SUCCESSFUL SEPARATION OF THE TRANSCRIPTIONALLY-ACTIVE CHROMATIN FRACTION FROM PIGEON RETICULOCYTES AND CALF THYMUS

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1. Introduction

Numerous procedures have been described for isolating transcriptionally-active portions of chromatin (reviewed [1]). The validity of most of these procedures has been called in question because hybridization experiments failed to demonstrate any enrichment of transcribed sequences in the DNA of putative active chromatin over bulk chromatin [2–7]. With few exceptions [5–6] only those fractionation procedures proved to be successful according to the hybridization criterion which included mild nuclease digestion as a means of chromatin fragmentation [8–11]. Alternatively, those procedures which dealt with mechanically sheared chromatin gave mainly negative results [2–5], also with some exceptions [12,13]. It is now generally believed that shearing ruins any attempt to isolate a template-active fraction of chromatin. In this report evidence is presented that a fractionation scheme based on differential solubility of sonicated chromatin under physiological ionic conditions yields a minor chromatin fraction enriched in transcribed DNA sequences. This is true for both highly specialized (pigeon reticulocytes) and unspecialized (calf thymocytes) cells.

2. Experimental

Erythrocytes were isolated from circulating blood of pigeons. For reticulocyte isolation, the blood was collected from the pigeons with anaemia induced by 7 daily phenylhydrazine injections. The cells were washed in reticulocyte standard buffer (RSB) (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4) 0.1 mM phenylmethylsulphonylfluoride). Cell lysis was effected by homogenization in RSB containing 0.5% Nonidet P40. The nuclei were washed several times in 100 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5) (buffer A). Sonication of the nuclei was done as in [14]. Isolation and sonication of calf thymus nuclei was done as in [14,15].

Nick-translation of isolated calf thymus nuclei in the presence of d[α-^{32}P]ATP and [α-^{32}P]TTP (Amersham, 2000–3000 Ci/mmol and 400 Ci/mmol, respectively) was performed according to [16], except that *Micrococcus luteus* DNA polymerase I was substituted for *Escherichia coli* DNA polymerase I. Pigeon erythrocyte globin c[^{3}H]DNA was synthesized by Dr L. Yu. Frolova by reversed transcription from globin mRNA.

DNA was isolated by repeated phenol extractions. Simultaneous DNA denaturation, its M_r reduction and RNA hydrolysis were accomplished according to [6].

Hybridization of chromatin DNA with globin c[^{3}H]DNA or ^{32}P-labeled nick-translated calf thymus nuclear DNA was performed in 0.24 M Na-phosphate buffer (pH 7), 0.1% SDS, 1 mM EDTA at 67°C in flame-sealed glass capillary tubes. After the desired C_0t values were reached the capillaries were emptied into 100 vol. 0.15 M NaCl, 30 mM Na-acetate (pH 4.5), 0.1 mM $ZnSO_4$ and the duplexes were challenged by S1 nuclease. S1 nuclease (24 IU/ml, Calbiochem) was added to 0.5 IU/ml and the samples were incubated for 3 h at 42°C prior to precipitation with 5% trichloroacetic acid. The precipitates were collected on membrane filters and counted in an SL-30 liquid scintillation counter.

DNase I digestion of chromatin was performed in buffer A at 10 μg enzyme/ml at room temperature. For kinetic measurements aliquots of the digestion

mixture were treated with 5% $HClO_4$ and the acid-soluble material was determined by the diphenylamine reaction and expressed as % of the input DNA.

3. Results and discussion

3.1. *Chromatin fractionation*

The fractionation scheme is rather simple. The chromatin is fragmented by sonication under ionic conditions of minimal chromatin solubility and the soluble fraction (further referred to as fraction S) is separated from the insoluble bulk by low-speed centrifugation. Fig.1 shows the time course of chromatin solubilization during sonication of the nuclei in buffer A. The yield of DNA in fraction S from pigeon erythrocytes reaches ~6% after the longest sonication time shown in fig.1. About 12% of the material is solubilized from calf thymus nuclei in this time. About twice as much material is solubilized upon sonication of pigeon and calf liver chromatin (not shown). Thus, it can be seen that the % of DNA which is found in fraction S roughly correlates with the transcriptional activity of the tissue which served as a source of chromatin.

3.2. *Enrichment of DNA from pigeon reticulocyte chromatin fraction S in globin-coding sequences*

The concentration of globin nucleotide sequences was measured in total chromatin DNA and fraction S DNA by DNA-globin c[^{3}H]DNA hybridization under conditions of moderate cDNA excess. The extent of the annealing reaction under saturation conditions was determined (fig.2). Of the cDNA 12% annealed to the DNA of total sonicated chromatin at saturation. In contrast, 55% of the globin cDNA formed hybrids with fraction S DNA under identical conditions. Application of the equations deduced in [17] to these data suggests 9-fold enrichment of globin sequences in S fraction DNA over total chromatin DNA. This is probably an underestimate because of the reduced reactivity of fraction S DNA due to its lower M_r as compared with total DNA [14]. No such enrichment is seen with chromatin fraction S from pigeon liver, a tissue not engaged in globin production.

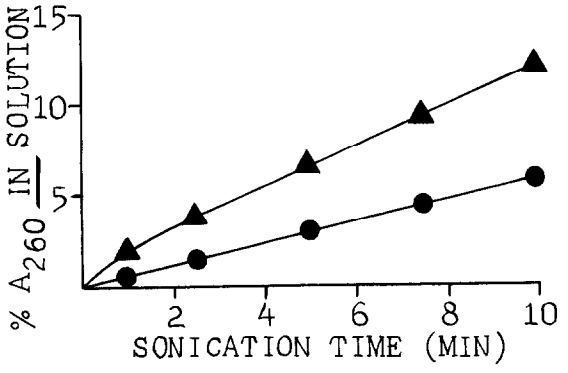

Fig.1. Kinetics of chromatin solubilization upon sonication. The nuclei (1.5 ml at 3.5 mg nuclear DNA/ml in buffer A) were sonicated in a tube oscillator of the ultrasonic disintegrator UZDN-1 (USSR) at a current of 0.3 A: (—●—) pigeon erythrocyte nuclei; (—▲—) calf thymus nuclei.

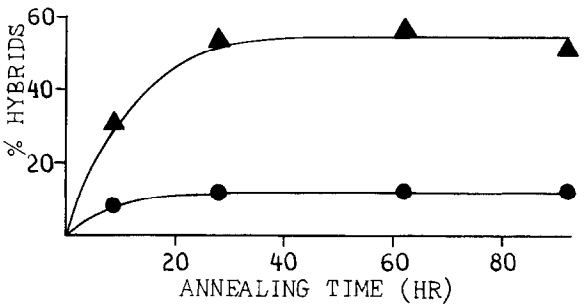

Fig.2. Time course of annealing of globin c[^{3}H]DNA to DNA from pigeon reticulocyte chromatin. For each sample constant amounts of DNA (5 μg) and cDNA (500 cpm at 4×10^6 cpm/μg) were taken and mixed in 10 μl. Experimental conditions for hybridization reaction and monitoring of hybrid formation are in section 2: (—●—) DNA from unfractionated chromatin; (—▲—) DNA from chromatin fraction S.

3.3. *Distribution of transcribed sequences in fractionated calf thymus chromatin*

Section 3.2 describes the successful application of the suggested fractionation procedure to the chromatin of highly specialized cells. It must be realized, however, that this is a simplified case as compared to the infinitely more complex situation characteristic of unspecialized tissues. Here, calf thymus was used as a representative of such tissues. A hybridization probe for the transcribed sequences in calf thymus DNA was prepared by the method in [16]. It consists in selective labeling of transcribed DNA by nick-translation of isolated nuclei. The specificity of labeling with respect to transcribed DNA is based on preferential susceptibility of this DNA to nicking by DNase I prior to label incorporation via DNA polymerase. This hybridization probe seems preferable to poly(A)-containing cytoplasmic mRNA labeled in vitro since

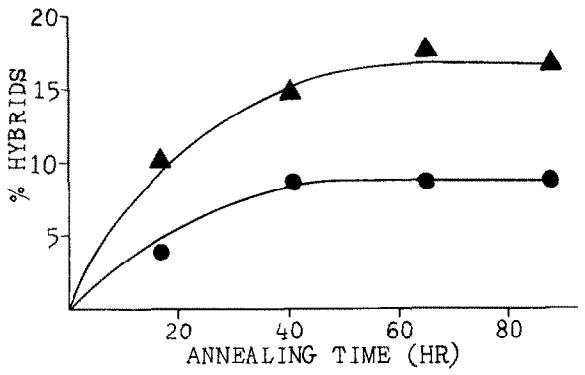

Fig.3. Time course of annealing of ^{32}P-labeled nick-translated nuclear DNA to DNA from calf thymus chromatin. DNA (80 μg) and 500 cpm nick-translated DNA were mixed in 30 μl. For details see section 2: (–●–) DNA from unfractionated chromatin; (–▲–) DNA from chromatin fraction S.

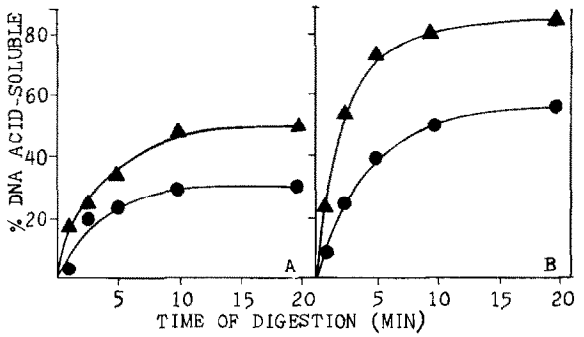

Fig.4. Kinetics of DNase I digestion of pigeon erythrocyte and calf thymus chromatin. Total sonicated chromatin and fraction S chromatin at 100 μg DNA/ml were digested by 10 μg/ml of DNase I in buffer A at room temperature. Aliquots were withdrawn and treated with 5% $HClO_4$. Acid-soluble DNA was determined by the diphenylamine reaction: (A) pigeon erythrocytes; (B) calf thymus; (–●–) total sonicated chromatin; (–▲–) fraction S chromatin.

it represents the whole set of transcribed sequences, whereas the poly(A)-containing cytoplasmic mRNA represents only a subset of transcribed sequences which are translated into proteins after mRNA processing especially splicing.

^{32}P-Labeled nick-translated calf thymus nuclear DNA (~500 cpm/μg) was annealed to fraction S DNA and DNA from total sonicated chromatin. The hybridization kinetics is shown in fig.3. The annealing reaction of nick-translated [^{32}P]DNA to the total DNA of sonicated chromatin attains a saturation value of 8.5%. The saturation value for fraction S DNA is 16%, i.e., this fraction is ~2-fold enriched in transcribed sequences [17].

3.4. *Increased susceptibility of fraction S chromatin to DNase I*

It is well established that the DNA of transcribed chromatin is preferentially digested by DNase I [18,19]. If chromatin fraction S obtained by the procedure outlined here, originates from transcribed regions of the genome, it can be predicted that this fraction will be digested by DNase I more readily than total chromatin. Indeed, as is seen from fig.4, both the initial rate and the extent of digestion are significantly higher for fraction S than for total sonicated chromatin. To rule out the possibility that the observed difference is due to preferential solubility of S chromatin in buffer A the same control experiment was done as in [20]. If the solubility of chromatin fraction S in buffer A is the single cause of its enhanced sensitivity to DNase then this fraction is expected to be more sensitive also to other nucleases, e.g., staphylococcal nuclease. The experiments reveal that this is not the case. Fraction S and total sonicated chromatin are digested by staphylococcal nuclease at the same rate under ionic conditions employed (not shown).

Thus, DNase I digestion studies further confirm the conclusion that the salt-soluble chromatin fractions from both tissues tested are derived from the transcribed regions of the genome.

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