclei (containing nucleoprotamine) yield no such subunits. In testis nuclei at an intermediate stage of development (containing both nucleohistone and nucleoprotamine), the yield of subunits correlates with the amount of nucleohistone originally present; these subunits contain histones but negligible amount of protamine.

These results suggest that at early stages of development trout testis chromatin has a subunit structure similar to that reported for other tissues, but that the structure of trout sperm nucleoprotamine differs, making it inaccessible to micrococcal nuclease digestion.

2. Materials and methods

2.1. *Preparation and micrococcal nuclease digestion of trout testis nuclei*

Nuclei were isolated from various stages of naturally maturing trout testis (obtained from Sun Valley trout farm, Mission, B.C. Canada) in a polyamine-containing buffer [1]. Digestion of nuclei by micrococcal nuclease (Sigma) was performed at 37°C in the same buffer made 1 mM in $CaCl_2$, as described by Noll [3]. It was found later that nuclei could be prepared by homogenizing testis in 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose, 15 mM 2-mercaptoethanol, 50 mM Tris pH 7.4 (TMKS) buffer. Nuclei, pelleted at 1 000 *g* for 10 min, were then washed twice more with TMKS buffer and digested with micrococcal nuclease at 37°C in TMKS–1 mM $CaCl_2$, 5×10^8 nuclei/ml.

To determine the content of nucleohistone and nucleoprotamine, nuclei were extracted with 0.4 N

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H_2SO_4, and basic proteins were precipitated with 3 vol of ethanol at −20°C overnight [4]. Proteins were electrophoresed on 15% urea–polyacrylamide gels (5), stained with Coomassie Blue and scanned at 600 nm using a Gilford spectrophotometer.

2.2. *Preparation of 'chromatin subunits' from digested nuclei*

Digested nuclei were homogenized in 0.2 mM EDTA, then centrifuged at 12 000 *g* for 30 min as described by Noll [3]. The resulting supernatant containing chromatin subunits was layered on a 1.5 X 9 cm, 16 ml, 10–30% linear sucrose–0.2 mM EDTA gradient and centrifuged at 25 000 rev/min for 18 hr. Twenty fractions of twenty drops each were then collected. Catalase, run in a separate tube, was found to be a convenient marker for the 11 S region.

Histone and protamine content was determined by precipitating chromatin subunits in sucrose gradient fractions with 2 mM Ca^{++} or 10 mM Mg^{++}, filtering them on GF/c paper (Millipore Corp.) washing with ethanol, then extracting the filters with 0.4 N HCl directly in the slot of a urea–lactate starch gel [6]. Starch gels were run and stained as described [6,7]. Alternatively, acid extracted protein was electrophoresed on 15% urea polyacrylamide gels as above.

2.3. *Analysis of DNA fragments from digested nuclei*

DNA from digested nuclei was prepared for polyacrylamide gel electrophoresis as described by Hewish and Burgoyne [1]. Chromatin subunits were first precipitated with Ca^{++} or Mg^{++}, then DNA prepared as above. DNA samples were electrophoresed on 2.5% acrylamide disc gels which were then stained with 'Stainsall' [8], and scanned at 550 nm in a Gilford spectrophotometer.

Diphenylamine estimations of DNA content were performed as described by Burton [9].

3. Results and discussion

When nucleohistone stage trout testis nuclei are digested with micrococcal nuclease, the DNA is reduced to fragments which are multiples of a unit length (fig. 1a) as determined by plotting log band number against the square root of band mobility [1,10]. Extended digestion of such nuclei converts

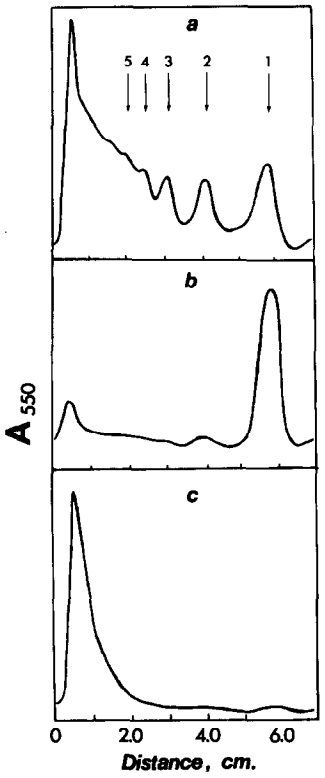

Fig. 1. 2.5% polyacrylamide gel scans of DNA: (a) from histone stage nuclei digested with 100 A_{260} units/ml of micrococcal nuclease, 3 min. (b) from histone stage nuclei digested with 600 units/ml of micrococcal nuclease, 6 min. (c) from late protamine stage nuclei digested with 600 units/ml of micrococcal nuclease, 6 min.

greater than 80% of the DNA to fragments corresponding to the smallest unit, (Fig. 1b) which has been determined to be approximately 200 base pairs long for the chromatin of other organisms [2,3].

Fig. 2 (solid black squares) shows the sucrose gradient sedimentation profile of chromatin prepared from nuclei digested with micrococcal nuclease, as described in 'Materials and methods'. As shown by Noll [3] for rat liver chromatin, trout testis chromatin is digested almost entirely to small fragments sedimenting at approximately 11 S. These chromatin subunit 'monomers' contain the smallest (200 base pair) DNA fragment, with some 'dimer' and 'trimer' at the heavy side of the peak.

This data further supports the model [1–3] that

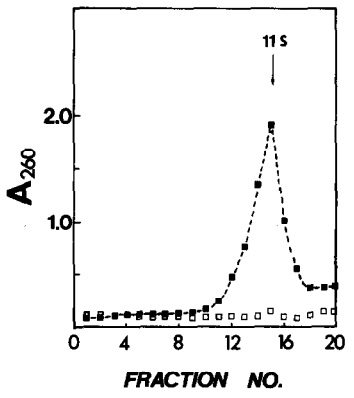

Fig. 2. Sucrose gradient centrifugation of chromatin subunits prepared as described in Materials and Methods from histone stage (■–■–■) and mature sperm (□–□–□) nuclei digested with micrococcal nuclease, 600 units/ml, 6 min.

Table 1

Developmental stage	% Nucleo-histone	% Nucleo-protamine	% DNA released into subunits
Histone	100	–	80
early protamine	95	5	80
mid protamine	35	65	28
late protamine	5	95	2
mature sperm	–	100	< 1

The % DNA in subunits from digested nuclei was determined from 2.5% polyacrylamide gels of extracted DNA. Alternatively, chromatin subunits were prepared and DNA determined by the diphenylamine reaction. Histones and protamine were quantitated from 15% urea-polyacrylamide gels.

chromatin consists largely of 200 base pair long segments of DNA, covered with proteins, and with a small 'spacer' region of DNA between subunits which is accessible to nuclease digestion.

Trout testis differs from other tissues studied however, since it undergoes a change from mitotic to meiotic tissue with subsequent replacement of nucleohistone by nucleoprotamine. It was of interest therefore to see how these changes would be reflected in the subunit structure of chromatin.

When sperm nuclei, which contain only nucleoprotamine, are digested with micrococcal nuclease, no DNA fragments are produced (fig. 1c) and no 11 S chromatin subunits are found on sucrose gradients (fig. 2, open squares).

We next examined trout testis at intermediate stages of development, containing both nucleohistone and nucleoprotamine.

In early protamine stage testis, much of the tissue is meiotic in origin and spermatid cells, which have just started making protamine, predominate [6]. The yield of chromatin subunits from such tissue is comparable to yields from histone stage mitotic testis, suggesting that meiotic and mitotic chromatin have a similar high proportion of subunit structure.

When mid-protamine stage nuclei are digested with micrococcal nuclease, 11 S chromatin subunits are produced in an amount roughly proportional to the amount of nucleohistone initially present (table I). The subunits from such tissue contained only histones, with negligible protamine. These results suggest that nucleoprotamine may not have a subunit structure analogous to nucleohistone, but instead may have all of the DNA covered with protamine molecules.

Alternatively, the failure to digest nucleoprotamine may occur because in tightly condensed nucleoprotamine, any spacer DNA between chromatin subunits may be sterically inaccessible.

The compact structure of nucleoprotamine is revealed when chromatin containing both nucleohistone and nucleoprotamine is sheared; only nucleohistone is solubilized into smaller fragments [11].

Trout testis provides an ideal system for the preparation and study of chromatin subunits. Testis cell suspensions can incorporate radioactively labelled precursors, and large amounts of nuclei free of cytoplasmic contaminants are readily prepared in TMKS buffer, without sedimentation through sucrose cushions. Furthermore, subunits can be concentrated from sucrose gradient fractions by precipitation with Mg^{++} or Ca^{++}, thus greatly facilitating their analysis.

Histone acetylation, phosphorylation and methylation have been extensively characterized in trout testis, and may be involved in the assembly and maintenance of chromatin structure [12]. The study of histone modifications in relation to isolated chromatin subunits will provide information on the role of these reactions in chromatin structure. Such studies are currently in progress.

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