Transcription of Fractionated Mammalian Chromatin by Mammalian Ribonucleic Acid Polymerase

DEMONSTRATION OF TEMPERATURE-DEPENDENT RIFAMPICIN-RESISTANT INITIATION SITES IN EUCHROMATIN DEOXYRIBONUCLEIC ACID

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The chromatin fractionation method of Frenster *et al.* (1963) as modified by Leake *et al.* (1972) was used to prepare fragments of euchromatin from rat liver nuclei. These remain soluble in 5mM-MgCl₂, and contain DNA of maximum mol. wt. 1×10^6 – 2×10^6 . The fragments were separated from condensable chromatin on a sucrose gradient. Euchromatin contains endogenous DNA-dependent RNA polymerase, and most of the nascent RNA labelled *in vivo* or *in vitro*. Euchromatin fragments allow initiation of transcription by added purified rat liver form-B RNA polymerase and contain temperature-dependent rifampicin-resistant initiation sites for the form-B enzyme. These findings indicate that transcription of the euchromatin regions of interphase chromosomes is not initiated in condensed chromatin, but is initiated within the euchromatin stretches. Condensable chromatin also contains most of these activities, but is not associated with nascent RNA.

Butterworth *et al.* (1971) showed that of the mammalian DNA-dependent RNA polymerase enzymes (EC 2.7.7.6) only one, the form B, can initiate RNA synthesis on chromatin. Since that report, considerable interest has been aroused by the speculative models of the eucaryotic transcriptional unit and its control mechanisms proposed by Georgiev *et al.* (1971), Crick (1971) and Paul (1972). These models are based on the idea that the initial transcript of the eucaryotic complementation group, giant heterogeneous RNA (HnRNA) contains one small region of actual mRNA coded by a polypeptide-specifying structural gene with the remainder being a non-protein-coding transcript of either redundant DNA or regulatory genes. The Georgiev *et al.* (1971) proposal is explicit in placing the structural gene transcript at the 3'-end of the HnRNA molecule. An important point raised by the models concerns the location of the initial binding site (promoter) for the RNA polymerase. Is the promoter located in the euchromatin or in the condensed region of the chromosome? To answer this question we have carried out transcription studies on fractionated chromatin.

Attempts to fractionate chromatin over the past 10 years have been moderately successful. The objective has been to separate that part of the chromatin which is involved in coding for RNA synthesis *in vivo*. The three stages of chromatin fractionation are as

follows: (1) cleavage of the chromosome fibres in such a way as to liberate the active cistrons; (2) separation of the active fragments from the rest; (3) experimental demonstration that (1) and (2) have been achieved. Perhaps the most spectacular success was achieved by McConaughy & McCarthy (1972) when they showed that the RNA found in chick erythrocytes would hybridize to only the low-temperature-'melting' fraction of chick embryo erythrocyte chromatin DNA. Unfortunately the separated product is single-strand DNA and is therefore useless for transcription studies. The method of Marushige & Bonner (1971) involves cleavage of chromatin by DNAase* treatment, a technique likely to introduce artificial initiation sites. Fractionation has also been reported by Chalkley & Jensen (1968) with calf thymus, Simpson & Reeck (1973) with rabbit liver, and Murphy *et al.* (1973) with mouse myeloma cells. These all involve extreme shearing treatments with a VirTis homogenizer or in a French pressure cell. It seems likely that these methods produce damaged template DNA in the fragments. Possibly the most interesting technique of chromatin fractionation so far devised is the most recent from McCarthy's laboratory (Janowski *et al.*, 1972). The fragments are separated

* Abbreviation: DNAase, deoxyribonuclease; RNAase, ribonuclease; HnRNA, heterogeneous nuclear RNA.

by gel filtration on an agarose column. The active fragments, which are eluted first, contain nascent RNA and will selectively bind ^3H -labelled RNA polymerase from *Escherichia coli*. However, again cleavage in a French pressure cell is used.

Because of the problems associated with these techniques we have reinvestigated that of Frenster *et al.* (1963) in which non-condensed euchromatin is isolated after a short sonication. Euchromatin from calf thymus nuclei was shown to contain nascent RNA and, unlike the condensed heterochromatin, to have a diffuse appearance under the electron microscope. We used this method, as modified by Leake *et al.* (1972) for rat liver nuclei, for the preparation of uncondensed chromatin fragments, and determined whether or not these contained initiation sites for the homologous DNA-dependent RNA polymerase.

Materials and Methods

Chemicals, enzymes and other materials were obtained or prepared as described previously (Butterworth *et al.*, 1971; Chesterton & Butterworth, 1971a; Chesterton *et al.*, 1972).

Preparation and fractionation of chromatin

Liver nuclei were prepared as before (Chesterton & Butterworth, 1971a) from one or two Wistar rats (300–400 g), i.e. from about 15 or 30 g of liver. The procedure described below is based on that of Leake *et al.* (1972), and it contains no wash stage in order to minimize loss of important proteins and factors from the chromatin. Also, no pelleting by centrifugation is used since we believe that resuspension from such pellets could damage or alter essential structural features. The nuclear pellet was resuspended in 10 ml of 0.25 M-sucrose containing 10 mM-Tes [*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid], pH 7.4 (sucrose-Tes buffer) and sonicated at 0°C for 10 s at maximum power with the medium probe of the Dawes Sonicator to lyse the nuclei and release uncondensed regions of the chromatin. The sonicate was layered on to a 50 ml sucrose gradient (15–50%) in 10 mM-Tes buffer, pH 7.4, and centrifuged at 50 000 g (20 000 rev./min) for 15 h in a 3 × 65 ml MSE swing-out rotor at 4°C. A hole was pierced just above the pellet and fractions (2.5 ml) were collected. The pellet was homogenized with 10 ml of sucrose-Tes buffer for DNA and protein analysis. Enzyme studies were not carried out with this fraction. DNA was extracted from condensable or soluble chromatin fragments with chloroform-phenol-sodium dodecyl sulphate as described by Bishop (1972). Protein and DNA were determined by the methods of Lowry *et al.* (1951) and Burton (1956) respectively. Histone and non-histone protein were measured as described by Davison *et al.* (1954). DNA size was determined as described by Studier (1965)

with a computer program prepared by Dr. David Watkins (M.R.C. Institute, Hammersmith Hospital, London, U.K.).

Assay for condensed and soluble chromatin fragments

Leake *et al.* (1972) showed that chromatin fragments derived from condensed chromatin will contract in the presence of 5 mM-MgCl₂ and pellet under light centrifugation. Therefore to determine the proportion of condensable and euchromatin fragments in each fraction, 0.2 ml was diluted to 2 ml with sucrose-Tes buffer containing 0 or 5.6 mM-MgCl₂ and subsequently centrifuged at 1900 g (2500 rev./min) for 20 min at 4°C in a swing-out rotor of the MSE Mistral. The E_{260} of each supernatant was determined and corrected for turbidity by deducting the E_{320} (Leake *et al.*, 1972). The value for the 5 mM-MgCl₂ supernatant represents the non-condensable euchromatin DNA present, whereas the difference between the 0 and 5 mM-MgCl₂ supernatant represents the condensable chromatin DNA present.

DNA-dependent RNA polymerase assays

RNA polymerase assays involving chromatin as a template were carried out as described previously (Butterworth *et al.*, 1971). Form-B rat liver RNA polymerase was purified as described by Chesterton & Butterworth (1971c). Determination of RNA polymerase activity from rifampicin-resistant initiation sites was by the method of Butterworth *et al.* (1972) as described below. Form-B RNA polymerase, normally at saturating amounts (over 40 units/incubation), was allowed to bind to DNA for 10 min at either 0°C or 37°C. The incubation volume at this stage was 0.15 ml and contained enzyme, 0.3 µg of DNA, 0.05 M-Tris-HCl, pH 8, 0.05 M-(NH₄)₂SO₄, 1 mM-MgCl₂ and 1 mM-dithiothreitol. Rifampicin AF/013 and the nucleotide mixture (Butterworth *et al.*, 1971) were added simultaneously in 0.1 ml, and the total mixture was incubated for 1 min at 37°C. The final incubation contained 0.04 mM-[^3H]UTP (2 µCi), 0.3 mM-ATP, GTP and CTP, 0.05 M-Tris-HCl, pH 8, 0.13 M-(NH₄)₂SO₄, 1 mM-MnCl₂, 1 mM-dithiothreitol, 200 µg of bovine serum albumin/ml, enzyme, 0.3 µg of DNA and 75 µg of rifampicin AF/013 in a final volume of 0.25 ml. RNA was precipitated, extracted and counted for ^3H as described before (Chesterton & Butterworth, 1971a). In calculating the results, the activity from incubations preincubated at 0°C was subtracted from similar incubations preincubated at 37°C. Thus only transcription from temperature-dependent rifampicin-resistant binding sites was assayed.

Labelling of nascent RNA

To label nascent RNA *in vivo* 2 mCi of [^3H]uridine was injected subcutaneously into each rat (about 300 g in weight). After 30 min the liver was excised and

the nuclear chromatin fractionated as before. To label nascent RNA *in vitro*, the nuclear pellet from 10 g of liver was resuspended in 3 ml of 0.01 M-Tris-HCl buffer, pH 8, containing 0.05 M-(NH₄)₂SO₄, 0.3 M-sucrose, 1 mM-MnCl₂, 5 mM-MgCl₂, 0.1 mM-EDTA and 1 mM-dithiothreitol. A 0.8 ml portion of 0.125 M-Tris-HCl buffer, pH 8, containing 1.5 mM each of ATP, GTP and CTP and 0.2 mM-[³H]UTP (sp. radioactivity 200 mCi/mmol) was added. After incubation at 37°C for 10 min, the nuclei were spun down at 700 g for 10 min at 4°C, resuspended in 3 ml of 0.25 M-sucrose containing 0.01 M-Tes buffer, pH 7.4, and sonicated as before except that the MSE microprobe was used. Chromatin was fractionated as described above.

Results

Chromatin fractionation

Fig. 1(a) shows the distribution of euchromatin and condensable chromatin fragments in the sucrose gradient. The fragments are distinguished by treating a portion of each fraction with 5 mM-MgCl₂ and centrifuging lightly. Condensable fragments pellet whereas euchromatin fragments stay in solution. As would be expected, the condensable fragments are larger and sediment faster than the 50S marker. Soluble fragments sediment at about 30S. Approx. 60% of the total chromatin is pelleted. Most of the DNA present is contained in condensable chromatin (Fig. 1b). However, about 15% of the total is found in

Fig. 1. Fractionation of rat liver chromatin fragments in a sucrose gradient

Chromatin derived from 25 g of liver was fractionated and assayed as described in the Materials and Methods section. The fraction volume was 2.5 ml. (a) Distribution of condensable (●) and euchromatin (○) fragments. (b) Distribution of DNA (■) and protein (□) and the maximum size of DNA present (▲) in the gradient fractions. The markers were rat liver ribosomal subunits generated by EDTA-treatment of polyribosomes precipitated at pH 5 as described by Monneron *et al.* (1971). These were sedimented in a parallel gradient. Values for the pellet fraction (18 ml) were as follows: soluble fraction, $E_{260} = 0$; condensable fraction, $E_{260} = 9.16$; protein content = 49 mg and DNA content = 6.48 mg.

euchromatin, which compares with 20% reported for rat liver nuclei by Leake *et al.* (1972) and 12.5% in calf thymus described by Frenster *et al.* (1963). A considerable amount of protein, presumably not bound to DNA, stays at the top of the gradient. The average DNA:histone:non-histone protein ratios for pelleted condensable and euchromatin fragments were 1.0:1.0:1.6 and 1.0:3.2:6.8 respectively. The high values of protein in the latter fragments probably reflect the fact that the chromatin was not washed free of 'non-specifically' bound protein.

It is noteworthy that the size of the DNA present in the unpelleted chromatin rises from a maximum of 1×10^6 – 2×10^6 daltons in the euchromatin fraction to 5×10^6 – 12×10^6 daltons in the condensable fragments (Fig. 1b). This suggests that euchromatin DNA could specify a polypeptide of about 50000 to 100000 daltons. The value agrees quite well with Crick's calculations (Crick, 1971), which suggest that each uncondensed interband region of the eucaryotic chromosome codes for only one protein. With these values there is not enough DNA to code for the giant heterogeneous RNA, thought to be the precursor of mRNA (Imaizumi *et al.*, 1973), which is generally well above 10^6 daltons in size. However, since our material was derived by sonication, these calculations must be regarded as minimal estimates. We have

tested the effect of further sonication on condensable chromatin. The bottom five fractions from a gradient similar to that shown in Fig. 1 were resonicated as before and re-run on a second sucrose gradient. Release of further euchromatin was not detected. These results confirm those of Leake *et al.* (1972) in proving that the release of euchromatin is not an artifact of the sonication procedure.

Endogenous template activity of fractionated chromatin

The RNA-synthesizing activity of the fragments in the sucrose gradient was determined at a fixed DNA concentration/assay (Fig. 2). At $0.15\text{M}-(\text{NH}_4)_2\text{SO}_4$ activity is seen in all fractions. At the top of the gradient, a peak of RNA polymerase activity is present, which can largely be inhibited by rifampicin AF/013. This indicates that the enzyme here has not initiated transcription, and is probably not bound to the chromatin template. Rifampicin only inhibits free or non-specifically-bound RNA polymerase (Butterworth *et al.*, 1971). This finding was confirmed (Fig. 2) by repeating the assays at $0.3\text{M}-(\text{NH}_4)_2\text{SO}_4$, which does not affect RNA polymerase in the process of transcription, but prevents new initiations (Butterworth *et al.*, 1971). At this higher concentration of salt, the peak of activity is virtually eliminated without affecting the endogenous bound activity which remains in all rifampicin-resistant fractions. The latter activity must therefore be generated by RNA polymerase in the process of transcription. It should be noted that the activity seen in these fragments at 0.15M - and $0.3\text{M}-(\text{NH}_4)_2\text{SO}_4$ is approx. 30 and 10% respectively of that of whole chromatin under these conditions. This effect is presumably due to the smaller template size present. These results show that euchromatin and condensable chromatin contain endogenous RNA polymerase. Specific assays for form-A RNA polymerase activity (Chesterton & Butterworth, 1971b) showed this to be located mainly in the pellet. This indicates the position of nucleoli in the fractionation system.

Template activity for added RNA polymerase

With assays containing purified rat liver form-B DNA-dependent RNA polymerase (Chesterton & Butterworth, 1971c) and a constant amount of chromatin (in terms of DNA), virtually all fractions in a similar gradient to that described above gave equivalent activities after deduction of activity in the absence of added enzyme (Fig. 3). This shows that transcription can be initiated to an approximately equivalent extent on both the condensable and euchromatin fragments. We have shown previously that the form-A DNA-dependent RNA polymerases had little activity on a chromatin template (Butterworth *et al.*, 1971). In experiments not reported here, we have confirmed that result for both purified rat

Fig. 2. *Endogenous DNA-dependent RNA polymerase activity in fractionated chromatin*

The samples assayed were derived from the fractionation shown in the legend to Fig. 1. Portions of each fraction were diluted to $100\mu\text{g}$ of DNA/ml with sucrose-Tes buffer, and 0.1ml was assayed for 40 min at 37°C at either 0.15M - or $0.3\text{M}-(\text{NH}_4)_2\text{SO}_4$ with preincubation for 5 min at 37°C plus or minus $100\mu\text{g}$ of rifampicin AF/013. $\circ$, $0.15\text{M}-(\text{NH}_4)_2\text{SO}_4$, no rifampicin; $\bullet$, $0.15\text{M}-(\text{NH}_4)_2\text{SO}_4$, plus rifampicin; $\square$, $0.3\text{M}-(\text{NH}_4)_2\text{SO}_4$, no rifampicin; $\blacksquare$, $0.3\text{M}-(\text{NH}_4)_2\text{SO}_4$, plus rifampicin.

Fig. 3. Distribution of template activity of fractionated chromatin for added form-B RNA polymerase

(a) Distribution of condensable (●) and euchromatin (○) fragments. (b) Distribution of template activity of chromatin fragments for 50 units of added enzyme/incubation (■) compared with the distribution of RNA-synthesizing activity already present in the gradient (□). The former is corrected for the latter. The chromatin was derived from 30g of liver. Each 0.25 ml incubation contained 0.1 ml of fraction diluted to 250 μg of DNA/ml with sucrose-Tes buffer and was carried out at 0.15M-(NH_4)₂SO₄ for 40 min at 37°C.

liver AI and AII enzymes (Chesterton & Butterworth, 1971a) by using chromatin fragments separated as shown in Fig. 1. It should be noted that saturating enzyme amounts were not used, owing to the difficulties pointed out by Butterworth *et al.* (1971). Hence the activities seen do not represent the relative numbers of initiation points present.

Location of nascent RNA

The results reported so far do not demonstrate a functional difference between the condensable and euchromatin fragments. Since the euchromatin, from its properties, should be the site of synthesis of RNA *in vivo*, we investigated the location of nascent RNA. Nascent RNA was labelled for 30 min *in vivo* as described in the Materials and Methods section, and its distribution between euchromatin and condensable chromatin was determined (Fig. 4). The gradients

Fig. 4. Association of labelled nascent RNA chains with euchromatin fragments *in vivo*

Nuclei from the liver (13 g) of a rat, which had been pulsed for 30 min with 2 mCi of [³H]uridine (sp. radioactivity 29 Ci/mmol), were fractionated as described in the Materials and Methods section, except that the sucrose gradient was centrifuged for 15 h at 65 000 g. The sucrose gradient fractions were assayed for condensable chromatin (●); soluble or euchromatin (○); and total acid-precipitable radioactivity present, i.e. labelled RNA *in vivo* (▲). The pellet fraction not shown in the figure contained 440 c.p.m. representing 14% of the total recovered. Total recovery of RNA loaded was 105%.

Fig. 5. Association of labelled nascent RNA chains with euchromatin fragments *in vivo*

Nuclei from rat liver (12 g) were pulsed with [³H]UTP and fractionated as described in the Materials and Methods section, except that the sucrose gradient was centrifuged for 15 h at 65 000 g. The sucrose gradient fractions were assayed for condensable chromatin (●); soluble or euchromatin (○); and total acid-precipitable radioactivity present, i.e. labelled RNA *in vitro* (▲). The pellet fraction not shown in the figure contained 22 060 c.p.m. representing 17% of the total recovered. Total recovery of RNA loaded was 102%. A, top fraction; B, euchromatin fraction; C, condensable chromatin fraction.

used were centrifuged faster than normal to move the euchromatin clear of the top quarter of the system. The results show that virtually all of the labelled RNA *in vivo* is associated with the euchromatin fragments. About 40% of nascent RNA labelled *in vitro* showed a similar pattern (Fig. 5). Much of the euchromatin was obviously degraded during the 10 min labelling period at 37°C, and therefore must have released nascent RNA. Possibly the released RNA bonds non-specifically to the remaining condensable fragments.

Fig. 6. Size of labelled nascent RNA chains associated with the various fractions of chromatin fragments separated *in vitro*

The distribution of ^3H -labelled RNA, from the fractions A (a), B (b), C (c) and D (pellet) (d), derived as shown in Fig. 5, sedimented through 5–20% sucrose gradients in 99% dimethylsulphoxide containing 2 mM-UMP and 1 mM-LiCl is shown. The 6 ml gradients were centrifuged at 24°C for 24 h at 260 000 g (60 000 rev./min) in the 3 × 6.5 ml MSE titanium swing-out rotor. ^3H RNA was extracted by the procedure of Ruiz-Carrillo *et al.* (1973) in the presence of 0.25 mg of yeast RNA/fraction, 0.5% sodium dodecyl sulphate and 2 mM-UMP. Fresh rat liver ribosomal RNA (1 mg), extracted with phenol from rat liver polyribosomes precipitated at pH 5, was run in a parallel gradient. The E_{260} of the fractions was determined on the alcohol-precipitable material present. Recoveries of ^3H RNA were greater than 80%. ○, labelled ^3H RNA *in vitro*; ●, ribosomal RNA.

To check that the labelled RNA was bound to euchromatin fragments, portions of the peak fraction were re-run in similar gradients with or without 0.1% sodium dodecyl sulphate. In the sodium dodecyl sulphate-containing gradient, the labelled RNA sedimented only one-half the distance of that moved by the intact chromatin fragments in the absence of sodium dodecyl sulphate. The size of the labelled RNA in fractions A, B, C and D (Fig. 5), i.e. the top, euchromatin, condensable chromatin and pelleted condensable chromatin fractions respectively, was determined on a 5–20% (w/v) sucrose gradient in 99% (v/v) dimethyl sulphoxide as described in the legend to Fig. 5. All fractions contained heterogeneously sedimenting nascent RNA from 5S to above 28S. Also, if purified labelled RNA is added to nuclei before sonication and fractionation, no acid-precipitable labelled material was detected in the fractionated chromatin. This indicates that the nascent RNA is in some way protected from RNAase. These findings show that the nascent RNA must be attached to the chromatin fragments in an RNAase-resistant form.

The fact that euchromatin contains only 15–20% of the total DNA whereas it is associated with at least 80 and 40% of the labelled RNA *in vivo* and *in vitro* respectively, clearly demonstrates a functional difference between the condensable and euchromatin fragments. The latter, as expected, are derived from the areas of the chromosome containing the genes that are being actively expressed.

Distribution of rifampicin-resistant initiation sites

Fig. 3 shows that added form-B RNA polymerase initiated transcription of chromatin DNA in both condensable and euchromatin fragments. However, it does not show whether or not this initiation occurred at true promoter sites and, unfortunately, at our present level of knowledge it is impossible to find out unequivocally. Nevertheless there is some evidence which suggests that initiation from so-called rifampicin-resistant sites may resemble that which occurs *in vivo* for the bacterial DNA-dependent RNA polymerase on bacteriophage templates (Bautz & Bautz, 1970; Dausse *et al.*, 1972). At these sites RNA polymerase can effect a temperature-dependent tight binding reaction to DNA, which renders it insensitive to rifampicin inhibition. Recent work by Chambon and co-workers (Meilhac *et al.*, 1972; Meilhac & Chambon, 1973) and in our laboratories (Butterworth *et al.*, 1972) has shown that such sites can be demonstrated for the mammalian form-B enzymes by using rifampicin AF/013. As it has been shown that temperature-dependent rifampicin-resistant sites cannot form at single-strand nicks in the template with either bacterial (Bautz & Bautz, 1970) or the mammalian (Meilhac & Chambon, 1973; P. H. W. Butterworth, unpublished work) RNA polymerases,

it was of great interest to determine whether or not these sites exist in euchromatin. Unfortunately rifampicin experiments cannot be carried out with chromatin owing to the stimulation of endogenous RNA polymerase caused by this drug (Butterworth *et al.*, 1971). In the present paper DNA extracted from the fragments was tested.

Fig. 7 shows rifampicin-resistant-enzyme-saturation curves obtained with DNA extracted from condensable and euchromatin fragments. Incubations for 1 min were used to eliminate effects of differing

template size, and only activity dependent on a pre-incubation at 37°C is shown. The curves indicate that DNA from the euchromatin contains these sites in numbers only slightly lower than is found in the condensable chromatin. To show that the rifampicin-resistant binding sites are specific and are not a characteristic of any DNA template, bacteriophage T4 DNA was also tested (Fig. 7) at a 30-fold higher concentration than was used with the above templates. In the presence of the drug virtually no activity was detected. Bacteriophage T4 DNA does contain rifampicin-resistant sites for *E. coli* RNA polymerase. Repeat experiments with sonicated or sheared chromatin (Butterworth *et al.*, 1971) confirmed this result (Table 1).

Discussion

In their respective models, Crick (1971) and Paul (1972) attempt to relate the transcriptional unit to the banding pattern seen in the giant polytene chromosome of diptera mainly because there is now some evidence that one band-plus-interband region corresponds to a complementation group (Judd *et al.*, 1972). Both accept the evidence (DuPraw & Rae, 1966) that a band region consists of the DNA strand condensed into some kind of superhelical configuration contrasted with a more relaxed, open conformation in the interband region. Crick (1971) suggests that the promoter for the DNA-dependent RNA polymerase is located in the band region, i.e. in the condensed chromatin, as an area of denatured DNA. This could be stabilized in a 'twisted hairpin' conformation by histone cross-linkage. Conversely Paul (1972) believes that the interband region can be created from a band region by specific unfolding at an 'address' site mediated probably by a non-histone protein 'destabilizer'. This exposes the promoter. Further unfolding would then be an automatic consequence of the transcription process. In other words, whereas Crick (1971) postulates that RNA polymerase binds in the condensed band region at some distance from the structural gene, Paul (1972)

Fig. 7. Saturation curves for the rifampicin-resistant initiation sites on DNA from condensable and soluble chromatin fragments and on bacteriophage T4 DNA

The initial rate of transcription of DNA from condensable chromatin fragments, euchromatin fragments or bacteriophage T4 is shown at various concentrations of form-B RNA polymerase. After extraction and purification, DNA was used at 0.3 µg/incubation (0.25 ml) for chromatin DNA, and 10 µg/incubation for bacteriophage T4 DNA. With chromatin templates, incubations were carried out for 1 min at 37°C with a preincubation for 10 min at either 0°C or 37°C. Values from the former incubations were subtracted from the latter to yield the values shown. With bacteriophage T4 DNA the procedure was identical, except that all incubations were preincubated at 37°C. Absence of rifampicin allowed approximately a fourfold higher rate of transcription. Condensable fragment DNA (●); euchromatin fragment DNA (○); bacteriophage T4 DNA plus rifampicin (■).

Table 1. Distribution of activity from temperature-dependent rifampicin-resistant sites between condensable and euchromatin fragment DNA

The results shown represent the temperature-dependent rifampicin-resistant activity of saturating amounts of form-B RNA polymerase on DNA derived from the chromatin fragments indicated. Nos. 1-5 indicate separate repeat experiments and the mean ± s.d. is shown.

Fragment	Expt.	...	Radioactivity (c.p.m.)					Mean	After shearing
			After sonication						
			1	2	3	4	5		
Condensable			764	761	749	697	689	732 ± 33	748
Euchromatin			534	460	519	643	589	549 ± 62	581

suggests that it occurs in a 'normal' fashion within the open non-condensed interband chromatin.

If Crick (1971) is correct, it might be expected that euchromatin would be deficient in promoters for the RNA polymerase. Also it would be a poor template on which RNA polymerase could only initiate non-specifically. Our results show that: (a) euchromatin contains endogenous RNA polymerase activity; (b) euchromatin is transcribed by added homologous form-B RNA polymerase; (c) euchromatin is associated with newly synthesized RNA chains; and (d) euchromatin DNA contains a number of rifampicin-resistant initiation sites for homologous form-B RNA polymerase which, in relation to DNA content, is equivalent to that found in the condensable chromatin DNA. Subject to the reservations discussed below, these findings indicate that euchromatin, as defined by Frenster *et al.* (1963), is derived from the genetically active regions of the chromosomes and does contain promoters for homologous RNA polymerase. Thus there seems no necessity to invoke special condensed-region promoters.

It is reasonable to ask certain questions about the nature of the techniques and products that we have used. First, can we be sure that euchromatin represents that part of the chromosome which is active *in vivo*? Frenster *et al.* (1963) isolated euchromatin by a method essentially similar to that which we used. They showed with calf thymus that the euchromatin was enriched for the products of RNA synthesis. Under the electron microscope, their euchromatin consisted of diffuse fibres in contrast with the condensed appearance of the rest of the chromatin. Leake *et al.* (1972) confirmed this result for rat liver euchromatin isolated by the Frenster *et al.* (1963) method, showing it to consist of threads (25 nm diam.) in contrast with the clumped appearance of condensed chromatin. They also showed that, in contrast with the remainder of the chromatin, the euchromatin was not precipitated at low $MgCl_2$ concentrations. These workers found, as we did, that euchromatin fragments are not released by further sonication, and showed that inactive hen erythrocyte chromatin does not produce the euchromatin fraction. Thus euchromatin does not consist merely of small condensable fragments, but is a special and characteristic fraction. Also we have shown here that approx. 80 and 40% of labelled nascent RNA *in vivo* and *in vitro* respectively is contained in euchromatin. From these results it is clear that the euchromatin described in this report corresponds to the euchromatin isolated by the other two groups, and that this almost certainly is derived from the regions of the chromosomes active in RNA synthesis.

Secondly, do rifampicin-resistant binding sites correspond to promoters *in vivo* in this eucaryotic system? Riva *et al.* (1972) have shown that rifampicin AF/013 binding to form-B RNA polymerase is not

stoichiometric, and further that the inhibitor will bind to other proteins. However, Chambon and co-workers (Meilhac *et al.*, 1972; Meilhac & Chambon, 1973) and ourselves (Butterworth *et al.*, 1972) have found that the tight-binding reaction which renders form-B rifampicin resistant is dependent on temperature, suggesting that a specific 'melting' or denaturing of the template occurs. Also, it has been shown that the sites are specific for the form-B enzyme. Neither form-A nor bacterial RNA polymerase will bind at these sites, and sites for form-B RNA polymerase are not found in bacteriophage T4 DNA. In addition, Meilhac & Chambon (1973) and P. H. W. Butterworth (unpublished work) have shown that form-B does not bind in a temperature-dependent rifampicin-resistant conformation at single-strand nicks in the template. Bautz & Bautz (1970) obtained similar results with *E. coli* RNA polymerase and bacteriophage T4 DNA. Our results therefore cannot be explained by random nicking of the chromatin template. As suggested by Meilhac & Chambon (1973), it thus seems probable that rifampicin-resistant sites do correspond to promoters *in vivo*.

The finding of equivalent numbers of rifampicin-resistant initiation sites in condensed and euchromatin fragments is explained by the fact that the former must contain repressed transcription units. The presence of endogenous RNA polymerase activity in condensed chromatin was not expected, but is probably explained by contamination with euchromatin, by the exposure of promoter sites during sonication which could bind free RNA polymerase, or, more likely, by the removal of repressor proteins during sonication, which would allow promoter-bound RNA polymerase on a previously inactive gene to begin transcription. Also, some chromatin loses protein at the concentration of salt used in the RNA polymerase assay (Butterworth *et al.*, 1971), and it may become partially decondensed and allow substantial read-through by the enzyme. The equivalent template activity for added form-B RNA polymerase on both types of fragment probably has a similar explanation. Also, it must be remembered that saturating amounts of enzyme were not used as explained earlier. So the activity seen is not strictly proportional to the amount of available template.

Bearing these points in mind, what overall conclusion can be reached? Our results concur with the most likely possibility for the activation and structure of a eucaryotic transcriptional unit, which may be described as follows. Inactive transcriptional units are contained in condensed chromatin. Of course those in frequent use may be transiently repressed by environmental or metabolic changes. Activation of a transcriptional unit involves the relaxation of condensed chromatin to form a euchromatin or interband region, which contains its own promoter for the transcribing enzyme and probably pre-bound RNA poly-

merase. The first point is supported by the equivalence of rifampicin-resistant activity on DNA from condensable and euchromatin fragments. The final point is supported by the remainder of our data.

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