Subnuclear Fractionation by Mild Micrococcal-Nuclease Treatment of Nuclei of Different Transcriptional Activities Causes a Partition of Expressed and Non-Expressed Genes

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Extremely mild treatment with micrococcal nuclease of isolated nuclei yields subnuclear fractions in which the majority of RNA polymerase II transcriptional complexes formed *in vivo* are segregated [Tata & Baker (1978) *J. Mol. Biol.* **118**, 249–272]. We now describe different approaches followed to establish whether or not the nuclei are thus resolved into transcribed and non-transcribed DNA. First, we have compared the sensitivity to deoxyribonuclease I, which is known to digest preferably expressed genes as present in nuclei or chromatin, of three micrococcal-nuclease-derived fractions from nuclei of different transcriptional activities. In transcriptionally active nuclei (rat liver, hen liver and oviduct, and *Xenopus* liver), the DNA in a polynucleosomal fraction comprising 6–15% of DNA and the majority of template-engaged RNA polymerase II (fraction P₂) was 10–50 times as sensitive to deoxyribonuclease I as the DNA in the other two fractions (fractions P₁ and S, comprising 78–88% of total nuclear DNA as large polynucleosomal aggregates and 2–6% of DNA mostly as mononucleosomes, respectively). In transcriptionally inactive nuclei obtained from hen erythrocytes, micrococcal nuclease did not separate DNA into fractions exhibiting such differential sensitivities. Second, we have monitored the partition of an expressed gene. Hybridization of complementary DNA to *Xenopus* albumin mRNA revealed a 5–10-fold enrichment of the albumin (but not the globin) gene in the P₂ fraction of nuclei from *Xenopus* liver in which this gene is fully expressed. Third, a large part of the nascent rapidly labelled RNA synthesized *in vivo* in rat liver nuclei was recovered in the micrococcal-nuclease-derived fraction that is more susceptible to digestion with deoxyribonuclease I. It is concluded that mild micrococcal-nuclease treatment of nuclei causes their separation into transcribed and non-transcribed DNA as determined by a number of very different criteria.

Although progress has been rapid in unravelling the basic structure of the nucleosome, with its invariable 140 b.p. of DNA in the core particle and the variable 20–60 b.p. of the linker DNA, relatively little is known about how the nucleosomal arrays would be organized into active and inactive genes within the nucleus (Kornberg, 1977; Felsenfeld, 1978; see Botchan & Watson, 1978). Biochemical and electron-microscopical data have suggested a heterogeneity of nucleosomes themselves or in the

internucleosomal arrangements which may be related to the transcriptional status of the DNA contained in them (Gottesfeld *et al.*, 1975; Foe *et al.*, 1976; McKnight & Miller, 1976; Gottesfeld & Butler, 1977; Franke *et al.*, 1978; Scheer, 1978; Botchan & Watson, 1978). Much of the knowledge on nucleosome structure and its organization into chromatin has been made possible by the use of various nucleases as probes, in particular micrococcal or staphylococcal nuclease, DNAases I and II and even restriction endonucleases (Noll, 1974*a,b*; Igo-Kemenes *et al.*, 1977; Kornberg, 1977; Lutter, 1977, 1978; Botchan & Watson, 1978; Felsenfeld, 1978; Sollner-Webb *et al.*, 1978). Preparative

Abbreviations used: DNAase, deoxyribonuclease; RNAase, ribonuclease; cDNA, complementary DNA; b.p., base-pairs.

methods have also been described for the separation of transcribed from non-transcribed chromatin by treating nuclei or chromatin with these enzymes (Gottesfeld *et al.*, 1976; see Gottesfeld, 1977; Hendrick *et al.*, 1977; Igo-Kemenes *et al.*, 1977; Bloom & Anderson, 1978; Levy & Dixon, 1978; Tata & Baker, 1978).

In an attempt to retain as far as possible the intranuclear configuration of polynucleosomal structures engaged or not in transcription, we have adopted the retention in polynucleosomes of endogenous RNA polymerase as transcriptional complexes as an index of separating transcriptionally active and inactive compartments of the nucleus (Tata & Baker, 1974*a,b*, 1978). On the basis of this stringent criterion, we have found that brief exposure of isolated nuclei to extremely low amounts of micrococcal nuclease offers the mildest possible procedure for resolving active from inactive fractions of nuclei, as judged by their transcriptional activity *in vitro* (Tata & Baker, 1978). The next step was to examine subnuclear fractions derived from a variety of cells with differing transcriptional activities in which the overall distribution of total expressed and unexpressed genes, as well as well-defined transcribed genes, are studied with hybridization probes. Towards this end, we have exploited the important discovery that DNAase I preferentially digests expressed genes (Berkovitz & Doty, 1975; Garel & Axel, 1976; Weintraub & Groudine, 1976; Flint & Weintraub, 1977; Garel *et al.*, 1977; Gottesfeld & Butler, 1977; Botchan & Watson, 1978) in order to establish whether or not the DNA in subnuclear fractions, separated by mild micrococcal-nuclease treatment of nuclei, exhibited differential sensitivity to DNAase I. Experiments were also designed to determine whether an expressed gene was unequally distributed in such subnuclear fractions and whether nascent RNA was non-randomly partitioned.

In this paper, we describe experiments in which three subnuclear fractions, obtained by mild micrococcal-nuclease digestion of nuclei from transcriptionally active (rat, hen and *Xenopus* liver, hen oviduct) cells, were compared with those from inactive (hen erythrocytes) cells for their sensitivity to DNAase I. In transcriptionally active nuclei, DNA in the fraction containing most of the RNA polymerase II as transcriptional complexes (fraction P₂) was 10–50 times as sensitive to DNAase I as the DNA in the fraction impoverished in endogenous RNA polymerase II transcriptional unit (fraction P₁); in transcriptionally inactive nuclei, micrococcal nuclease does not separate DNA into fractions exhibiting similar differential sensitivities to DNAase I. cDNA–DNA hybridization analysis revealed that the gene coding for albumin in *Xenopus* liver was substantially enriched

in fraction P₂. Most of the nascent nuclear RNA in rat liver was also recovered in the subnuclear fraction P₂.

Materials and Methods

Preparation of nuclei

Rat liver. Liver nuclei from Sprague–Dawley rats (200–220 g), bred at the National Institute for Medical Research, were prepared in a sucrose medium by the method of Blobel & Potter (1966). The nuclei from a batch of 18 g of liver were suspended in 20 ml of TKNaM buffer [50 mM-Tris/HCl (pH 7.6)/12.5 mM-KCl/12.5 mM-NaCl/5 mM-MgCl₂] and the suspension was vortex-mixed after addition of Triton X-100 to a final concentration of 0.1%. The nuclei were centrifuged at 600 g for 10 min and washed with 10 ml of TKNaM medium containing 0.1% Triton X-100, and then three times with detergent-free TKNaM buffer. The procedure was carried out at 2°C.

Xenopus liver. Nuclei from livers of adult male *Xenopus laevis* (purchased from the South African Snake Farm, Fishoek, South Africa) were prepared by a procedure at –20°C in a glycerol medium (Tata & Baker, 1975). The nuclear pellet was suspended in the glycerol medium containing 0.2% Triton, centrifuged at 1200 g for 10 min and the pellet was washed once with glycerol medium containing 0.1% Triton and three times with glycerol medium free of Triton.

Hen liver. Nuclei from liver, oviduct and erythrocytes were prepared simultaneously from the same Rhode Island Red laying hens from the National Institute for Medical Research Farm. Minced liver (20–30 g) was homogenized at 4°C in 2.5 vol. of 0.25 M-sucrose in TKNaM medium by using a Teflon/glass Potter–Elvehjem homogenizer. After filtration through 110-mesh nylon bolting cloth, the homogenate was diluted 1:1 (v/v) with 0.25 M-sucrose in TKNaM medium and centrifuged at 900 g for 10 min. The pellet was suspended in 60 ml of 0.25 M-sucrose in TKNaM medium containing 0.2% Triton and homogenized again as described above. To the homogenate 100 ml of 2.0 M-sucrose in TKNaM medium was added; 25 ml portions were placed into SW27 tubes and underlayered with 10 ml of 1.65 M-sucrose in TKNaM medium before centrifugation in a Beckman SW27 rotor at 27000 rev./min at 4°C for 1 h. The nuclear pellets were washed once with 0.25 M-sucrose containing 1 mM-MgCl₂ and 0.1% Triton and three times with 0.25 M-sucrose containing 1 mM-MgCl₂ only.

Hen oviduct. The oviduct was removed, slit longitudinally, and the tubular-gland cells were scraped off with a microscope slide and homogenized at 4°C with four strokes in a Teflon/glass homogenizer in 5 vol. of 0.25 M-sucrose in TKNaM

medium. The suspension was dispersed for 1 min in an Ultra-Turrax disintegrator and further homogenized with ten strokes in the Potter/Elvehjem homogenizer. The homogenate was filtered through two layers of bolting cloth and centrifuged at 1200 *g* for 10 min. The pellet was suspended in 0.25 M-sucrose in TKNaM medium containing 0.2% Triton, and 2 vol. of 2.0 M-sucrose in TKNaM medium were added. Further centrifugation and washes with Triton were performed as for chicken liver nuclei.

Hen erythrocytes. Erythrocyte nuclei were prepared in a glycerol/saponin medium as described by Hentschel & Tata (1978). The nuclear pellet was washed once with the glycerol medium without saponin and twice with 0.25 M-sucrose/1 mM-MgCl₂, in a volume equivalent to that of blood, followed by centrifugation at 800 *g* for 10 min.

Fractionation of nuclei by mild micrococcal-nuclease digestion

Nuclei from the different tissues were resolved into three components with different transcriptional characteristics by a procedure based on very limited digestion with extremely small amounts of micrococcal nuclease, as described previously (Tata & Baker, 1978). It has been our experience that each batch of commercially obtained micrococcal nuclease exhibits different efficiencies of nuclear breakage, and therefore produces variable sub-nuclear fractionation. Also, nuclei from different tissues exhibit different sensitivities to the same batch of micrococcal nuclease. For these reasons, optimum incubation conditions had to be determined for nuclei from different sources by monitoring complete (>95%) nuclear breakage microscopically and arresting further nuclease digestion with EGTA so that less than 5% of DNA (except for *Xenopus* liver nuclei) appeared in 105 000 *g* supernatant (fraction S), as described by Tata & Baker (1978). The final nuclear pellet was suspended in micrococcal-nuclease digestion buffer [10 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/1 mM-dithiothreitol/8% (v/v) glycerol/0.25 mM-CaCl₂] at a concentration of 3–5 mg of DNA/ml, usually determined by a nuclear count in a light microscope or by light scattering in a spectrophotometer at 550 nm, and incubated at 25°C for various periods of time and amount of enzyme, according to the source of nuclei. For nuclei from rat, *Xenopus* and chicken liver, incubation usually lasted 90 s at 0.2 unit of the enzyme/ml, and for chicken oviduct nuclei 90 s at 0.1 unit/ml of the enzyme, whereas chicken erythrocyte nuclei were incubated for 3 min at 0.35 unit/ml. Digestion was stopped by the addition of 2 mM-EGTA and chilling in ice. The suspension was centrifuged at 1200 *g* for 10 min to yield a low-speed pellet (fraction P₁) and a supernatant which was centrifuged in a Beckman

SW60 Ti rotor at 150 000 *g* at 4°C for 1 h to yield a second pellet (fraction P₂) and a further supernatant fraction (fraction S).

DNAase-I digestion of nuclei and subnuclear fractions

For pancreatic-DNAase-I-digestion experiments, the pellets of whole nuclei or fractions P₁ and P₂ were suspended in 50% (w/v) glycerol/10 mM-Tris/HCl (pH 7.4)/10 mM-NaCl/3 mM-MgCl₂ and the DNA content was determined on a sample by the Burton (1956) procedure. Dilutions of whole nuclei or fractions P₁ and P₂ at different DNA concentrations were made in the 50%-glycerol medium. Digestion was carried out at 37°C and its kinetics were expressed in terms of the amount of 260 nm-absorbing material solubilized in 0.5 M-HClO₄ (Noll & Kornberg, 1977).

Complementary DNA to Xenopus albumin and globin mRNA

Total cytoplasmic polyadenylated RNA was extracted from male *Xenopus* liver and a radioactive (labelled with [³H]dCTP) DNA complementary to total mRNA prepared as described by Smith *et al.* (1979). Since albumin mRNA is the most abundant messenger in male *Xenopus* liver (Farmer *et al.*, 1978), the cDNA for albumin was purified by back-hybridization with an excess of crude mRNA preparation to a very low R₀t value along the lines of the method described for enrichment of vitellogenin (Smith *et al.*, 1979) and albumin cDNA (Gordon *et al.*, 1978). The hybrid, which contained over 90% albumin cDNA, was treated with alkali to destroy the mRNA and the residual radioactive cDNA analysed by agarose-gel electrophoresis. ³H-labelled *Xenopus* globin cDNA was prepared by Dr. C. C. Hentschel (Imperial Cancer Research Fund) by reverse transcription of adult *Xenopus* globulin mRNA and characterized by hybrid-arrested translation assay (Hentschel *et al.*, 1979).

cDNA–DNA hybridization

DNA from *Xenopus* liver nuclei and their sub-fractions was extracted by the method of Gross-Bellard *et al.* (1973), the preparation being especially exhaustively treated with pancreatic RNAase followed by an additional cycle of deproteinization. The high-molecular-weight DNA was sheared in a VirTis disintegrator to a mean length of 600 b.p. (range 200–1200 b.p.) as described by Britten *et al.* (1974). Hybridization with [³H]cDNA to *Xenopus* albumin and globulin mRNA was carried out in 1 mM-Tris/HCl (pH 7.9)/0.2 mM-EDTA/0.4 M-NaCl at 69°C under paraffin oil, at ratios (w/w) of DNA:cDNA of 2 × 10⁶–8 × 10⁶. The extent of hybrid formation was monitored by S₁-nuclease digestion and the kinetics of

hybridization were expressed both as the usual saturation analysis and as initial rates of reaction as described by Garel & Axel (1976).

Labelling of RNA *in vivo*

Rats (120 ± 10 g) were injected intraperitoneally with 300–500 μ Ci of [$5\text{-}^3\text{H}$]orotic acid (15 Ci/mmol) at intervals between 7 and 100 min before killing. Nuclei and subnuclear fractions were prepared from livers pooled from three rats per batch. The procedure for determining specific radioactivity of RNA has been described by Tata & Baker (1974b). For determining the radioactivity associated with nascent RNA in polynucleosomes (fraction P_2) after sucrose-density-gradient fractionation, six-drop fractions were collected while the A_{260} was being automatically recorded in a Gilford 240 spectrophotometer fitted with an automatic density-gradient-scanning device (Tata & Baker, 1978). The sucrose in the fractions was diluted with 2.0 ml of water containing 100 μ g of total yeast RNA as carrier and the RNA was precipitated with 5% trichloroacetic acid. The precipitates were washed twice with 5% trichloroacetic acid, trapped on Whatman GF/C glass-fibre filters and the radioactivity was measured in a toluene-based scintillation fluid in a Beckman LS 250 scintillation counter.

Gel-electrophoretic analysis of RNA

RNA labelled *in vivo* was extracted with phenol/sodium dodecyl sulphate as described by Tata & Baker (1975). In order to overcome problems of RNA aggregation, electrophoresis was carried out on 3.5% polyacrylamide gels in 99% (v/v) formamide (Duesberg & Vogt, 1973). The distribution of RNA was recorded in a Gilford 240 spectrophotometer fitted with a gel scanner before measurement of the radioactivity in 2 mm sections as described by Tata & Baker (1975).

Protein analysis and measurement of radioactivity

Protein determination and procedures for measurement of radioactivity were as described by Tata & Baker (1974a,b).

Materials

Micrococcal nuclease was obtained from Sigma (London) Chemical Co., London S.W.6, U.K.; DNAase I was from Worthington Corp., Freehold, NJ, U.S.A.; proteinase K and S_1 nuclease were from Boehringer Corp., Lewes, Sussex, U.K. [^3H]dCTP and [$5\text{-}^3\text{H}$]orotic acid were from The Radiochemical Centre, Amersham, Bucks., U.K. Avian-myeloblastosis-virus reverse transcriptase was kindly supplied by Dr. J. W. Beard, Life Sciences Inc., St. Petersburg, FL, U.S.A. All other reagents were of analytical grade and were purchased from Sigma or BDH, Poole, Dorset, U.K.

Results

Fractionation of nuclei

After treatment of nuclei with extremely low amounts of micrococcal nuclease for very brief periods of time, most of the DNA (80–90%) was recovered in all cases in the P_1 fraction, which is known from our previous studies to represent most of the nucleoli and transcriptionally inactive non-nucleolar DNA (Tata & Baker, 1978). Taking into account the varying amounts of micrococcal nuclease and times of incubation, the sensitivity to the enzyme, as judged by the release of DNA into P_2 and S fractions, is a function of relative transcriptional activity of different tissues (Table 1). This enzymic sensitivity can only be a rough guide to transcriptional status of nuclei of very divergent activities, but does not provide an accurate means of comparing transcriptional activities of nuclei from different organisms, as, for example, between rat, chicken and *Xenopus* nuclei. It was shown earlier (Tata & Baker, 1978) that fraction P_2 prepared in this way comprises nearly 85% of endogenous transcribing RNA polymerase II in large oligomeric polynucleosomal complexes, whereas much of the DNA in fraction S is degraded to about 200 b.p. Our next step was to test the

Table 1. Recovery of DNA in different subnuclear fractions obtained after treatment with micrococcal nuclease of nuclei from rat, *Xenopus* and chicken liver and chicken oviduct and erythrocytes

Nuclei were prepared from the different tissues as described in the text and treated with micrococcal nuclease as follows: liver nuclei, 0.2 unit/ml for 1.5 min; oviduct nuclei, 0.1 unit/ml for 1.5 min; erythrocyte nuclei, 0.3 unit/ml for 3.0 min. The different subnuclear fractions were separated and DNA contents measured as described by Burton (1956).

Source of nuclei	Subnuclear fraction	DNA recovered (% of total)
Rat liver	P_1	88.3
	P_2	8.8
	S	2.5
<i>Xenopus</i> liver	P_1	78.5
	P_2	8.1
	S	12.0
Chicken liver	P_1	78.5
	P_2	15.0
	S	5.9
Chicken oviduct	P_1	87.4
	P_2	6.6
	P_2	5.1
Chicken erythrocyte	P_1	93.4
	P_2	2.3
	S	3.1

sensitivity to DNAse I of each of the subnuclear fractions derived from different types of nuclei by mild and limited micrococcal-nuclease digestion.

Digestion of subnuclear fractions with DNAse I

Figs. 1 and 2 show the remarkable differences in the kinetics of DNAse-I digestion of the subnuclear fractions P_1 and P_2 isolated from different transcriptionally active nuclei (liver and oviduct) as a function of amount of enzyme and time of digestion, especially at low amounts of DNAse I (50–200 units/mg of DNA) or short times of incubation (5–10 min). Under these conditions the fraction P_2 was digested to a much greater extent than fraction P_1 , from which it is reasonable to conclude

that the 6–10% of total DNA recovered in fraction P_2 represents expressed non-nucleolar genes. The variability of results with fraction S may arise from the facts that the DNA in this fraction is already highly degraded and may be derived to variable extents from both P_1 and P_2 fractions as a result of micrococcal-nuclease treatment of nuclei.

Quite different results were, however, observed when similar experiments were carried out with the transcriptionally inactive nuclei from chicken erythrocytes (Fig. 3). Not only did more vigorous micrococcal-nuclease digestion produce less DNA in the P_2 fraction, but it did not exhibit a higher sensitivity to DNAse I than did DNA in fraction P_1 . Indeed, in some experiments (Fig. 3*b*), the DNA in the latter fraction was more sensitive to digestion.

Fig. 1. Different sensitivities to DNAse I of subnuclear fractions obtained by micrococcal nuclease treatment of nuclei from (a) rat liver, (b) *Xenopus* liver, (c) hen liver and (d) hen oviduct, as a function of amount of DNAse I added. Nuclei from the different tissues were incubated for 1.5–2.5 min with 0.1 or 0.2 unit of micrococcal nuclease (Table 1) and separated into fractions P_1 (●), P_2 (■) and S (▲), as described in the Materials and Methods section. Each subnuclear fraction, corresponding to 40–100 μ g of DNA measured by the method of Burton (1956), was incubated in a total volume of 0.1 ml with different amounts of DNAse I, as indicated, for 5 min at 37°C. Digestion of DNA was expressed as acid-soluble material released as described by Noll & Kornberg (1977).

Fig. 2. Different sensitivities to DNAase I of subnuclear fractions obtained by mild micrococcal nuclease treatment of nuclei from (a) rat liver, (b) *Xenopus* liver, (c) hen liver and (d) hen oviduct, as a function of time of incubation

The three subnuclear fractions, P₁ (●), P₂ (■) and S (▲), were obtained and treated with DNAase I, as indicated in Fig. 1. The samples were incubated with 44 units (a and b), 250 units (c) or 150 units (d) of DNAase I/mg of DNA, for the different periods of time indicated. The kinetics of digestion of DNA were expressed as the fraction of DNA solubilized. Other details are described in the text.

This finding is in accord with the conclusion that fraction P₂ in transcriptionally inactive nuclei may be derived from fraction P₁ as a result of the harsher enzymic treatment of erythrocyte nuclei with micrococcal nuclease that is required to disrupt these nuclei.

Enrichment of expressed gene in fraction P₂

In order to draw a definitive conclusion that the higher sensitivity of the fraction P₂ to DNAase I reflected a selective fractionation of expressed and unexpressed genes, it was essential to demonstrate an enrichment in fractions P₂ and P₁ of specific gene sequences that are or are not actively transcribed in a given cell. Towards this end, we followed by cDNA–DNA hybridization kinetics the distribution of sequences coding for *Xenopus* albumin and globin in the two subnuclear fractions from *Xenopus* liver.

Albumin is perhaps the most actively transcribed gene in adult male *Xenopus* hepatocytes, whereas there is virtually no globin-gene transcription in the majority of non-erythroid cells (Thomas & Maclean, 1975; Farmer *et al.*, 1978). Fig. 4 summarizes the results of titration of DNA from liver P₁ and P₂ fractions against cDNA to *Xenopus* albumin and globin mRNA species, which are depicted both in the conventional log C₀t plot (Fig. 4a) and as a linear representation of the kinetics at early times of hybridization (Fig. 4b). An enrichment of 5–10-fold of the albumin gene was thus recorded in fraction P₂ relative to fraction P₁. A similar enrichment was found when cDNA molecules to *Xenopus* vitellogenin (a protein synthesized exclusively in the liver) mRNA as well as to total poly(A)-containing RNA from liver were used as probes (J. R. Tata, B. S. Baker & J. V. Deeley, unpublished work). As also shown in Fig. 4, a similar enrichment

Fig. 3. Accessibility to DNAase I of DNA in subnuclear fractions P₁, P₂ and S derived from hen erythrocyte nuclei, expressed (a) as a function of time of incubation and (b) as a function of amount of enzyme

Hen erythrocyte nuclei were treated with 0.6 unit of micrococcal nuclease/mg of DNA for 2.5 min in order to resolve them into fractions P₁ (●), P₂ (■) and S (▲), as described in the text. In the experiments shown in (a), each fraction, corresponding to 50 μ g of DNA, was incubated with 15 units of DNAase I for the different periods of time indicated. In the experiments in (b), the same amount of the different fractions was incubated for a fixed time of 5 min with the different amounts of DNAase I indicated. Other details are as described in the text and Figs. 1 and 2.

Fig. 4. Kinetics of hybridization of *Xenopus* albumin and globin cDNA with DNA and P₁ and P₂ fractions derived from *Xenopus* liver nuclei

DNA was extracted from subnuclear fractions P₁ (Δ, ○) and P₂ (▲, ●) and sheared to an average size of 600 b.p. Annealing reactions were carried out under conditions described by Garel & Axel (1976) with 30 μ g of DNA and 1000 c.p.m. of [³H]CTP-labelled albumin (▲, Δ) or globin (●, ○) cDNA (approx. 10⁷ c.p.m./ μ g) at a ratio of cDNA to DNA of 2 × 10⁶–8 × 10⁶ in 2 μ l duplicate samples removed at each time interval. The reaction of input cDNA as duplex was measured by digestion of one half of the reaction mixture with S₁ nuclease. All other procedures were as described by Garel & Axel (1976). (a) Conventional log C₀t plot; (b) kinetics at early times of hybridization (H = fraction of cDNA hybridized).

was not observed with *Xenopus* globin cDNA, which hybridized less readily to DNA from P₂ than from P₁ fractions. Thus it can be concluded that limited treatment of nuclei with micrococcal nuclease causes a substantial, but not complete, separation of expressed and unexpressed genes.

Distribution of newly synthesised RNA in vivo in subnuclear fractions

If the subnuclear fraction P₂ is enriched in expressed genes, it would be expected, but not obligatory, that this fraction P₂ retains some of the nascent or rapidly labelled nuclear RNA synthesized

Table 2. *Distribution of RNA labelled in vivo in subnuclear fractions*

RNA was labelled with 330 μ Ci of [3 H]orotic acid injected 8 and 100 min before death to each of a group of three rats. Nuclei were prepared from the pooled livers and organelles from 15g equivalent of tissue resolved into subnuclear fractions P_1 , P_2 and S. Each value is the mean of four determinations, with variation within $\pm 10\%$.

Fraction	Duration of labelling (min)	$10^{-3} \times$ Total [3 H]RNA recovered (c.p.m.)	Specific radioactivity (c.p.m./ μ g of RNA)
Nuclei	8	40.2	17.1
P_1		16.1	13.7
P_2		7.6	44.8
S		10.4	46.9
Nuclei	100	461.0	189.3
P_1		301.5	164.6
P_2		60.6	361.1
S		108.1	445.3

in vivo. We therefore studied the subnuclear distribution of rapidly labelled RNA in rat liver nuclei. Table 2 shows that, with an 8 min pulse of [3 H]orotic acid, the specific radioactivity of RNA labelled *in vivo* was 3–4 times higher in fractions P_2 and S than in fraction P_1 . This result would be expected if polynucleosomes in fraction P_2 are preferentially derived from euchromatin or expressed genes (Frenster, 1974; Tata & Baker, 1974b, 1975). With longer pulses the difference in specific radioactivity of RNA between the fractions tended to diminish, presumably a reflection of rRNA synthesis building up in the nucleoli-enriched fraction P_1 with time.

On sucrose-density-gradient centrifugation of the fraction P_2 , some of the newly synthesized RNA co-sedimented with the large polynucleosomal aggregates accumulating at the interface with the 2.0M-sucrose cushion (results not shown), although a significant amount of radioactive RNA of polynucleosomes migrated in the mononucleosome–pentanucleosome region. Whether or not this radioactive RNA component is bound to nucleosome particles is not certain, and the possibility cannot be ruled out that the RNA may be present as discrete messenger ribonucleoprotein particles. The size distribution of the RNA molecules from the P_2 and S fractions on deproteinization was in any case quite different. Fig. 5 shows that on polyacrylamide-gel analysis under denaturing conditions the bulk of labelled RNA in fraction S was 4–7S in size, much of it presumably representing degradation products of larger species. The RNA from fraction P_2 was heterodisperse, ranging from 6S to 50S in size (most of it 12–35S), and would be expected to represent nascent non-ribosomal RNA (Tata & Baker, 1978). If some of this RNA is still attached to the chromatin–RNA polymerase complex, whether as messenger ribonucleoprotein particles or not, then it further supports the idea that polynucleosomes in fraction P_2 , as isolated by

Fig. 5. Polyacrylamide-gel electrophoresis in 99% formamide of nascent RNA labelled *in vivo* associated with fractions P_2 and S

RNA was labelled *in vivo* with a 10 min pulse of 450 μ Ci of [3 H]orotic acid injected to each of three rats. Nuclei from pooled livers were fractionated by the standard procedure by using 0.2 unit of micrococcal nuclease/ml for 90 s and the P_2 and S fractions were separated. RNA was extracted by the hot-phenol/SDS procedure. Samples corresponding to 6g (P_2) (●) and 3g (S) (○) of tissue were loaded on 3.5% polyacrylamide disc gels in 99% formamide and electrophoresed as described by Duesberg & Vogt (1973). The distribution of RNA was recorded in a Gilford spectrophotometer fitted with a gel scanner before measurement of the radioactivity in 2 mm slices as described by Tata & Baker (1975). Arrows indicate the migration of rat liver 28S and 18S rRNA and 4S tRNA as markers.

us, represent transcriptional units as they existed *in vivo*.

Discussion

There is now firm evidence that expressed genes are more rapidly digested by DNAase I added to

nuclei or chromatin from cells in which they are actively transcribed than are genes that are not expressed (Garel & Axel, 1976; Weintraub & Groudine, 1976; Flint & Weintraub, 1977; Garel *et al.*, 1977; Panet & Cedar, 1977; Miller *et al.*, 1978). In our earlier studies, disruption of rat liver nuclei by brief exposure to extremely small amounts of micrococcal nuclease permitted a separation by differential centrifugation of the bulk of heterochromatin and nucleoli (fraction P_1) from about 10% of DNA, which carried 85% of RNA polymerase II transcriptional complexes (fraction P_2) and was considered to be transcribed chromatin (Tata & Baker, 1978). What was not established was whether or not such a distribution was typical of nuclei from other transcriptionally active cells similarly treated with micrococcal nuclease, or whether transcriptionally inactive nuclei would behave differently. Also, it was not demonstrated that DNA in the active and inactive components was in a different transcriptional configuration, as regards sensitivity to DNAase I, nor whether a specific expressed gene would be non-randomly distributed. The experiments reported in this paper have resolved these uncertainties to a large extent.

The recovery of a buoyant fraction of 5–12% of nuclear DNA in fraction P_2 , as summarized in Table 1, turns out to be a characteristic of several transcriptionally active cells, i.e. rat liver, *Xenopus* liver and chicken liver and oviduct. The much smaller amount of DNA that was recovered as fraction P_2 from the transcriptionally inactive nuclei of adult hen erythrocytes resulted from the more extensive digestion with micrococcal nuclease needed to obtain this fraction (results not shown), which also caused an accumulation in the supernatant (fraction S) of monomeric nucleosomes and degraded DNA released from fraction P_1 . Similar results (J. R. Tata & B. S. Baker, unpublished work) have been obtained with adult *Xenopus* erythrocyte nuclei. Although laying-hen liver and oviduct would be expected to be transcriptionally extremely active (because of the intensive synthesis of vitellogenin and ovalbumin, respectively, in these tissues), it is difficult to establish a quantitative relationship between the absolute rate of transcription *in vivo* and the recovery of subnuclear fraction (P_2) active in transcription *in vitro*, on the basis of the six types of nuclei that we have studied. In this context, Garel *et al.* (1977) observed no difference in the DNAase-I sensitivity of genes that are transcribed at extremely different rates, so that the maintenance of an active conformation around specific genes does not represent RNA polymerase distribution. Miller *et al.* (1978), studying the globin gene in nuclei of induced and uninduced mouse erythroleukaemia cells, also concluded that the conformation of specific genes as reflected by

their DNAase-I sensitivity is necessary to permit transcription of these genes, but the rate of transcription must depend on other factors.

The results in Figs. 1 and 2 reveal a most dramatic difference in the DNAase-I sensitivities of DNA in the three subnuclear fractions from transcriptionally active cells. At low enzyme concentrations, DNA in P_2 fraction from such nuclei is digested 10–50 times faster than that in the P_1 fraction. On the other hand, with transcriptionally inactive avian erythrocyte nuclei, not only is there little DNA appearing in the P_2 fraction but the small amount of such DNA is equally, or even less, sensitive to DNAase I as the bulk of the heterochromatic DNA (Table 1, Fig. 3). Gottesfeld & Butler (1977), using the DNAase-II/Mg²⁺ solubility procedure to fractionate chromatin, also found that the 'active' fraction was much more sensitive to DNAase I than was total chromatin. The difference that we observe is considerably greater than that reported in other studies for either individual (globin, ovalbumin and viral) or unspecified genes that are transcribed or not (Weintraub & Groudine, 1976; Flint & Weintraub, 1977; Garel *et al.*, 1977; Chae *et al.*, 1978; Miller *et al.*, 1978). The reason for the magnitude of the differences that we observe in DNAase-I sensitivity between the 'active' and 'inactive' fractions must lie in the fact that the template-active and -inactive compartments are tested separately, whereas the total nuclear or chromatin DNA was digested in the other studies. Since the differential rate of DNAase-I digestion is a quantitative difference and the bulk of the DNA is in the transcriptionally inactive configuration, the net effect of digesting total DNA would be to give an underestimate of the extreme sensitivity of transcribed genes to DNAase I.

The results in Fig. 4 of the hybridization analysis of DNA from P_1 and P_2 fractions with cDNA probes corresponding to a gene known to be expressed (albumin) or not expressed (globin) in adult *Xenopus* liver further establish that mild micrococcal-nuclease treatment of nuclei causes a non-random separation of active and inactive genes. The differential distribution of the albumin gene in the two subnuclear fractions is not an all-or-none phenomenon, but is comparable with the extent of separation achieved by other workers using micrococcal or other nucleases under different conditions (Gottesfeld *et al.*, 1976; Gottesfeld, 1977; Bellard *et al.*, 1978; Bloom & Anderson, 1978). The less-marked separation of globin gene in P_1 and P_2 fractions may result from the presence of a small number of active erythroid cells in amphibian liver. It is also noteworthy that, with more extensive digestion of nuclei with micrococcal nuclease than used by us, Bellard *et al.* (1978) observed that the ovalbumin gene was released into monomeric

nucleosomes more readily from chicken oviduct nuclei, in which the gene is actively expressed, than from erythrocyte nuclei, in which it is not. We did not carry out more extensive digestion of nuclei with micrococcal nuclease in order to allow only the minimal release of DNA from the P_2 fraction to the non-sedimentable fraction S (Tata & Baker, 1978). In any event, our hybridization data confirm that the differential DNAase-I digestibility of different subnuclear fractions reflects the non-random distribution of expressed and unexpressed genes by the micrococcal nuclease procedure used by us to fractionate nuclei.

From our previous findings on the distribution of actively transcribing RNA polymerase II complexes (Tata & Baker, 1978) and the present findings of differential DNAase-I sensitivities, one would expect to recover the bulk of the nascent RNA in the P_2 fraction. It is therefore significant that in rat liver the bulk, but not all, of rapidly labelled nuclear RNA co-sedimented with the P_2 fraction (Table 2). Gel-electrophoretic analysis (Fig. 5) showed the heterodisperse nature of rapidly labelled RNA associated with the P_2 fraction, which would be expected for non-ribosomal RNA synthesized on extranucleolar chromatin (Darnell *et al.*, 1973), and the presence of degraded RNA (most of it $<7S$) in the supernatant is compatible with the earlier suggestion made by us (Tata & Baker, 1974b, 1975) from analysis of labelling kinetics that much of the rapidly labelled RNA in nuclear sap is part of the RNA that turns over rapidly within the nucleus. Indirect evidence from different approaches also suggests some non-random association of non-nucleolar nascent RNA, even polyadenylated, with fractionated chromatin (Tata & Baker, 1974b, 1975; Berkovitz & Doty, 1975; Gottesfeld, 1977; Gottesfeld & Butler, 1977). More recently, using a similar mild micrococcal-nuclease-digestion procedure as used by us in this work, Djondjurov *et al.* (1979) have also found an enrichment of heterogeneous nuclear RNA (hnRNA) in a fraction corresponding to our fraction P_2 after fractionation of chromatin from Friend erythroleukaemic cells and Chinese-hamster fibroblasts.

The mechanisms underlying the fractionation of nuclei by mild micrococcal-nuclease treatment and the subsequent behaviour of the subnuclear fractions with DNAase I remain unknown. However, there is much evidence confirming that the two nucleases attack DNA, as organized in nuclei or whole chromatin, in entirely different ways (Noll, 1974a,b; Kornberg, 1977; Lutter, 1977, 1978; Axel, 1978; Felsenfeld, 1978; Rill *et al.*, 1978; Sollner-Webb *et al.*, 1978). It is also relevant to note that, by analysing restriction-enzyme-cleavage patterns of fragments generated by DNAase I and micrococcal nuclease, Wu *et al.* (1979) found that

both types of nucleases cleave chromatin at position-specific sites. There seems to be no gross difference in the nucleosome size or polynucleosomal arrangement between active and inactive chromatin fractions (Oudet *et al.*, 1975, 1978; Bostock *et al.*, 1976; Kornberg, 1977; Tata & Baker, 1978; Rill *et al.*, 1978), so that the differential sensitivities to these nucleases must reside in some subtle aspect of chromosome organization such as the length of linker DNA between nucleosomes, and variations or post-translational modifications in histones and non-histone chromosomal proteins. Indeed, much interest has been recently expressed in the possibility that the post-translational modifications, particularly phosphorylation and acetylation, of not only the core histones but also histone H1 and non-histone proteins, including 'high-mobility-group' (HMG) proteins, may enhance the transcriptional potential of the genome and render it more accessible to nucleases (Marushige, 1976; Allfrey, 1977; Botchan & Watson, 1978; Cole *et al.*, 1978; Davie & Candido, 1978; Goodwin *et al.*, 1978; Mathews & Bradbury, 1978; Mathis *et al.*, 1978; Neumann *et al.*, 1978; Ord & Stocken, 1978; Sealey & Chalkley, 1978; Simpson, 1978; Vidali *et al.*, 1978; Weisbrod & Weintraub, 1979). Another report describes a 40% deficiency in histone H1 in a fraction corresponding to P_2 from Friend erythroleukaemia and Chinese-hamster fibroblasts (Djondjurov *et al.*, 1979); it would be interesting to determine if a selective deficiency in one of the variants was responsible for this finding.

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