Characterization of chick liver chromatin and analysis of its in vitro transcription products

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ABSTRACT

Carefully controlled preparation of chromatin from purified chick liver nuclei yielded over 50% native chromatin as shown by the analysis of the nucleosome pattern after micrococcal nuclease digestion. The size of DNA in this chromatin as analyzed on alkaline sucrose gradients varied from 10S to 19S, the majority being 14S. All endogenous RNA polymerases were represented in the chromatin preparation although to different extents: RNA polymerase I was the most and RNA polymerase II the least abundant. Initiation studies showed that endogenous RNA polymerase II was capable of initiating RNA chains during 5 min. Saturation of chromatin with purified homologous RNA polymerase II increased initiation to 10 min. The addition of heparin caused the RNA transcribed to be larger in size and of increased yield. Chromatin transcription with added purified RNA polymerase II in the presence of heparin produced RNA as large as 32S. A chromatin preparation of this kind would therefore be suitable to transcribe any eukaryotic gene in vitro provided additional homologous RNA polymerase II is used.

INTRODUCTION

Transcription of specific genes in chromatin followed by identification of the product has been previously reported by several laboratories¹⁻⁶. In only one instance² was a chromatin preparation obtained in which endogenous RNA polymerase activity was maintained in sufficient quantity to enable transcription of a specific gene. In all other instances E. coli polymerase was used as the transcribing enzyme. In none of the reports, however, were the chromatin preparations defined and the methods (except ref. 2) employed extensive shearing leading to a total loss of endogenous RNA polymerase activities. Such

chromatin does not resemble the normal in vivo state any longer.⁷ When the gene to be transcribed is of relatively small size, then shearing may not be harmful but with large size genes the risk of even a partial destruction by shearing⁸ is greatly enhanced. Our work is concerned with the specific transcription of such a large size gene, i.e. the vitellogenin gene. We have isolated extremely large messenger RNA of vitellogenin which is 2.3×10^6 daltons⁹. Since the gene is presumably at least the size of the messenger, chromatin DNA must be at least 2.3×10^6 daltons to enable in vitro transcription of the gene. It is evident that in our case the length of chromatin fibres is critical. Ideally, the chromatin should be preserved in its native state. However, native chromatin is a thick gelatinous stringy material which cannot be pipetted accurately, hence not defined chemically with precision. We have now worked out a method which yields a chromatin preparation largely native and yet pipettable which we currently employ to transcribe the vitellogenin gene in vitro. In this paper our method is being detailed and the resulting chromatin preparation is evaluated in terms of native state, size, endogenous RNA polymerase II activity, capacity to initiate RNA chains and analysis of its products of transcription. We shall show that with a rigorous methodology it is possible to obtain a defined chromatin which satisfies our requirements to a great extent and which therefore might lend itself to the faithful transcription of any eukaryotic gene.

MATERIALS

Unlabelled nucleotide triphosphates (ATP, CTP, UTP and GTP) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany), [$5\text{-}^3\text{H}$]cytidine 5'-triphosphate (spec.act. 17 Ci/mmol) and [$6\text{-}^3\text{H}$]thymidine (spec.act. 26 Ci/mmol) were from The Radiochemical Centre (Amersham, U.K.) and [$\gamma\text{-}^{32}\text{P}$]guanosine 5'-triphosphate (spec.act. 15-25 Ci/mmol) was from the New England Nuclear Corporation (Boston, Mass.). All other chemicals not mentioned were of analytical grade. All glassware was steri-

lized and all plastic ware was diethylpyrocarbonate-treated. White leghorn chicks were supplied locally. α -Amanitin and micrococcal nuclease were from Sigma Biochemicals.

METHODS

Preparation of chromatin

Chromatin was prepared from chick liver nuclei whose purification and properties have been described^{10,11}. Nuclei (equivalent to 5 mg DNA) were suspended in 2 ml of ice-cold buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5) according to Paul and Gilmour¹² and immediately centrifuged for 3 min at 700 x g in a Sorvall RC-5 centrifuge after which the supernatant solution was decanted. Exposure to this buffer must not exceed 3 min to prevent RNA polymerases detaching from chromatin. The pellet was suspended in 2 ml of ice-cold distilled water and quickly centrifuged as above. This step was repeated once. The resulting chromatin pellet was now gelatinous. It was resuspended in 2 ml of distilled water by very gentle mixing and passed slowly, i.e. using very light pressure, 5 times through an 18 gauge needle and twice through a 23 gauge needle using a 2 ml syringe. The DNA content was determined at $A_{260\text{ nm}}$ and the preparation was diluted with distilled water to 500 μg DNA/ml. The entire procedure should take no longer than 30 min and the chromatin must be used within the following 30 min to avoid loss of endogenous RNA polymerase activities. For establishing the DNA-RNA-protein ratio, separate aliquots were used. DNA was determined according to Burton¹³, RNA with the method of Fleck and Munro¹⁴ and proteins according to Ehresman *et al*¹⁵. Our preparation gave a DNA-RNA protein ratio of $1:0.16 \pm 0.01:1.44 \pm 0.12$ (means of 30 determinations $\pm$ S.D.). During chromatin preparations 50% of the nuclear proteins and 80% of the nuclear RNA were lost. This cannot be avoided².

Determination of chromatin structure by means of degradation with micrococcal nuclease

Chromatin and nuclei as a control were dispersed in 20 mM Tris-HCl, pH 7.8, 60 mM KCl, 15 mM NaCl, 1 mM CaCl_2 at a DNA

concentration of 250–300 $\mu\text{g/ml}$. Digestion was for 4 min at 37°C after addition of micrococcal nuclease (0.5 $\mu\text{g/ml}$). After phenol extraction DNA was concentrated by ethanol precipitation. Aliquots of 2 μg DNA were then electrophoresed in a 3 mm thick slab gel of 2% polyacrylamide–0.5% agarose at 60 V for 4 h. The DNA bands were visualized after staining with ethidiumbromide under UV light and scanned in a Beckman spectrophotometer^{7,16}.

Sizing of chromatin DNA

Chromatin was prepared in the described way from liver nuclei of a 200 g chick which 30 min prior to sacrifice had received 1 mCi [$6\text{-}^3\text{H}$]thymidine (spec. radioact. 26 Ci/mmol) intraperitoneally. In parallel, a non-radioactive chromatin preparation was made. Both were analyzed on an alkaline sucrose gradient after alkali digestion on the gradient as previously reported¹⁷. S values were calculated¹⁷ and molecular weights estimated according to Studier¹⁸.

RNA polymerase assay

Enzyme activities were determined by measuring the incorporation of [^3H]CTP into acid-precipitable material essentially as described by Kedinger *et al*¹⁹ and modified recently by us²⁰. Aliquots of chromatin containing 25 μg of DNA were incubated for 10 min at 30°C in a total volume of 0.25 ml consisting of 12% glycerol, 80 mM Tris-HCl, pH 7.9, 1 mM each of ATP, UTP and GTP, 0.2 mM CTP, 1 μCi [^3H]CTP, 0.1 M dithiothreitol and different concentrations of ions¹⁹ to satisfy the requirements of the individual RNA polymerases I, II and III. These were 10 mM Mg^{++} plus 50 μg α -amanitin for measuring polymerase I and 2 mM Mn^{++} plus 1 μg α -amanitin for measuring polymerase I + III together, polymerase III was then obtained by difference. Polymerase II was determined in the presence of 2 mM Mn^{++} plus 100 mM NH_4^+ and also by difference after measuring polymerase I + II together in the presence of 3 mM Mn^{++} plus 10 mM NH_4^+ + 1 μg α -amanitin.

Purification of RNA polymerase II

The details of the purification procedure have already been

published^{11,21}. Basically, it involved solubilization of the enzyme from purified chick liver nuclei by sonication, $(\text{NH}_4)_2\text{SO}_4$ precipitation and separation of RNA polymerase II from I and III by DEAE-cellulose chromatography. The enzyme was electrophoretically pure. To achieve increased transcription of our chromatin preparation, 40 μl of purified polymerase II was added to 25 μg of chromatin-DNA (saturation).

Analysis of RNA transcripts from polymerase II

Since our interest concerns only transcripts from RNA polymerase II the incubation conditions were optimized as follows: Aliquots of chromatin containing 25 μg DNA were incubated for 30 min at 30°C in a total volume of 0.1 ml consisting of 20 mM Tris-HCl, pH 7.9, 0.01 M dithiothreitol, 0.6 mM each of ATP, UTP and GTP, 0.05 mM CTP, 1 μCi [^3H]CTP, 2 mM Mn^{++} and 100 mM NH_4^+ and stopped with 10 volumes of 1% SDS in 10 mM EDTA. Isolation of the RNA synthesized in vitro after deproteinization by hot phenol-chloroform was according to Marzluff et al²². The RNA was analyzed on an exponential gradient of 5.1%-28.6% sucrose in buffer (0.1 M LiCl, 1 mM EDTA, 0.2% SDS and 10 mM Tris-HCl, pH 7.4) in a SW 60 rotor for 90 min at 60,000 rpm. Standards were 18S and 28S purified ribosomal RNA (a gift of Dr. G. Thomas) and purified 5 S yeast RNA. Gradients were collected by puncturing the bottom of the tubes. Of each fraction TCA-precipitable counts were determined.

For studying initiation of transcription, the incubation medium contained in addition 150 μCi of [$\gamma^{32}\text{P}$]GTP (spec.act. 23.9 Ci/mmol) omitting unlabelled GTP.

RESULTS

Chromatin contains nucleosomes

To investigate whether the structure of the chromatin was still intact after isolation, we digested the isolated chromatin with micrococcal nuclease and compared the released DNA fragments with DNA fragments obtained by incubation of whole nuclei under the same conditions. Although during the isolation

chromatin was passed through a narrow gauge needle, a large part of its structure remained intact in terms of leaving discrete DNA fragments after nuclease digestion (Fig. 1). Compared with nuclear digests (A), more interband staining was obtained with isolated chromatin (B). This interband fluorescence also revealed on the scans represents less intact parts of chromatin, probably due to the isolation procedure. Although chromatin and nuclei were digested under identical conditions, isolated chromatin was digested faster than chromatin in intact nuclei. Photographs of the gels were scanned using a writer equipped with an integrator. For both nuclear and chromatin digests, the portion of the DNA in the bands was determined. Taking nuclear digests as 100% native chromatin, it could be calculated that more than 50% of the DNA fragments of isolated chromatin were in the same band positions as DNA fragments from nuclear digests. Therefore, we conservatively estimate that at least 50% of the isolated chromatin has an intact structure.

Chromatin consists of large size DNA

Fig. 2 shows the results of chromatin DNA sized on an alkaline sucrose gradient. DNA varied from 10S (0.5×10^6 daltons to 19S (2.5×10^6 daltons) the majority being of 14S (1.1×10^6 daltons) size.

Fig. 1

Micrococcal nuclease digestion patterns of chick liver nuclei and chromatin. Digestion of nuclei (A) and chromatin (B) was done as described in the method section. DNA ($2 \mu\text{g/slot}$) was analyzed on a polyacrylamide-agarose slab gel, stained with ethidium bromide under UV light, scanned and photographed.

Fig. 2

Sedimentation profile of DNA in alkaline sucrose gradients.

0.5 ml of chromatin solution ($\sim 10 \mu\text{g}$ of DNA) labelled with ^3H was mixed to a lysis solution on top of a 5-20% alkaline sucrose gradient. Centrifugation was for 25 h at 20,000 rev/min at 20°C in a SW 27 rotor. Fractions were collected from the bottom of the tube. One fraction covers a range of 1.5 S. Calibration of the gradient was as described.

Chromatin contains endogenous RNA polymerase I, II and III activities

We tested the individual RNA polymerase I, II and III activities in 8 different chromatin preparations as described in the method section. The amounts of pmoles of [^3H]CTP incorporated into RNA obtained for each polymerase were added and taken as 100% (Table I). A repartition of the enzyme activities shows that there was 2-3 times more RNA polymerase I present than RNA polymerase II or III.

Since we are interested in the transcription of the specific messenger RNA for the vitellogenin gene, we studied only chromatin transcription by RNA polymerase II in the following experiments.

Initiation of RNA chains

For studying initiation chromatin transcription was carried out with [^3H]CTP as well as with [$\gamma\text{-}^{32}\text{P}$]GTP. Fig. 3 shows that the endogenous RNA polymerase II was capable of initiating (and maybe reinitiating) for 5 min.

Fig. 4 shows that initiation could be extended to 10 min if chromatin was saturated with additional purified RNA polymerase II. The insert shows 4 min pulse-labellings with

Table I. Endogenous RNA polymerase activities of chick liver chromatin

	Per cent $\pm$ S.D.
RNA polymerase I	58 $\pm$ 12
RNA polymerase II	18.6 $\pm$ 10
RNA polymerase III	23.2 $\pm$ 4

Fig. 3

Initiation of RNA synthesis on chromatin by endogenous RNA polymerase II. The incubation conditions for RNA synthesis using [$\gamma^{32}\text{P}$]GTP and [^3H]CTP are mentioned in the experimental section. 20 μg of chromatin DNA were used, triplicates were done for each time point when acid-precipitable counts were determined.

[$\gamma^{32}\text{P}$]GTP and demonstrates that the rate of pppGp formation falls to zero within 10 min.

To prove that the [$\gamma^{32}\text{P}$]GTP was indeed used to initiate RNA chains and not to phosphorylate proteins, the transcription product was deproteinized by hot phenol-chloroform extraction and the RNA purified as described in the experimental section. The purified product was then analyzed on sucrose gradient for size (Fig. 5) and revealed RNA chains from 5 S to larger than 28 S. Another aliquot of the purified product after digestion with alkaline phosphatase produced mostly acid-precipitable ^3H counts and the majority of the ^{32}P counts in the supernatant

Fig. 4

Initiation of RNA synthesis on chromatin saturated with homologous purified RNA polymerase II. The experimental conditions were as for Fig.3, except that to chromatin (20 μ g DNA) a previously determined amount of homologous purified RNA polymerase II was added. Insert: The labelled GTP was added as 4 min pulses while the labelled CTP was present from time 0 on. Rates of [γ ³²P]GTP incorporation and total counts of [γ ³²P]GTP incorporation are plotted as function of time of incubation. Note that the rates of GTP incorporation fall to negligible values within 10 min in correspondence to the large graph.

Fig. 5

Analysis of RNA transcripts.

The experimental conditions were as for Fig. 3, but scaled up 15-fold. After phenol extraction and ethanol precipitation, the RNA was dissolved in 0.2 ml H₂O and placed on top of the sucrose gradient as described in the method section. Ribosomal RNA (28S and 18S) and yeast RNA (5S) were used as markers.

solution (results not shown). These results prove that initiation took place.

Sizing of transcribed RNA

Since RNA transcribed from endogenous RNA polymerase II in our chromatin preparation was quantitatively speaking mostly of 5S size (Fig. 5), the transcriptional system needed improving in order to obtain larger size transcripts. A major problem, the breakdown of RNA due to RNases can be overcome by the well known use of heparin. In Fig. 6 we show that heparin addition (insert: dose-response curve from which 25 μg heparin per μg DNA was taken as optimum) increased the acid-precipitable counts from chromatin (endogenous RNA polymerase II activity) 5 times within a 30 min incubation period. Similar increases were obtained when chromatin was first saturated with purified RNA polymerase II (results not shown), here the RNA transcribed within 30 min was mostly 5S - 20S, but larger than 32S was also made (Fig. 7).

DISCUSSION

Faithful transcription of a eukaryotic gene involves correct strand selection, correct initiation, termination and pro-

Fig. 6

Effect of heparin on RNA synthesis.
 Insert: To chromatin (20 μg DNA) increasing amounts of heparin were added and the endogenous RNA polymerase II activity was determined after 30 min incubation. Heparin in a concentration of 25 $\mu\text{g}/\mu\text{g}$ DNA was considered optimum and was therefore used in the experiment shown in the large graph.

cessing of the RNA made. In vitro, it is quite possible to have most of these conditions fulfilled if nuclei are used². With chromatin, a number of problems arise. Most chromatin preparations involve high speed centrifugations and extensive mechanical shearing, they are time consuming and provide at best templates to be transcribed by exogenous RNA polymerases due to loss of the endogenous enzymes. The complexity may have suffered to the point where incorrect reading occurs. Harsh shearing methods cause also nicking of DNA⁷ which would make initiation studies meaningless. In this context we feel that Reeder's^{23,24} interpretation of incorrect sequence reading of xenopus chromatin 5S and rRNA by exogenous purified RNA polymerase I and II is probably a misjudgement. Extensive shearing of the chromatin preparation was more likely the underlying cause.

Now that the structure of chromatin is becoming understood²⁵⁻²⁹, it should be a major concern to preserve the structure for the study of its function, yet we were unable to find from an extensive literature search a chromatin preparation of optimum function with its structure intact. The best preparation

Fig. 7

Analysis of RNA transcripts after the use of heparin.

RNA produced during a 30 min incubation by the endogenous RNA polymerase II of chromatin (4 mg DNA) in the absence of heparin (○—○) and RNA produced by saturating amounts of purified RNA polymerase II added to chromatin (2 mg DNA) in the presence of 50 mg heparin (●—●) were analyzed on sucrose gradients as in Fig. 5.

(in terms of template activity and product size) made by gentle lysing of rat liver nuclei³⁰ when used with chick liver nuclei produced a chromatin preparation inferior to the one obtained with our method. The preservation of the endogenous RNA polymerases seems to remain problematic and may vary with the method as well as with the tissue. Thus, de Pomerai *et al*³⁰ who used rat liver chromatin and Cox³¹ who worked with chick oviduct chromatin reported partially preserved activity of RNA polymerase II, but Huang² using mouse myeloma chromatin partially preserved activities I and III. Percentages of the preserved activities were not given. Our method of chromatin preparation preserved mostly RNA polymerase I (Table I). Of the total nuclear RNA polymerase activities, 55% were recovered in chromatin. This figure was obtained after determining the RNA polymerase I, II and III activities in a given amount of nuclei and then repeating these enzyme assays at each step during the chromatin preparation in the supernatant fluids. Of the 45% of RNA polymerases lost most was RNA polymerase II. This enzyme seems to be less firmly bound to chromatin in case of chick liver. For this reason we added purified enzyme in saturating amounts to obtain increased transcription. Initiation could nevertheless be effectively studied with our chromatin preparation and we demonstrated that the endogenous RNA polymerase II (Fig. 3) was capable of initiating RNA for 5 min, with additional purified enzyme for 10 min (Fig. 4). Since the size of the RNA transcripts was smaller than anticipated, we suspected breakdown of RNA by RNases contained inevitably in chromatin preparations. While RNA transcripts became indeed larger after the use of an RNase inhibitor such as heparin, initiation was most likely reduced to a minimum as we were not able to measure it anymore in the presence of heparin.

Adding both saturating amounts of purified polymerase II and heparin to our chromatin, RNA transcripts extended beyond the size of 28S RNA. This result shows that there was no limitation to the size of RNA obtained. We therefore feel that our method of chromatin preparation, which results in chromatin

with intact nucleosomes, large size DNA and only 45% loss of endogenous polymerases represents a significant improvement over previous preparations. Our preparation is also capable of endogenous transcription and must therefore represent a better preparation close to the natural in vivo state. We believe that our method represents an improvement for selecting chromatin for whole gene studies and that it would lend itself to the in vitro studies of any eukaryotic genes.

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