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MODULATION OF THE INITIATION OF MOUSE β-GLOBIN TRANSCRIPTION BY NON-HISTONE PROTEINS PURIFIED FROM MOUSE ERYTHROPOIETIC FRIEND CELLS

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Summary : By comparing the initiation of transcription on mouse β-globin DNA and λ-bacteriophage DNA we have studied the specificity of initiation of β-globin transcription. With both E. coli and calf thymus RNA polymerases, the specificity of initiation of β-globin transcription is equally poor and depending on the RNA polymerase : DNA ratios. Non-histone proteins (NHP) from erythrogenic Friend cell nuclei, partially purified on hydroxylapatite, modulate the initiation of β-globin transcription. Some subfractions stimulate specifically the transcription of β-globin. NHP which induce a specific β-globin transcription are also present in the cytoplasm. The specific stimulatory NHP are two-fold more abundant in uninduced than in DMSO induced erythrogenic Friend cells. These proteins may play a general role in vivo in modulating the transcription during the terminal differentiation of erythropoietic cells.

Introduction :

During the last ten years, many different experimental approaches have been made to the problem of transcriptional regulation by the non-histone proteins (NHP)* of eucaryotic cells. The principle of most of these experiments is to transcribe chromatin and compare the in vitro transcript with the in vivo synthesized nuclear RNA or with RNA transcribed in vitro from a naked DNA template (1-6). The conclusions from these experiments were that RNA transcribed from chromatin by E. coli RNA polymerase is very similar to in vivo RNA made in the same cell, and the RNA synthesis depended on the accessibility of the DNA to the polymerase. DNA accessibility to RNA polymerase is reduced by histones but this inhibitory effect is reversed by components of the non-histone protein fraction. We have previously shown that a non-histone fraction from mammary cells stimulates the in vitro transcription of the mouse mammary viral DNA integrated in the chromatin of these cells (7). Recent observations have shown that the nucleosome structure of chromatin is not necessary

* NHP = Non Histone Protein

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for correct RNA synthesis and a whole cell extract without histones permits a normal specific messenger RNA transcription from naked DNA (8,9). In order to see selective and accurate initiation of transcription such a soluble cell extract is necessary. Now that it is possible to measure initiation more precisely, it is evident that with the purified polymerase initiation is not correct (8). It is possible that factors required for specific initiation were either lost during purification of the polymerase or that cytoplasmic and nuclear proteins are necessary for correct RNA initiation.

Recently, we have demonstrated that the mouse β-globin DNA can be transcribed with either E. coli RNA polymerase or calf thymus RNA polymerase B from initiation sites at the 5' end of the gene (10). In order to clarify the role of the non-histone proteins in regulating transcription we have partially purified non-histone proteins and compared the effect of subfractions on the initiation of in vitro transcription on λ-bacteriophage and the mouse β-globin gene. These results permit us to distinguish non-histone proteins, present both in the cytoplasm and nuclei, which have two types of activities on the initiation of mouse β-globin transcription.

Material and Methods :

Preparation and analysis of recombinant mouse β-globin DNA fragments : A recombinant of λgtWES bacteriophage and mouse Eco RI β-globin fragment was obtained as described by Tilgham et al.(11). The certified EK_2 recombinant λgtWES G_2 bacteriophage was propagated in E. coli C 600 using a P2 facility (19). Recombinant bacteriophage mouse β-globin DNA was prepared by phenol extraction. Restriction endonuclease cleavage of λgtWES G_2 was performed with 1.5 µg of DNA in 50 µl under standard conditions for Eco RI (13). Restricted DNA was than prepared by phenol-chloroform extractions as previously described (13). The fragments were analyzed on a 1 % agarose gel (17) and visualized by ethidium bromide staining.

Preparation of non-histone proteins (NHP) : Friend erythroleukemic cells (7071 C) were innoculated at 10^5 cells per ml in Dulbecco's modified Eagle's medium supplemented with 15 % horse serum (GIBCO) and incubated at 37°C for 5 days. Induction was carried out by the addition of (DMSO)* to a final concentration of 1.6 %. From these cells chromatin was prepared using a method previously described (15). The 50 mM phosphate non-histone protein fractions were prepared according to MC Gillivray et al. (16). To prepare subfractions, the 50 mM fraction was applied to a column of hydroxylapatite previously equilibrated with 10 mM phosphate buffer (pH 6.8). Subfractions were obtained by eluting the colum with a 1 to 100 mM phosphate buffer gradient.

* DMSO = dimethylsufoxide

Preparation of transcription complexes : DNA-RNA polymerase RNA ternary complexes on DNA fragments obtained by cleavage with Eco RI endonuclease were analyzed by agarose gel electrophoresis as described by Chelm et al. (17). Components were added in a final volume of 30µl containing : 50 mM Tris HCl pH 7.9 ; 10 mM $MgCl_2$; 0.1 mM EDTA ; 1 mM DTT, 100 µg/ml bovine serum albumin (Sigma) ; 1 µg of DNA fragments and usually 0.075 µg of E. coli RNA polymerase (Boehringer). The mixture was preincubated at 30° C for 5 minutes and RNA synthesis was started by adding 10 µl of ribonucleotide triphosphates to yield a final concentration of 250 µM ATP and GTP, 25 µM CTP and 2,5 µM UTP. Under these conditions, RNA chain elongation is very slow. (α-^{32}P) UTP had a specific radioactivity of 20 µCi/mM. RNA synthesis was allowed to proceed at 30°C for 5 minutes unless otherwise indicated. Synthesis was stopped by addition of 10 % glycerol 20 mM EDTA plus heparin (100 µg/ml). A 1 µl aliquot was precipitated with 5 % trichloracetic acid ; the precipitate was then passed on a glass filter and counted in a scintillation counter to measure RNA synthesis. The remainder of the sample was loaded on a 1 % agarose gel (15 x 10 x 0.5 cm horizontal agarose gel in 40 mM Tris acetate pH 7.9, 20 mM acetate, 1 mM EDTA). Electrophoresis was performed at 4°C for 6 hrs at 100 volts. After removing the dye front containing the free ribonucleotides, the gel was stained with ethidium bromide (1 µg/ml) for 20 minutes at room temperature. The gel photographed with a polaroid camera was dried under vacuum on a Whatman 3 MM peper at room temperature. Several autoradiographies of the same gel were carried out with a Kodirex film (Kodak) for various periods of time to determine the relative intensity of each band.

Results and Discussion :

Numerous results indicate that bacterial RNA polymerase is able to bind and correctly initiate RNA synthesis at initiation sites of eukaryotic viral DNA (18-20). This specific initiation of transcription was shown to depend on the ratio of RNA polymerase : template DNA (18). In order to analyze the specificity of initiation on the β-globin gene we have compared the initiation of β-globin transcription with the transcription of the λ early gene in the presence of various amounts of E. coli RNA polymerase and calf thymus RNA polymerase B. Using both types of RNA polymerase, analysis of the nascent RNA-DNA complexes performed as described in Material and Methods clearly shows that the β-globin DNA fragment is slightly more effectively transcribed than the λ early gene and λ late gene DNA fragments (Fig. 1). The high molecular weight DNA fragment corresponds to the unrestricted β-globin DNA recombinant. After scanning with a Vernon photometer, autoradiographs with 3 different exposure times, it can be calculated that the optimal ratio for the initiation of transcription is 20. At this DNA : RNA polymerase ratio (W/W) we can estimate that approximately four and eight RNA polymerase molecules are bound respectively to the DNA fragments and the β-globin DNA fragment.

This transcription system should allow us to investigate a possible regulatory function of non-histone proteins (NHP). In order to test an

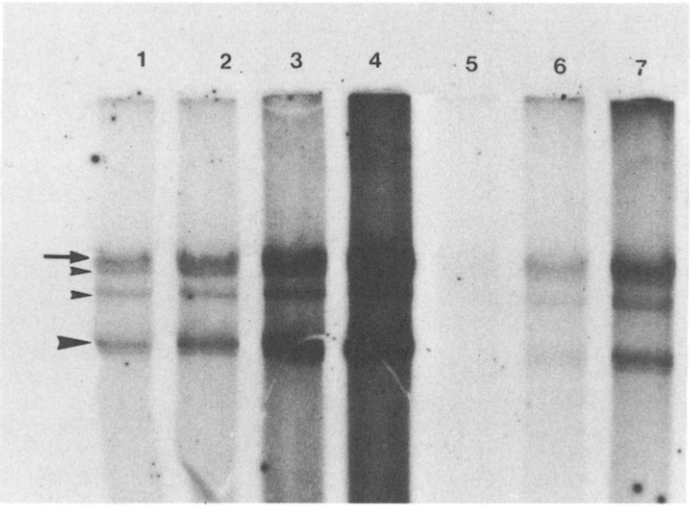

Figure 1 : Initiation complexes of E. coli RNA polymerase and calf thymus RNA polymerase II on Eco RI cleaved λglobin recombinant DNA. Ternary initiation complexes were formed at various enzyme : DNA weight ratios and electrophoresis was performed as described in Material and Methods. For 1 µg of DNA, lanes 1-4 correspond respectively to 12,5 ng, 25 ng, 50 ng, 100 ng of E. coli. RNA polymerase (specific activity : 2000 units/µg). For the same amount of DNA lanes 5-6, 7 correspond to 24 ng, 50 ng, 100 ng of calf thymus RNA polymerase II (specific activity 500 units/µg). Arrows, ►, ►, ►, respectively indicate λ bacteriophage DNA fragments, β-globin DNA insert and whole unrestricted β-globin recombinant DNA.

NHP effect on specific β-globin transcription we have partially purified NHP from uninduced and DMSO-induced Friend cells on a hydroxylapatite column (16). This purification leads to the isolation of a NHP chromatin subfraction free of histones and nucleic acids. Complexes between various amounts of NHP and λβ-globin fragments obtained by Eco RI digestion were formed by dialysis against the transcription buffer and transcribed as previously described. Figure 2 shows the agarose gel electrophoresis of the transcription complexes with three concentrations of uninduced Friend cell NHP (lanes 2, 3, 4) and DMSO-induced cell NHP (lanes 5, 6, 7). As compared to the naked DNA control (lane 1) NHP-DNA complexes are much more transcribed using both types of NHP. The total transcription is increased up to one hundred fold depending on the NHP-DNA ratio. In this experiment, the two λ DNA fragments which migrate very closely are differently transcribed. Whereas the fragment with late genes which does not contain in vitro promotor is not transcribed, the λ early gene DNA fragment with P_R and P_L promotors is significantly transcribed.

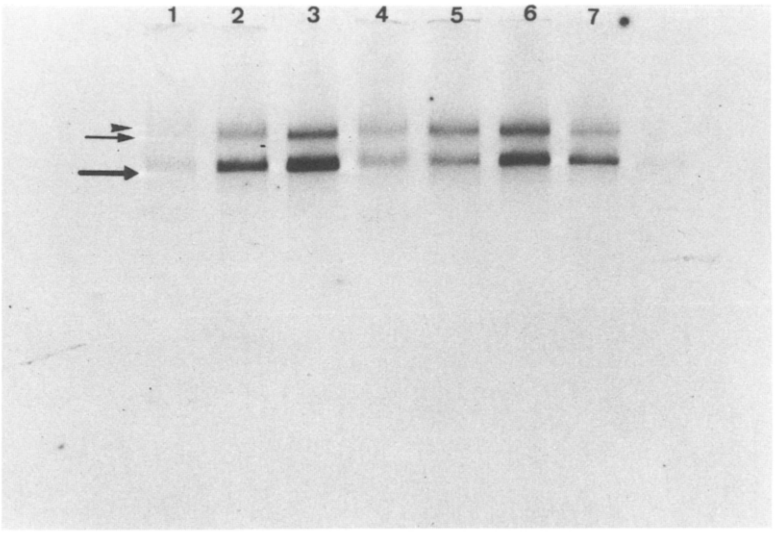

Figure 2 : Modulation of the initiation of transcription by the 50 mM phosphate NHP fraction from uninduced Friend cell nuclei and DMSO induced Friend cell nuclei.
Three different concentrations of nuclear NHP eluted from the hydroxylapatite column at 50 mM phosphate (16) were dialyzed with 1 µg of Eco RI cleaved λ globin DNA against the transcription incubation mixture for 1 hour at room temperature. DNA protein complexes were then transcribed with 12,5 ng of E. coli RNA polymerase under similar conditions described in Figure 1. Lanes 1 to 7 of the autoradiograph of the agarose gel electrophoresis correspond respectively to the transcription without NHP transcription with 1 µg, 2 µg, 3 µg of uninduced Friend cell NHP (lanes 2, 3, 4) and 1 µg, 2 µg, 3 µg of DMSO induced Friend cell NHP (lanes 5, 6, 7). Arrow, ► ➤➤ show respectively the λ late genes, λ early gene DNA fragments and the β-globin DNA fragment.

The stimulatory effect of NHP is more or less specific for the β-globin DNA fragment depending on the NHP-DNA ratio. Thus, the scanning with a Vernon photometer of each band in three independant experiments shows that the specific initiation of β-globin transcription is differently modulated by Friend cell NHP (see figure 4). NHP from uninduced as well as induced Friend cell nuclei are capable of specifically stimulating the β-globin DNA transcription. Thus, for a NHP-DNA ratio of 1 (uninduced Friend cell nuclei) and 3 (uninduced Friend cell nuclei) the β-globin initiation of transcription is stimulated more that the λ initiation of transcription. For a higher protein-DNA ratio the specific stimulatory effect was almost completely abolished. Even inhibitory effect was observed with higher concentrations (Fig. 3, lanes 3 and 5). We have also tested the effect of cytoplasmic protein similarly purified on hydroxyl-

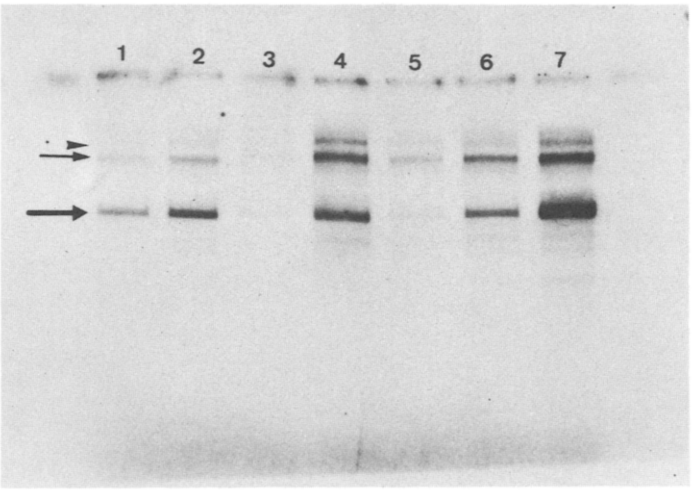

Figure 3 : Effect of the cytoplasmic NHP fraction on β-globin transcription initiation compared to that of the nuclear NHP fraction.
NHP from uninduced Friend cell cytoplasm were purified on hydroxylapatite column as described for the nuclear NHP (16). The fraction eluted at 50 mM phosphate was compared to the nuclear fractions eluted under the same conditions. Lanes 1 to 7 of the autoradiograph of the agarose gel electrophoresis correspond respectively to the transcription without NHP, transcription with 3 µg, 6 µg of uninduced Friend cell nuclear NHP, 3 µg, 6 µg of induced Friend cell nuclear NHP, 4 µg, 6 µg of uninduced Friend cell cytoplasmic NHP. Arrows correspond respectively to λ DNA and β-globin DNA fragments. Minor bands correspond to Eco RI star digestion.

apatite column on the specificity of transcription (Fig. 3, lane 6 and 7). It is clear that cytoplasmic proteins stimulate both the total transcription and the initiation of specific β-globin transcription. Proteins from induced Friend cell cytoplasm are as efficient as proteins from uninduced Friend cell cytoplasm (results not shown). In order to precisely compare the β-globin to the λ early gene initiation of transcription we have quantified the amount of initiation complexes by measuring the band intensities with a Vernon integrator photometer. By calculating the β-globin : λ early gene transcription ratio we can estimate the effect of NHP on the specificity of the β-globin initiation of transcription. Thus, the composite curves (Fig. 4) suggest that several stimulatory and inhibitory activities co-exist in the 50 mM phosphate NHP fraction from uninduced and DMSO induced Friend cell chromatin. All these results suggest that one or several proteins more abundant in uninduced Friend cell nuclei stimulate specifically the transcription of an eucaryotic gene.

We have fractionated the 50 mM phosphate protein fraction by eluting sub-fractions with an phosphate gradient from the hydroxylapatite

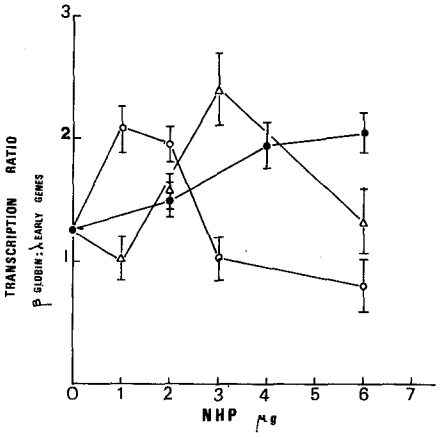

Figure 4 : Variations of the β-globin : early gene transcription ratio with the nuclear and cytoplasmic NHP fraction from uninduced and DMSO induced Friend cell.
Several autoradiographs exposed for different times were quantified with a Vernon integrator photometer. The amount of initiation complexes on each DNA fragment was estimated by measuring the band intensities in three independant experiments : The β-globin/λ early gene transcription ratios were quantified for the uninduced Friend cell NHP (—o—), DMSO induced Friend cell NHP (—Δ—) and cytoplasmic proteins from uninduced Friend (—●—).

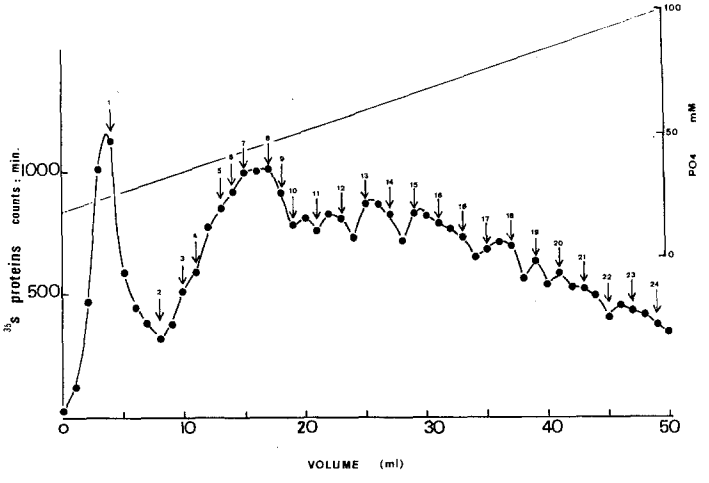

Figure 5 : Non-histone proteins eluted from the hydroxylapatite column
Proteins were labelled with ^{35}S methionine (specific activity : 500 mCi/mM) in cell culture for 12 hours. ^{35}S labelled proteins have a specific activity of 120 counts per minute and per µg of protein. The 50 mM phosphate labelled protein fraction from uninduced Friend cell chromatin was loaded on a hydroxylapatite column as already described (16). Proteins were eluted from the column with a phosphate gradient from 1 mM to 100 mM phosphate.

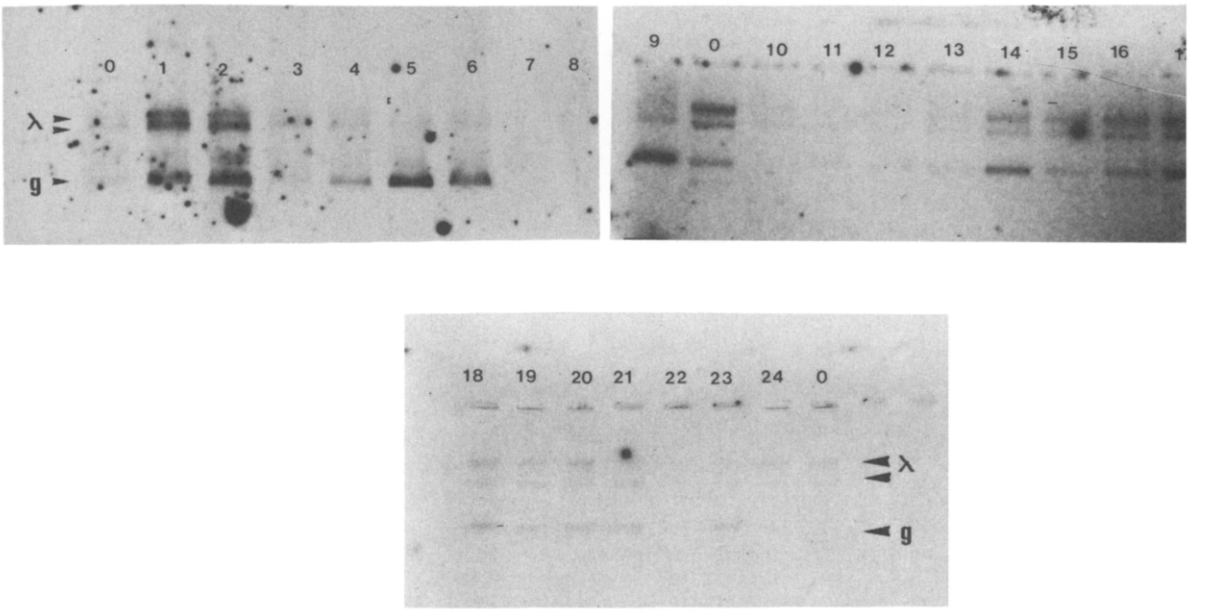

Figure 6 : Modulation of β-globin transcription initiation by nuclear NHP subfractions eluted from hydroxylapatite column with NaCl.
The nuclear NHP fraction of uninduced Friend cell eluted at 50 mM phosphate was loaded on a second and similar hydroxylapatite column. Proteins were eluted with a phosphate (1-100 mM) in 50 different subfractions. One protein amount (1 µg) of every fraction in two was tested on the β-globin transcription initiation under conditions described in Figure 2. Number 0 to 8, 9 to 17, 18 to 24 correspond to three different test series of fractions from the same column. Lanes 0 are control tests without NHP subfractions. λ and g show respectively the λ bacteriophage DNA fragments and the β-globin DNA fragment.

column (Fig. 5). These NHP subfractions were tested at the same NHP-DNA ratio as previously described. From Fig. 6 it can be concluded that fraction n° 5 and 6 stimulate exclusively the initiation of β-globin transcription. In contrast, fraction n° 1 and 2 stimulate non specifically the initiation of λ and β-globin transcription. It has been noted that other protein fractions inhibit non specifically the λ DNA and β-globin DNA transcription (for example, fraction n° 10-13). These subfractions analysed on two dimensional gel still contain about 20 species of NHP (unshown results).

A regulatory function in transcription has been demonstrated for DNA binding NHP (1-3). These proteins are involved in the in vitro activation of total DNA transcription. We describe here a NHP fraction which contains this type of protein. Furthermore, we can distinguish a subfraction which stimulates globin gene transcription specifically as compared to the λ bacteriophage transcription. In Friend cells the globin gene is transcribed both in the uninduced and induced Friend cell cultures (22) although the number of copies of globin mRNA present in the latter is much higher. We would suggest that the NHP subfraction which has a specific stimulatory effect on the initiation of globin of globin gene transcription in vitro has a general stimulatory effect on the initiation of transcription in Friend cells. Its higher level in non-induced cultures reflects the higher stimulatory activity in these cells (21). Implicit with a regulatory role, the NHP should possess components that can interact specifically with homologous DNA. This transcription system constitutes an assay to purify proteins which can interact with site specific regions in the genome.

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