

# Regulation of globin genes expression: New findings made with the chicken domain of $\gamma$ globin genes

## Research Article

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**Abbreviations:** Avian erythroblastosis virus, (AEV); chloramphenicol-acetyl-transferase, (CAT); isoquinolinesulphonil-2-methylpiperazine-dichloride, (IMD); Locus Control Region, (LCR)

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## Summary

The domain of chicken  $\gamma$  globin genes represents one of the best studied genomic domains in higher eukaryotes. Nevertheless, many questions concerning the nature of mechanisms regulating coordinated expression of globin genes in the course of development remain open. Here we show, that the whole cluster of  $\gamma$  globin genes is preceded by a CpG-rich region which colocalises with the replication origin and the permanent site of DNA attachment to the nuclear matrix. In non-erythroid cells the upstream CpG-rich area of the  $\gamma$ -globin gene domain is selectively methylated. In model experiments, methylation of this sequence element exerted a strong negative effect on the activity of globin gene promoters. We suggest that the upstream CpG rich area of the  $\gamma$ -globin gene domain constitute a molecular switch which regulate expression of  $\gamma$  globin genes in cells of different lineage.  $\gamma$ -globin genes are found to be transcribed in both proliferating (premature) and differentiated (mature) erythroid cells. However, only the latter express globins at a protein level. We found that the block of productive expression of globins in premature erythroid cells occurs at post-transcriptional level. In these cells the transcripts of  $\gamma$ -globin genes are retained in nuclei. Induction of proliferating erythroid cells to differentiation is accompanied by a release of globin gene transcripts to the cytoplasm.

## I. Introduction

Recent evidence suggests that clusters of  $\alpha$  and  $\beta$  globin genes in vertebrates are organized and regulated in a different fashion. The  $\gamma$ -globin genes domains in mammals and chickens are packed in closed (DNase I "non-sensitive") chromatin in all cells except the erythroid ones where the globin genes are expressed (Forrester et al, 1990; Felsenfeld, 1993; Craddock, 1995). The transcription status of  $\gamma$ -globin clusters in all organisms studied so far is regulated by so-called Locus Control Region (LCR) (Forrester et al, 1987, 1993; Grosveld et al, 1987; Li et al, 1990; Moon and Ley, 1990). Naturally occurring deletions of the LCR block expression of  $\gamma$ -globin genes. At the same time the replication timing and the mode of  $\gamma$ -globin genes packaging in chromatin is

changed (Forrester et al, 1990). The necessity of the LCR for maintaining the open chromatin configuration of the mouse  $\gamma$ -globin gene domain has been, however, questioned by some recent results (Epner et al, 1998; Higgs, 1998; Bender et al, 2000).

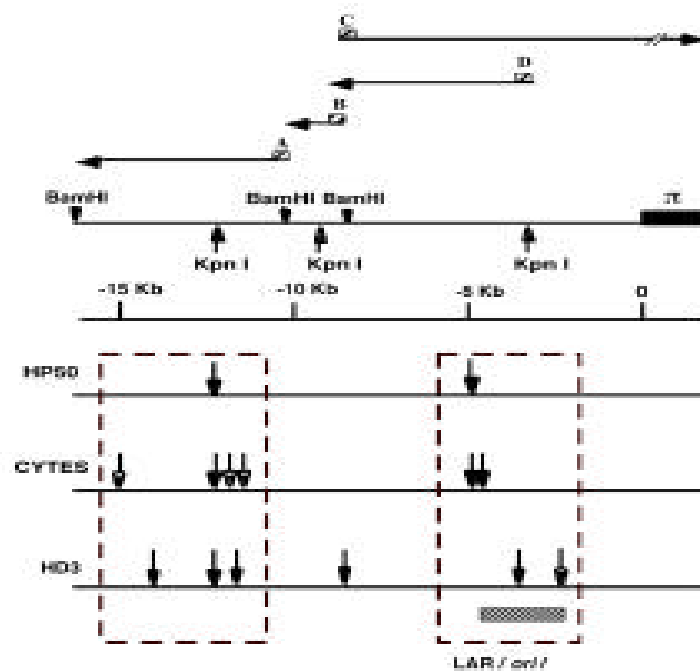
As for domains of  $\gamma$ -globin genes, they were found in open ("DNase-sensitive") chromatin configuration in both erythroid and non-erythroid cells (Vyas et al, 1992; Craddock, 1995). The fact possibly reflects the presence of an apparently house-keeping gene that overlaps the upstream part of the  $\gamma$ -globin genes domains in mammals and chickens (Vyas, 1995; Razin et al, 1999; Sjakste et al, 2000). This gene is transcribed opposite to the direction of globin gene transcription (Vyas, 1995). The major positive regulatory elements of human and mouse  $\gamma$  globin gene

domains (Higgs et al, 1990; Gourdon et al, 1995) reside within one of the introns of the above gene (Vyas, 1995). Although the major positive regulatory elements of the globin gene domains (in human known as HS-40) share some properties of the LCR of  $\gamma$ -globin genes, as shown in experiments with transgenic mice (Sharpe et al, 1992), the similarity is limited. Mammalian  $\gamma$ -globin genes reside within CpG islands which are not methylated in a variety of cells (Bird et al, 1987; Shewchuk and Hardison, 1997). Being transfected to cultured cells, the  $\alpha$ -globin genes are expressed in erythroid and non-erythroid cells both transiently (even in the absence of enhancer) and after integration into genome (Whitelaw et al, 1989; Brickner et al, 1991; James-Pederson et al, 1995; Shewchuk and Hardison, 1997). Hence, the promoters of  $\gamma$ -globin genes are not tissue-specific. Yet, normally, these genes are expressed only in erythroid cells. Thus, it is likely that there should be some negative regulators which suppress the expression of  $\gamma$ -globin genes in non-erythroid cells. The aim of our study was to identify and to characterise the regulatory elements of this kind.

## II Results

### A. Mapping of DNase I hypersensitive sites in the upstream area of the domain of $\gamma$ -globin genes

Positions of DNaseI hypersensitive sites in chromatin usually mark the sites of interaction of different regulatory factors with the target sequences on DNA. analysis of distribution of DNaseI hypersensitive sites in a given genomic area may thus give good indications of the positions of regulatory elements. To map the positions of DNase I hypersensitive sites in the upstream area of the domain of  $\gamma$ -globin genes, an indirect end-labelling approach was used. In parallel experiments, positions of DNase I hypersensitive sites were mapped in chicken erythroid cell (line HD3; clone A6 of line LSCC (Beug et al, 1979)) and chicken lymphoid cell (line HP50 (Dhar et al, 1990)) nuclei. The strategy of the labelling experiments and the results obtained are represented schematically in **Figure 1**. Two clusters of DNase I hypersensitive sites were identified in the upstream area of the domain of  $\gamma$ -globin genes at distances of 11-16 and 3-6 kb

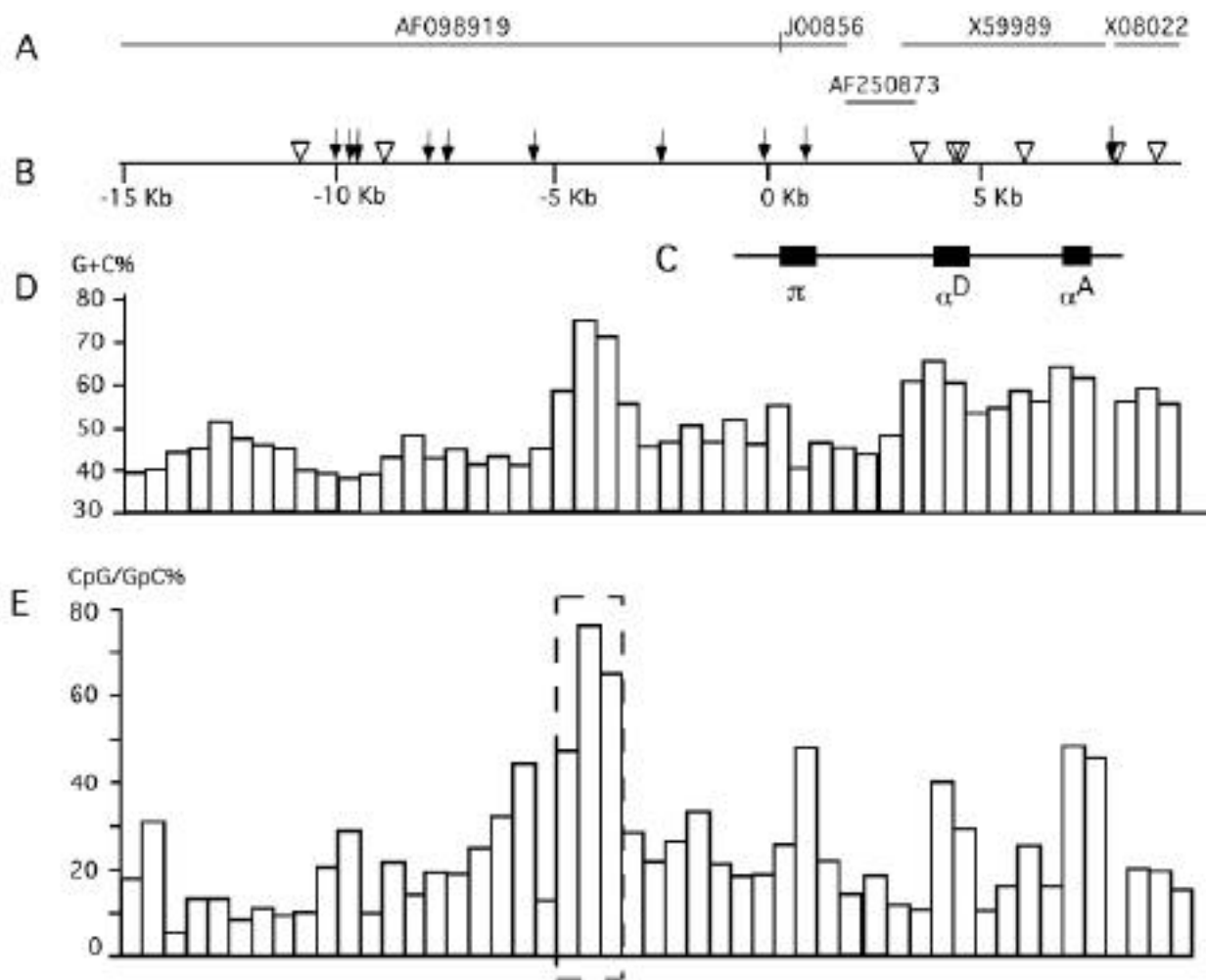


**Figure 1.** Distribution of the DNase I hypersensitive sites in the upstream area of the chicken domain of  $\gamma$ -globin genes. In the upper part of the figure, the restriction map of the area under study and the strategy used to map DHSs are shown. The rectangles indicated by capital letters A - D show positions of hybridization probes. The horizontal arrows show the full-sized restriction fragments recognised by each of the probes. Positions of DNase I hypersensitive sites found in cultured lymphoid cells (HP50), normal chicken erythrocytes (CYTES) and cultured chicken erythroid cells (HD3) are shown below the restriction map. The two clusters of DNaseI hypersensitive sites are outlined by broken line rectangles.

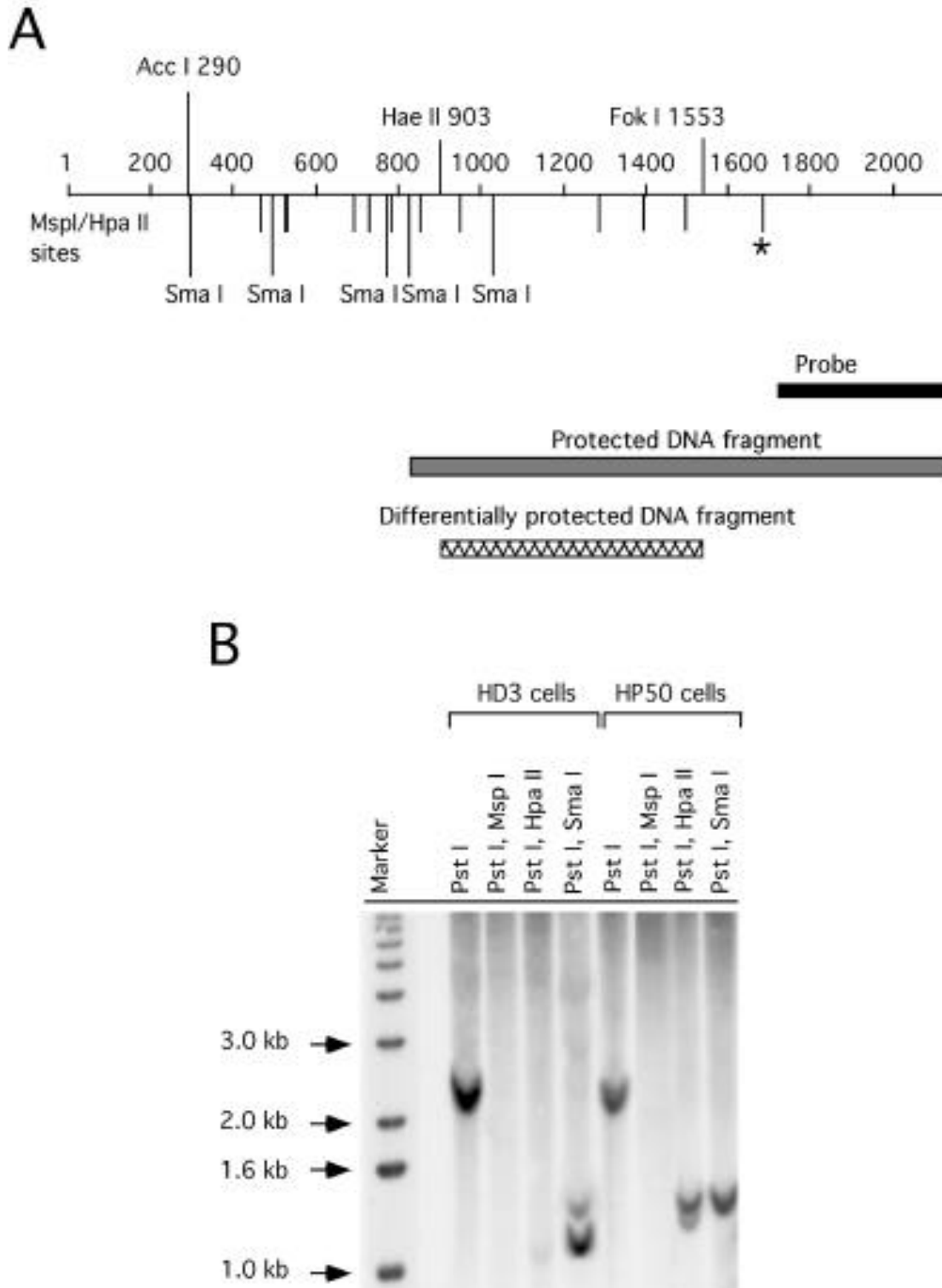
upstream to the pi gene. The DNA region including the first cluster of DNase I hypersensitive sites have been studied in our previous work (Razin et al, 1999). This region was found to contain several transcription silencers, MAR element and transcription termination sites. The group of DNase I hypersensitive sites located 3-6 kb upstream to the gene mark positions of the DNA loop anchorage site (Razin et al, 1991) and of the replication origin (Razin et al, 1986; Verbovaia and Razin, 1995). The data presented below suggests that a negative regulator of transcription may also be located in this area.

**B. The cluster of DNase I hypersensitive sites located 3-6 kb upstream to the gene colocalizes with a CpG island which is partially methylated in non-erythroid cells**

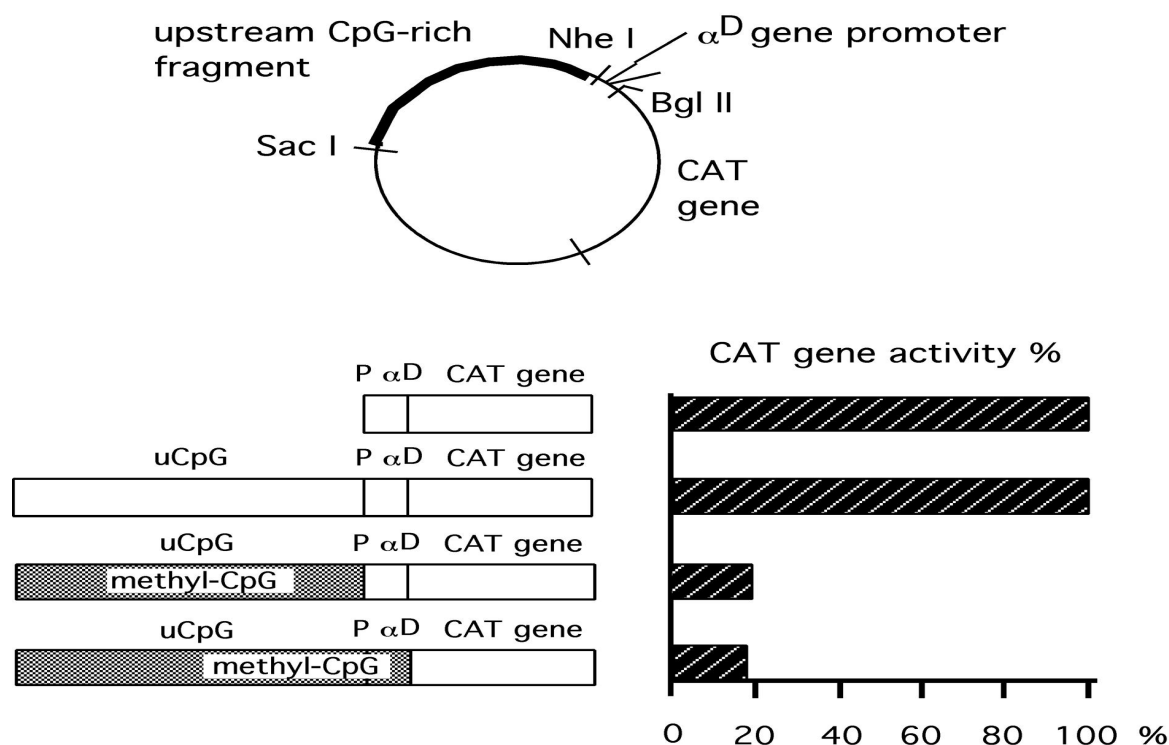
Computer analysis of the DNA sequence of the upstream area of chicken  $\alpha$ -globin gene domain has permitted to identify a CpG-rich region in about the same position where one of the clusters of DNase I hypersensitive sites was found (Figure 2).



**Figure 2.** Compositional characteristics of the chicken domain of  $\alpha$ -globin genes. (A) Accession numbers of DNA sequences analysed; (B) The scale of distances (in Kb). (C) Positions of globin genes. (D) Percentage of GC pairs Each column shows composition of a 0.5 kb fragment. (E) Ration of CpG to GpC. The CpG rich area is shown by broken line rectangle



**Figure 3.** Analysis of methylation pattern of  $\gamma$  globin gene domain upstream CpG reach region. **(A)** A scheme demonstrating distribution of Msp I/Hpa II recognition sites within the area under study. Below the scheme the position of the probe used and the position of the area showing selective methylation in non-erythroid cells are shown. The Msp I/Hpa II recognition site methylated at all C residues is shown by asterisk. **(B)** Results of hybridization. Note different patterns of Hpa II and Sma I digestion products visualised upon hybridization of the probe with HD3 and HP50 DNA digested by the above mentioned enzymes



**Figure 4.** Analysis of the activity of CAT gene present in constructs with methylated and non-methylated CpG-rich fragment inserted upstream to the non-methylated promoter of the  $\alpha$  D gene. The figures were normalised versus activity observed in experiment with transfection of construct without CpG-rich fragment. All figures represent an average of results obtained in five independent transfection experiments. In the upper part of the figure the map of the construct is shown

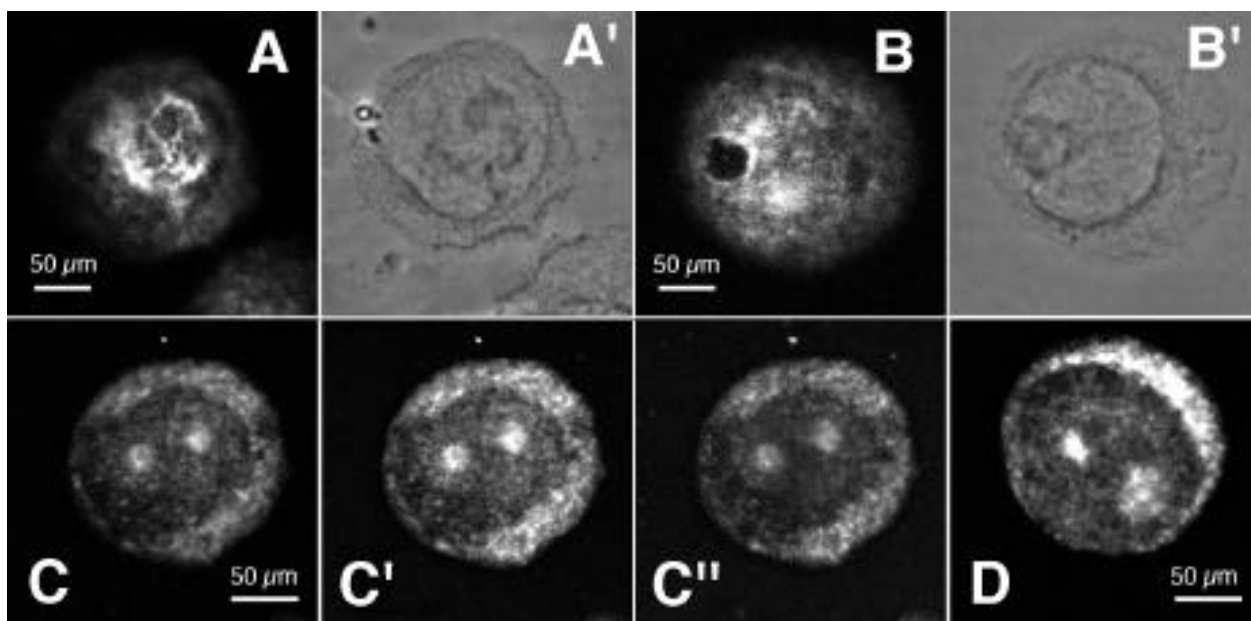
In order to check whether CpG dinucleotides within this region are non-methylated (as normally in CpG islands), the digestion with isoschizomers of restriction enzymes sensitive and non-sensitive to the CpG methylation was carried out. The results of this analysis (**Figure 3**) have permitted to conclude that the downstream part of the CpG area under study is selectively methylated in non-erythroid cells.

The obvious correlation between the expression of globin genes in erythroid cells and the demethylation of the 0.65 Kbp CpG-rich DNA fragment, in the upstream area of the domain of  $\alpha$ -globin genes made it reasonable to check whether the DNA sequence elements present in this fragment (either methylated or non-methylated) can influence the activity of globin gene promoters. With this aim we constructed a recombinant plasmid with the CpG-rich fragment inserted upstream to the CAT reporter gene expressed from the promoter of the chicken  $\alpha$  D globin gene. The CpG-rich fragment was cut from this construct, methylated *in vitro*, using the Sss I methylase, and religated back. In control experiments, the same manipulations were carried out with the mock-methylated fragment, which was incubated with Sss I methylase in the absence of S-adenosylmethionine. The same amounts of methylated and non-methylated construct were then transfected into HD3 cells, and the activity of the CAT gene was assayed after 72 h of cell cultivation. The results

presented in **Figure 4** show, that methylation of the cytosine residues within all CpG dinucleotides of the CpG-rich fragment suppresses significantly (5 times) the activity of the CAT gene, expressed from the non-methylated promoter

### C. The globin genes are transcribed in non-differentiated cultured erythroid cells, but their transcripts are not transported to cytoplasm

The HD3 cells used in the present study (AEV-transformed chicken erythroblasts) can normally proliferate for many generations. In this state they do not express haemoglobin's, although previous observations indicate that the globin genes are transcribed in proliferating AEV cells (Therwath, 1982). Under special conditions (cultivation at elevated temperature and treatment with an inducer of differentiation) these cells undergo typical steps of terminal differentiation into erythrocytes, resulting in proliferation arrest and the start of haemoglobin synthesis. As the globin genes are transcribed both before and after the terminal differentiation of AEV cells, the possibility exist that important steps of erythroid cell differentiation are controlled at post-transcriptional level. In order to test this supposition we have studied the cellular distribution of the



**Figure 5.** *In situ* hybridization of the globin probe on exponentially growing (**A, B**) and induced to differentiation (**C-C''**, **D**) AEV cells. Cells cultivated *in vitro* to medium density were deposited in cytospin centrifuge onto microscopical slides and processed for *in situ* hybridisation as detailed in the Methods section. All micrographs shown are single confocal sections. Panels (**A,A'**) show a typical uninduced cell with a very large and diffuse nucleolus (see phase contrast in **A'**). Panels **B,B'** show a partially differentiated cell with a more compact nucleolus (see phase contrast in **B'**) and an already formed "nuclear spots" where the globin transcripts are accumulated. Panels **C-C''** show three consecutive confocal sections of a differentiated cell. Another typical differentiated cell is shown in panel **D**. Note the intensive spots in cell nuclei.

transcripts of  $\alpha$  A globin gene in proliferating and differentiated AEV cells. To induce differentiation, the proliferating HD3 cells were incubated with the IMD inducer at 42°C (see Methods section for details). Aliquots were taken from the cell suspension at different time intervals after the beginning of induction and the percentage of cells producing haemoglobins was calculated using benzidine staining. It was found to be correspondingly 1%, 18%, 30% and 42% in the samples taken 24, 48, 52 and 76 h after the beginning of induction. The percentage of the dead cells present at the same samples was calculated using trypan blue staining and was found to be correspondingly 9%, 11%, 18% and 40%. Hence, four days after the start of induction, about 70% of cells still alive produced haemoglobins. The morphology of these cells was checked by light microscopy after staining with Giemsa. Proliferating cells are big, with huge nuclei, and more or less oval in shape. After incubation for 4 days under conditions favouring differentiation, one could observe a number of differentiated (small almost square) cells with picnotic nuclei originating, apparently, from large (precursor ?) cells and from cell clusters (not shown).

The RNA probe recognising the transcripts of the  $\alpha$  A globin gene was prepared as described in "Methods" and used for hybridisation *in situ* on the non-induced cultured chicken erythroblasts (line HD3). The results of

our experiments show that the  $\alpha$  A gene is indeed transcribed in proliferating HD3 cells (**Figure 5 A, B**). This globin RNA is not randomly distributed within the nuclear volume. There is a clear concentration of the hybridisation signal around nucleoli clearly visible in phase contrast (**Figure 5 A', B'**) and also, in some instances, at the nuclear periphery, in addition to faint tracks in the nucleoplasm.

When HD3 cells were induced to differentiate the situation changed drastically. The strongest signal was then present in the cytoplasm (**Figure 5 C-C'', D**) in an apparently homogeneous manner. The pattern of nuclear transcripts had also changed: their distribution differed extensively from that observed in non-differentiated cells. Most of the RNA identified by the  $\alpha$  A globin probe could be observed within one or two intensively stained spots (**Figure 5 C-C'', D**). These might represent the processing centres of pre-mRNA. The confocal series (**Figure 5 C-C''**) and DAPI staining (not shown) indicate that these, apparently spherical bodies are distinct from the nucleoli, outlined by globin RNA in the uninduced cells (**Figure 5 A,A', B,B'**).

It seems evident that the *amount* of nuclear staining in proliferating and differentiated HD3 cells is almost the same, whereas the *pattern* of distribution changes drastically. It is hence likely that after induction, the expression of globin proteins commences in AEV cells, because the pre-mRNA is released from the nuclei.

### III. Discussion

Although the domains of  $\beta$ -globin genes in human and chicken have been extensively studied over past 25 years, many questions concerning the regulation of  $\beta$ -globin gene expression (including switching from the embryonic to the adult expression pattern) remain unsolved. Here we have demonstrated that in AEV-transformed erythroid cells expression of globins is regulated in both transcriptional and post-transcriptional levels. Although it was shown previously, that an LCR-like positive regulatory element (in human cells known as HS -40 (Higgs et al, 1990)) is essential for expression of  $\beta$ -globin genes, the correct expression in erythroid cells may depend on a negative regulatory element described here. We have demonstrated that, being methylated, this regulatory element ensure 5X suppression of the activity of  $\beta$  D gene promoter in a model experiment with  $\beta$  promoter-CAT gene cassette transfected to cultured chicken erythroid cells. In a normal chromatin context this effect is likely to be even more profound. Indeed, the gene silencing by CpG methylation may depend on a certain reorganization of the chromatin structure (Razin, 1998) and it is known that transfected plasmids do not fully acquire the normal chromatin organization.

An interesting observation made in the present study is that  $\beta$  A gene-specific transcripts are virtually absent from cytoplasm of immature erythroid cells although they can be easily detected in the nuclei. Hence it is likely that beginning of expression of globin proteins upon differentiation of erythroid cells becomes possible because pre-m-RNA is released to undergo processing and to leave nuclei. This suggests that there is a special control mechanism that regulates the destination of the primary transcripts, by targeting them to special nuclear compartments placed, possibly, on the processing and transport pathways. Looking at the distribution of globin RNA in non-differentiated cells, one can propose that the peri-nucleolar area serves for the temporary storage (and possibly for subsequent destruction) of transcripts that were unable to pass to the processing centres. Conversely one may simply observe a compartment on the normal processing pathway, which is blown-up due to a block in the transport system downstream. Indeed, Chan and Ingram [Chan, 1973 #509] showed many years back that nuclei of *in vitro* cultivated chicken blood islands contain globin mRNA many hours before the latter starts to show up in the cytoplasm. Furthermore, there was always a suspicion that the nucleoli might be involved somehow in gene expression, beyond the mere contribution of ribosomes to the protein biosynthesis machinery (e.g.: cf. [Deak, 1972 #511; Pederson, 1999 #527])

### IV. Materials and Methods

#### A. Cell culture and DNA transfection

Avian erythroblastosis virus (AEV)-transformed chicken erythroblasts of the line HD3 (clone A6 of line LSCC (Beug et al, 1979)) and chicken lymphoid cells (line Hp50) were grown in

suspension in Dulbecco modified Eagle's medium supplemented with 8% fetal bovine serum and 2% chicken serum. Transfection of DNA into these cells was performed with the "lypofectin" transfection reagent (Gibco-BRL), as described in the manufacturer's manual. In standard experiments,  $10^6$  cells were transfected with either 1  $\mu$ g of methylated or non-methylated constructs or with equimolar amount of vector DNA. To monitor the efficiency of transfection, the cells were cotransfected with pSV- $\beta$ -Galactosidase Control Vector (Promega). The activity of the CAT (chloramphenicol-acetyl-transferase) and  $\beta$ -gal genes was assayed in cellular extracts 60 h after transfection. To induce erythroid differentiation of growing HD3 cells, the inducer (IMD, isoquinolinesulphonil-2-methylpiperazine-dichloride) was added to the cell suspension up to a final concentration of 20 mM and, thereafter, the cells were cultivated at elevated temperature (42°C instead of 37°C). At the beginning of differentiation the concentration of cells in suspension was adjusted to  $5 \times 10^5$  per ml.

#### B. Preparation of DNA constructs

All manipulations with recombinant DNA were carried out as described (Maniatis, 1982). The initial vector containing the CAT gene expressed under the control of  $\beta$  D globin gene promoter  $\beta$  pCAT3 has been described previously (Razin et al, 1999). The DNA fragment showing different methylation pattern in erythroid and non-erythroid cells (0.65 kb fragment of the upstream area of chicken  $\beta$ -globin gene domain) was excised from the insertion of the previously described recombinant clone  $\beta$  5HR ((Razin et al, 1991), Gene Bank accession number: X54965) by double digestion with *Fak* I and *Hae* II restriction endonucleases. This fragment was inserted into *Mlu* I site of  $\beta$  pCAT3 vector after the ends of both the vector and the insertion were made blunt. The clone bearing the recombinant plasmid with the CpG-rich DNA fragment inserted in the same orientation (versus promoter) as in the genomic chicken DNA was selected and used in further experiments. From this recombinant construct the CpG-rich DNA fragment was excised by cleavage with *Sac* I and *Nhe* I restriction endonucleases. The excised fragment was methylated *in vitro* using *Sss*I methylase (Biolabs). The degree of methylation was monitored by digestion of aliquots of reaction mixture with *Hpa* II restriction enzyme. Methylated DNA fragment was reinserted into dephosphorylated vector DNA and circular DNA was purified by preparative agarose gel electrophoresis.

#### C. Analysis of chloramphenicol-acetyl-transferase and $\beta$ -galactosidase activities in cell extracts

Promega assay systems were used in both cases, and enzyme activity was determined exactly as described in the manufacturer's manuals. To determine the activity of chloramphenicol-acetyl-transferase, thin layer chromatography was used. After chromatographic separation, the spots containing non-modified chloramphenicol and butyrylated forms were scraped from the chromatographic plate and the radioactive signal was quantified in a liquid scintillation counter.

#### D. DNA hybridization (Southern analysis)

Chicken genomic DNA from HP50 or HD3 cells was digested with either *Msp* I or *Hpa* II or *Sma* I restriction enzymes, and additionally with *Pst* I. The digestion products were separated in 1.5% agarose gels and transferred on nylon

filters "NYTRAN-PLUS" (Schleicher&Schuel). Hybridization was carried out in "Rapid-hyb" buffer (Amersham), as described in manufacturer's manual. The probes were labelled with <sup>32</sup>P dCTP using Megaprime DNA labelling system (Amersham).

### E. *In situ* hybridization

To prepare a strand-specific probe, the 1.8 kb chicken genomic DNA fragment containing the  $\alpha$  gene (for a genomic map see [Recillas Targa, 1994 #459]), was cloned into the pSP73 Vector (Promega). The fragment was then transcribed in the direction opposite to that of globin gene transcription with the T7 RNA polymerase, using the Boehringer (Mannheim) kit for preparation of digoxigenin-labelled RNA. Hybridization *in situ* was carried out according to the Boehringer (Mannheim) manual as described before [De Conto, 1999 #502]. Briefly, HD3 cells were fixed in 1% paraformaldehyde in PBS for 20 min at r.t. before treatment with a solution of 70% ethanol and 3% H<sub>2</sub>O<sub>2</sub>, to suppress endogenous peroxidases. Cells were then permeabilized with 0,2% Triton X-100 in PBS for 10 min, washed carefully in PBS and immersed in 0,1 M glycine in PBS for 5 min. After rinsing in PBS, cells were treated with 0,25% acetic anhydride in 0.1M triethanolamine buffer for 10 min, prior to incubation at 42°C for 16 hours with the ribo-probe (0.5 ng/ml) in hybridization buffer (50% de-ionized formamide, 5x SSC, 10% dextran sulphate, 2.5x Denhardt's solution, 10 mM dithiothreitol, 20 mM vanadyl ribonucleotide complex). After hybridization, the digoxigenin-labelled probe was detected by incubation with anti-digoxigenin-AP, FAB fragments (Boehringer (Mannheim)), followed by incubation with tyramide, as described in the manual for the TSA-DIRECT (tyramide signal amplification) kit (DuPont, NEN).

### F. Confocal Laser Scanning Microscopy and image analysis

Analysis of patterns of globin RNA localization in HD3 cells was performed using the TCS (Leica Germany) confocal imaging system, equipped with a 63X objective (plan apo; NA 1.4). For Cy3 excitation, an Argon-Krypton ion laser adjusted at 488 nm was used. The signal was treated using line averaging, to integrate the signal collected over 8 lines in order to reduce noise. For high resolution, we defined a set of acquisition parameters, which took into account Nyquist's principle. The confocal pinhole was closed to yield a minimum field depth (about 0.6  $\mu$ m), and focal series were collected for each specimen. The focus step between these sections was generally 0.3  $\mu$ m and the XY pixelization was set to 100 nm.

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