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Clustering of RNA polymerase B molecules in the 5' moiety of the adult β-globin gene of hen erythrocytes

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ABSTRACT

Nuclei were prepared from mature and immature hen erythrocytes and incubated for RNA synthesis in the absence or in the presence of Sarkosyl. The *in vitro* labelled synthesized RNA was hybridized to specific 5' and 3' fragments of the chicken adult β-globin gene to investigate the possible presence of RNA polymerase molecules bound to this gene in the form of transcriptional complexes. Surprisingly, such RNA polymerase B molecules were found located preferentially in the 5' end moiety of the β-globin genes of mature erythrocytes, although they are apparently evenly distributed along the β-globin genes of immature polychromatic erythrocytes. The significance of these observations with respect to (1) preferential DNaseI sensitivity of "genes which have been transcribed" and (2) control of transcription in eukaryotic cells is discussed.

INTRODUCTION.

The differentiating avian erythrocyte provides an interesting model for the study of programmed events which lead to the almost complete switch-off of DNA transcription (for refs., see 1 and 2). Since changes in chromatin organization are likely to be involved in these events, DNaseI digestion studies (3) have been performed to analyze the chromatin structure of the adult globin genes in mature chicken erythrocytes. It has been found that these genes are in active "open" configuration characterized by an increased sensitivity to digestion by DNaseI (3, 4). From such studies it has been proposed that transcription is not a prerequisite for the existence of an "active" chromatin configuration and more specifically that preferential digestion by DNaseI is typical not only of genes which are actively transcribed, but also of genes which

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have been transcribed (3, for review and additional refs., see 5). This conclusion rests on the assumption that the adult globin genes are no longer transcribed in the mature chicken erythrocytes. In the present study we have tested the validity of this assumption and found that, in fact, RNA polymerase B molecules are bound in the form of transcriptional complexes to the adult β-globin genes of mature and immature erythrocytes. Surprisingly, these RNA polymerases B molecules are located preferentially in the 5' end moiety of the β-globin genes of mature erythrocytes, although they are apparently evenly distributed along the β-globin genes of immature polychromatic erythrocytes. The significance of these observations with respect to the control of transcription in eukaryotic cells is discussed.

MATERIALS AND METHODS.

Nuclei were prepared as described (4) from mature hen erythrocytes and polychromatic erythrocytes after acetyl-phenylhydrazine treatment (6). After this treatment more than 90 % of the cells are immature erythroïd cells as judged from staining with brilliand cresyl-blue (7) (there were less than 2 % polychromatic erythrocytes in the normal untreated hens). HeLa cell nuclei were obtained by homogeneization in the hypotonic buffer of Wu (8). Nuclear pellets were stored at -90°C in one volume 20 mM Tris HCl pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 8.5 mM dithiothreitol (DTT), 0.125 mM phenylmethylsulfo-nylfluoride (PMSF) and 50 % glycerol. No loss of transcriptional activity occured during storage for several weeks and the results obtained were identical with freshly prepared or stored nuclei. DNA was assayed as described (9).

Plasmid pβ1BR15 which contains the BamHI-EcoRI chicken adult β-globin segment present at the right of λCβG1 (10) was a gift of Dr. Engel.

All other Materials and Methods are described in the legends to figures.

RESULTS.

A. RNA synthesis in isolated erythrocyte nuclei.

We first analyzed the transcription capability of nuclei isolated from mature hen erythrocytes and compared it with that of nuclei from immature polychromatic erythrocytes (termed reticulocytes in this paper for the sake of simplicity) and HeLa cells. Fig. 1 shows the time-course of RNA synthesis in isolated nuclei in the absence (panel a) or in the presence (panel b) of Sarkosyl. Measurements of RNA synthesis in the presence of Sarkosyl provide a way to indirectly quantitate the amount of RNA polymerase molecules actually engaged in transcription complexes (engaged molecules), since it is known to release most of the chromatin bound proteins, including RNA polymerase molecules unless they are bound to DNA in the form of transcription complexes (11, 12). In agreement with previous studies on avian (see for instance 13) or amphibian (14) erythrocytes, RNA synthesis in chicken erythrocyte nuclei, in the absence of Sarkosyl, is about 4 fold lower than in reticulocytes nuclei and 13 fold lower than in HeLa cell nuclei. The presence of Sarkosyl stimulates RNA synthesis by 14-fold in mature erythrocyte nuclei, whereas RNA synthesis is stimulated 10-fold in reticulocyte nuclei and only 5-fold in HeLa cell nuclei. Similar stimulations have been found in the case of amphibian erythrocytes after addition of 0.4 M ammonium sulfate (13) which is known to act like Sarkosyl by releasing most of the chromatin-bound proteins, but not transcriptionally engaged RNA polymerase molecules (15). The addition of α-amanitin (1 μg/ml, results not shown) to the incubation medium resulted in a 80-90 % inhibition of RNA synthesis in the absence of Sarkosyl and to a 95 % inhibition in its presence, indicating that in both cases transcription was mostly catalyzed by RNA polymerase B (16). We conclude from the above results that although transcriptionally relatively inactive, the mature erythrocyte nuclei contain a substantial number of engaged RNA polymerase B molecules (only five times less than HeLa cell nuclei as judged from RNA synthesis in the presence of Sarkosyl), which are in some way preferentially prevented from transcribing efficiently in the absence of Sarkosyl (the HeLa cell nuclei are 13-fold more active than the mature erythrocyte

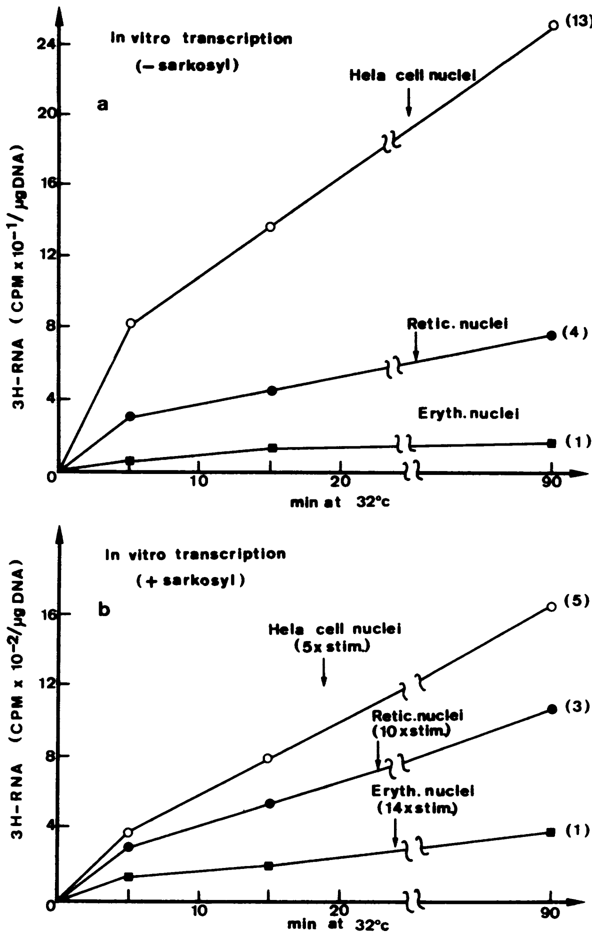

Fig. 1. : Endogenous RNA polymerase activity of purified nuclei in the absence (a) and in the presence of Sarkosyl(b). Nuclei (100-150 μg DNA) were incubated at 32°C in a reaction mixture (125 μl) containing 125 μM [^{3}H] UTP (20 μCi) and ATP, CTP and GTP 1 mM each, 95 mM Tris-HCl pH 7.8, 54 mM NaCl, 50 mM ammonium sulfate, 4 mM $MnCl_2$, 1.2 mM DTT, 0.4 mM EDTA, 0.1 mM PMSF and 29 % glycerol. Sarkosyl, when used, was at a final concentration of 0.6 %. Reactions were stopped by addition of EDTA to 5 mM. After incubation with 20 μg/ml of DNaseI (Worthington) in the presence of E.coli tRNA (40 μg) and 10 mM $MgCl_2$, aliquots (in duplicate) were spotted onto DE/81 filters which were treated and counted as described (17). Blank values were obtained from reactions run in the presence of EDTA. In panels a and b the numbers in parentheses indicate the RNA polymerase activity of HeLa cell and chicken reticulocyte (retic.) nuclei, relative to mature erythrocyte (eryth.) nuclei. In panel b the extent of the stimulation (stim.) of transcription in the presence vs absence of Sarkosyl is indicated in parentheses over the lines.

nuclei in the absence of Sarkosyl).

B. Transcription of the β-globin gene in isolated erythrocyte nuclei.

The molecular cloning of the adult chicken β-globin gene (10) offers a possibility to investigate whether some of these engaged RNA polymerase molecules are present on this gene in

mature and immature chicken erythrocytes. RNA synthesized *in vitro* in the presence or absence of Sarkosyl was hybridized with the 5' (fragments b and c), or the 3' (fragments a and d) moieties of the chicken adult β-globin gene (Fig. 2) bound to diazobenzyloxymethyl (DBM)-paper. When RNA synthesis was carried out for relatively short period of times in the absence (Fig. 2A and B) or in the presence (Fig. 2C-E) of Sarkosyl under conditions were the elongation rate was kept low (by decreasing the concentration of [^{32}P]-α-CTP down to 5 μM), it is clear that the RNA synthesized *in vitro* with adult erythrocyte nuclei hybridizes preferentially to the 5' moiety of the gene in the absence (A) or in the presence of Sarkosyl (C), whereas the RNA synthesized under the same conditions by reticulocyte nuclei appear to hybridize equally well to both 5' and 3' fragments (B, D and E). An almost identical pattern was observed after 15 min of incubation in the presence of Sarkosyl and 5 μM [^{32}P]-α-CTP (Fig. 3, A and B).

When incubations were carried out for longer times in the presence of Sarkosyl and 125 μM [^{32}P]-α-CTP (i.e. under conditions more favorable for chain elongation) there is a trend for the labelled RNA to hybridize preferentially with the 3' moiety of the gene (Fig. 3 C, panel C). (Identical results were obtained when the amount of DBM-paper bound cloned DNA fragments was doubled - not shown ; it should be noted that the size of the hybridizing RNA is small, due to a treatment with a DNaseI preparation which is not RNAse-free, see legend to Fig. 2). This observation which is in agreement with the previous conclusion (11) that initiation of transcription is completely blocked in the presence of Sarkosyl, excludes that the RNA chains transcribed on the β-globin genes of mature erythrocyte nuclei could be initiated during the incubation period. In addition the hybridizing RNA is transcribed by RNA polymerase B, since no labelled hybrids were found when α-amanitin was added to the RNA reaction (not shown). That there is no general transcription of the chicken genome under our incubation conditions is indicated by the inability of the synthesized RNA to hybridize with cloned DNA fragments of the ovalbumin gene (Fig. 3 C and D).

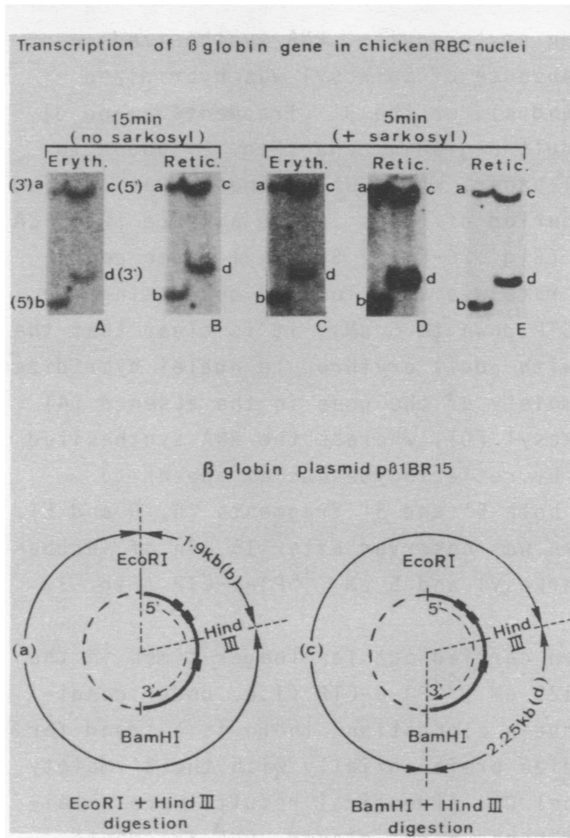

Fig. 2 : Hybridization of [^{32}P]-labelled RNA synthesized *in vitro* by mature erythrocyte and reticulocyte nuclei. RNA was prepared from reaction carried out as described in legend to Fig. 1, but in the presence of 5 µM [^{32}P]-α-CTP (125-250 µCi), with or without Sarkosyl for the time indicated. After DNaseI treatment (see legend to Fig. 1) the samples were diluted with 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and brought to 0.5 % SDS and 100 µg/ml proteinase K (preincubated for 20 min. at 37°C). After 2 hours at 37°C, the samples were extracted once with phenol (pH 5), twice with chloroform isoamylic alcohol (24:1) and precipitated with ethanol at -20°C in the presence of 0.2 M sodium acetate. After dissolution of the RNA pellets in 200 µl of 100 mM Tris-HCl pH 7.5, 1 mM EDTA, the remaining free nucleotides were removed by filtration through a Sephadex G50 column. The plasmid pβ1BR15 which contains the 4.2 kb BamHI-EcoRI chicken adult β-globin segment (thick line ; the closed squares indicate the approximate position of the globin exons and the dashed line corresponds to the large EcoRI-BamHI fragment of pBR322) was cut with EcoRI and HindIII, or HindIII and BamHI, to generate the fragments a, b, c and d which are specific for the 5' or 3' moiety of the β-globin gene (10). Samples (400 ng DNA) of each digest were then electrophoresed in adjacent lanes of agarose gels and transferred to DBM-paper as previously described (4). The hybridization conditions between DNA bound to DBM-paper and the *in vitro* synthesized [^{32}P]-labelled RNA were as described (18). Each paper (A-E) contains on the left side the DNA fragments (a) and (b) and on the right side the DNA fragments c and d. Paper E is the same as D, but autoradiography was for a shorter time. Eryth. = mature erythrocyte nuclei ; Retic. = reticulocyte nuclei.

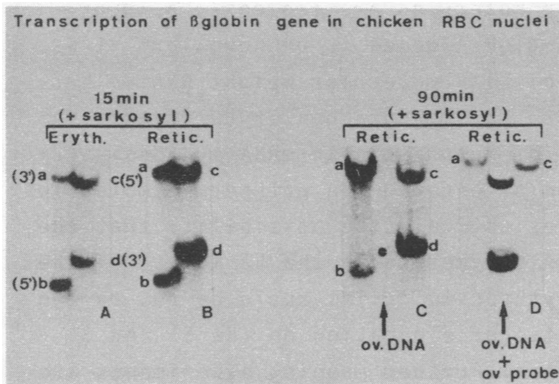

Fig. 3 : Hybridization of [^{32}P]-labelled RNA synthesized *in vitro* by mature erythrocyte and reticulocyte nuclei. RNA synthesis and hybridization conditions were as described in legend to Fig. 2. The DNA fragments immobilized on paper A and B are as in Fig. 2. For paper C and D, the left lane contains the globin fragments a and b, the middle lane contains 100 ng of two ovalbumin gene fragments (ov. DNA) and the right lane contains the globin fragment c and d. The reactions for RNA synthesis were run in the presence of Sarkosyl for the time indicated with 5 μM [^{32}P]-CTP in the reactions used for papers A and B, and 125 μM [^{32}P]-CTP in the reactions used for papers C and D. Paper D is the same as C, but after dehybridization (4) it was rehybridized as a control with nick-translated labelled ovalbumin gene fragments (Ov. probe). The two upper bands a and c which are visible in paper D are due to a slight contamination of the ovalbumin nick-translated fragments with pBR322 sequences. Abbreviations are as in the legend to Fig. 2.

DISCUSSION.

Our present studies demonstrate that, in contrast with a widely spread belief, RNA polymerase molecules, most likely engaged in transcription complexes, are present on or close to the adult β-globin genes in hen mature erythrocytes (we cannot formally exclude the unlikely possibility that another unknown form of complex between RNA polymerase B molecules and DNA could protect them against inactivation by Sarkosyl). Therefore the DNaseI sensitivity of the β-globin genes in chromatin of mature chicken erythrocytes cannot anymore be taken as evidence of preferential DNAseI sensitivity in the absence of the transcriptional machinery, since it cannot be ruled out that transcription is not occuring *in vivo* at a very low rate throughout the entire gene, even though most of the polymerase molecules are clustered at the 5' moiety of the gene. Engaged RNA polymerases molecules are also present on the β-globin genes in immature erythrocytes (reticulocytes) but they appear to be evenly distributed along the

gene ; whether *in vivo* these molecules are frozen *in situ* or actively transcribing the β-globin genes is unknown, but it is worh noting that synthesis of high molecular weight RNA molecules has been observed *in vivo* in immature hen erythrocytes, in the absence of any evidence of mature globin mRNA synthesis (2). In any case the distribution of engaged RNA polymerase molecules in reticulocytes nuclei makes unlikely the possibility that the preferential location of RNA polymerase in the 5' moiety of the β-globin genes of mature erythrocyte nuclei could be due to two widely different *in vivo* rates of elongation on the 5' and 3' moieties of the β-globin genes. Further mapping experiments are in progress to more precisely localize the engaged RNA polymerase molecules in the 5' moiety of β-globin genes of mature erythrocytes.

Our present results can be correlated with previous studies which indicate that engaged RNA polymerase B molecules, which can be stimulated by Sarkosyl, polyanions or high ionic strength, are present in several other *in vivo* reputed transcriptionally inactive systems : e.g. amphibian adult mature erythrocytes (14), the paternal chromosome set of the male mealy bug Planococcus citri (19), the inactive nuclei from Artemia dormant gastrulae (20) and mitotic mammalian chromosomes (21, 22). Moreover, the work of Hameister (23) and Sass (24) has shown that engaged RNA polymerase molecules are present not only at the gene sites of Chironomus tentans polytene nuclei which are transcribed *in vivo* at a given stage, but also at all the puff sites which are ever formed in the salivary glands. It appears therefore that RNA polymerases B molecules can be bound to DNA in the form of transcriptional complexes, not only at the loci actively being transcribed *in vivo*, but also at loci which either have been or will be transcribed.

In the light of our present results it is tempting to interpret all of these previous observations as an indication that the elongation step of transcription could be controlled in eukaryotic cells through a regulatory step equivalent to the attenuation phenomenon in prokaryotes (25). On some genes which have the potential to be transcribed in a given tissue, RNA polymerase B molecules would be allowed to initiate, but not to

elongate, and would therefore be clustered around the 5' end of the genes. Full transcription of the gene would occur *in vivo* only when the blockage would be relieved (for instance by modification of the chromatin structure or by the release of a particular protein from the DNA template) and this activation would be mimicked *in vitro* by addition of Sarkosyl or polyanion or by increasing the ionic strength. Such a mechanism leads to the testable prediction that, for a given cell type, clustering of RNA polymerase B molecules should be found in the 5' moiety of many genes which are not being actively transcribed, but have the potential to be transcribed. It is worth noting that premature termination of viral (26) and cellular (27) hnRNA transcripts has been previously reported to occur in mammalian cells and that engaged RNA polymerase B molecules have been found clustered in some SV40 minichromosomes in the 5' end region of the SV40 late genes (28).

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