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Functional Analysis of DNA Sequences Located Within a Cluster of DNase I Hypersensitive Sites Colocalizing With a MAR Element at the Upstream Border of the Chicken α-Globin Gene Domain

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Abstract We have cloned and sequenced a genomic DNA fragment of chicken containing a cluster of DNase I hypersensitive sites (DHS) located 11–15 kb upstream from the first gene of the α-globin gene domain and including a constitutive DHS flanked by two erythroid-specific ones. A 1.2-kb subfragment of the DNA fragment under study located upstream to the constitutive DHS and colocalizing roughly with one of the erythroid-specific DHS was shown to possess the properties of a matrix association region (MAR). The cloned DNA sequences were tested for their ability to serve as promoters and/or influence transcription from the promoter of the α^D globin gene. In the region studied, we did not find any promoters or enhancers that were active in erythroid cells. The whole DNase I hypersensitive region and some of its subfragments showed a silencing effect when placed downstream from the reporter gene. The expression of the reporter gene was completely abolished, however, when these DNA fragments were placed between the α^D promoter and the reporter gene. Thus, they seem to act as transcription "terminators." Numerous polyadenylation signals (AATAAA) and an AT-rich palindrome were found within the sequenced DNA fragment. These observations are discussed within the frame of the hypothesis postulating that continuous transcription is essential for maintaining the active status of genomic domains. Furthermore, it is suggested that the DNA fragment studied contains a negative control element that keeps globin genes silent within the chromatin domain permanently open in nonerythroid cells. J. Cell. Biochem. 74:38–49, 1999.

Key words: domain of chicken α-globin genes; DNase I hypersensitive sites; enhancers; silencers; transcription termination signals; matrix association regions

The domain of the chicken α-globin genes represents, perhaps, one of the best-studied areas of the genomes of higher eukaryotes [for a review, see Recillas Targa et al., 1995]. This domain includes the three α-type globin genes (the embryonic π gene and adult α^D (minor) and α^A (major) genes, in the 5′ to 3′ direction) flanked upstream and downstream by permanent sites of DNA attachment to the nuclear matrix [Farache et al., 1990] and by blocks of AT-rich sequences [Moreau et al., 1982]. A strong erythroid-specific enhancer is located at a short distance downstream from the last gene of the domain [Knezetic and Felsenfeld, 1989; Kretsovali et al., 1988]; in the chicken, it is flanked by a silencer [Recillas Targa et al., 1993]. Another weak and cell type nonspecific enhancer was mapped within a CpG island located about 2.5 kb upstream from the first gene of the domain [Razin et al., 1991]. This enhancer element colocalizes with the origin of DNA replication [Razin et al., 1986, 1991; Verbovaia and Razin, 1995]. Along with transcripts of individual genes, a long transcript starting ≥8 kb upstream to the first gene and spanning the whole domain has been detected in the immature AEV-transformed erythroid cells [Broders et al., 1990]; it is present in normal erythroblasts as well [Broders and Scherrer,

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1987]. The functional significance of this full domain transcript (FDT) is unclear.

In the human genome, the upstream area of the domain of α-globin genes contains the so-called "−14" gene that is transcribed, in erythroblasts and in all other cells tested, in the direction opposite from globin gene transcription [Vyas et al., 1995]. This "−14" gene seems to be a typical housekeeping gene that is permanently transcribed, whereas the expression of the partly overlapping globin gene domain is restricted to erythroid cells at later stages of development. The locus control region (LCR)-type elements controlling the expression of the human α-globin gene domain are located in one of the introns of the "−14" gene [Jarman et al., 1991; Vyas et al., 1995]. It is of interest that, in chicken cells, a sequence corresponding (with more than 70 % homology) to the exon 2 of the "−14" gene has been found about 2.5 kb upstream from the π gene [Vyas et al., 1995], i.e. in the region containing a replication origin and a constitutive site of DNA attachment to the nuclear matrix [Razin, 1987].

It is unclear whether the coordinate expression of the chicken α-globin genes is controlled by dominant positive regulatory elements similar to those in the LCR of the human domain of β-globin genes [Grosveld et al., 1987]. In an attempt to find such regulatory elements, we have previously mapped several DNase I hypersensitive sites (DHS) in the upstream area of the domain. A constitutive DHS flanked by two erythroid-specific DHS was found at a distance of 12.5 kb upstream from the first gene of the domain [Razin et al., 1994]. DHS usually mark positions of various regulatory DNA sequences [Gross and Garrard, 1988], but no particular function could be ascribed a priori to the above cluster of DHS. In order to elucidate possible functions of DNA sequences underlying the identified DHS, we have now cloned and sequenced the corresponding fragment of chicken genomic DNA. The fragment has been screened for the presence of matrix association regions (MARs), which are believed to mark the borders of genomic domains [for a discussion, see Razin, 1996]. Using transient transfection experiments, we analyzed the ability of this whole fragment and of its subfragments to influence expression of a reporter gene driven by the strong promoter of the chicken α^D gene. In addition, the fragments under study were tested for the presence of erythroid-specific promoters and polyadenylation signals. It appears that these DHS demarcate the upstream border of the α-globin domain and might possibly act as negative control elements ("negative LCR") maintaining the globin genes in an inactive state in nonerythroid cells.

MATERIALS AND METHODS

Cell Culture and DNA Transfection

Avian erythroblastosis virus (AEV)-transformed chicken erythroblasts of the line HD3 (clone A6 of line LSCC [Beug, 1979]) were grown in suspension in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS) 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 3 μg of pCAT3 basic vector DNA, or equimolar amounts of DNA of different constructs. The activity of the chloramphenicol acetyltransferase (CAT) gene was assayed in cellular extracts 60 h after transfection.

Preparation of DNA Constructs

Standard protocols were used to clone DNA [Maniatis, 1982]. The chicken genomic DNA library was purchased from Promega (Madison, WI). Plasmid DNA was isolated with the "Wizard Plus" DNA purification system (Promega). DNA sequencing was carried out with an automatic ABI PRISM (model 373) DNA sequencer.

Binding In Vitro of Cloned DNA Fragments to Nuclear Matrices

To isolate nuclear matrices, the cells were washed with cold TM buffer (10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM PMSF) supplemented with 0.2 mM $CuSO_4$ and resuspended in the same buffer. Nonidet P-40 (NP-40) was then added (5% solution up to a final concentration of 0.1%), and the suspension was incubated on ice for 10 min. This was followed by two washes with TM buffer. Permeabilized nuclei were then resuspended again in TM buffer and DNase I was added ≤100 μg/ml. After incubation for 30 min at 37°C, an equal volume of ice-cold extraction buffer (4 M NaCl, 20 mM EDTA, 20 mM Tris-HCl (pH 7.4) was added. After incubation for 20 min at 0°C, the nuclear matrices were precipitated by centrifugation

for 15 min at 1,000*g* and 4°C. The pellet was washed one time with 0.5× extraction buffer and 2× with TM buffer supplemented with 0.25 mM sucrose. The matrices were stored at −20°C in the above buffer supplemented also with 50% glycerol. The MAR assay was carried out exactly as described by Cockerill and Garrard [1986]. The matrix bound DNA was purified by conventional procedure and analyzed using electrophoresis in 1% or 1.5% agarose gels. Digestion of cloned DNA by restriction enzymes and labeling of the DNA fragments were carried out as described [Maniatis, 1982].

Analysis of Chloramphenicol Acetyltransferase and β-Galactosidase Activities in Cell Extracts

Promega assay systems were used in both cases, and enzymatic activity was determined exactly as described in the manufacturer's manuals. To determine the activity of chloramphenicol acetyltransferase (CAT), thin-layer chromatography (TLC) was used. After chromatographic separation, the spots containing nonmodified chloramphenicol and butyrylated forms were scraped from the chromatographic plate, and the radioactive signal was quantified in a liquid scintillation counter.

RESULTS

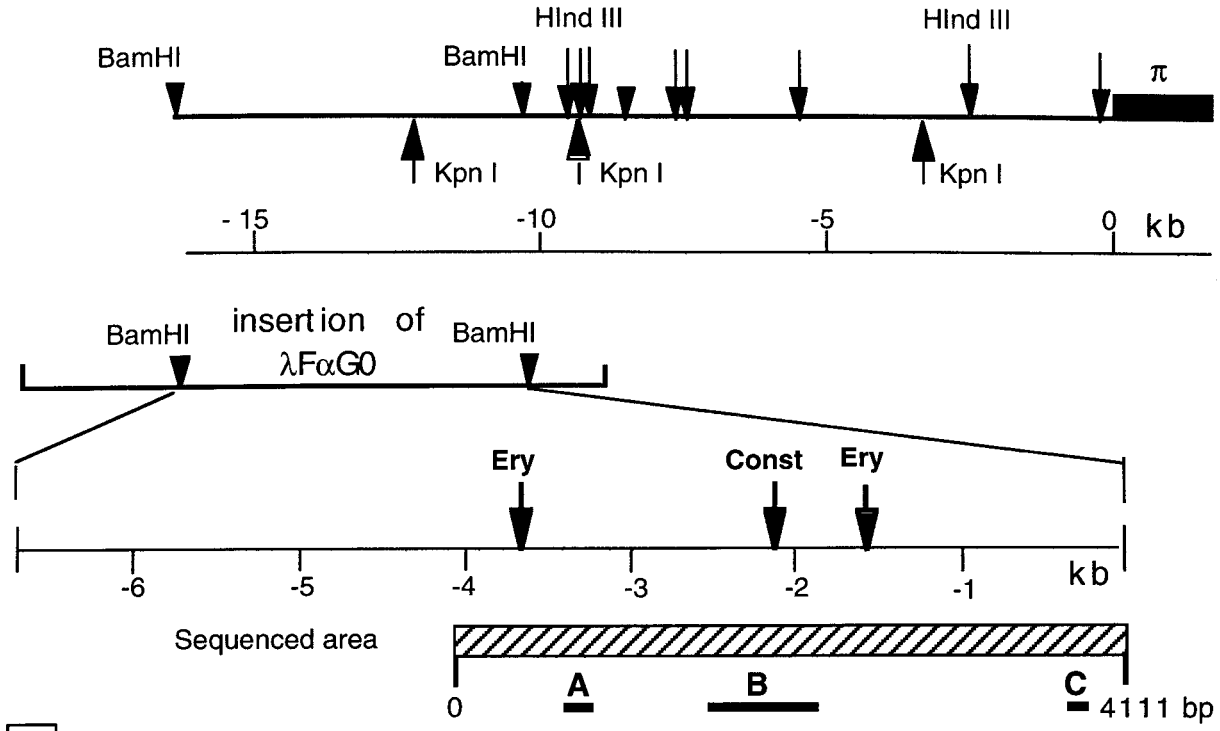
Cloning and Sequencing of the DNA Fragment Containing a Cluster of DNase Hypersensitive Sites Located 11–15 kb Upstream from the Chicken Embryonic α-Type π Gene

The domain of the chicken α-globin genes was originally cloned by Engel and Dodgson [1980]. We have used the upstream part of the insertion of their λCαG6 clone as a probe to screen a genomic DNA library of chicken. Several clones were isolated and one of them (λFαG0) contained the DNA sequences that included the entire cluster of DHS studied in the present work (Fig. 1). The 6.6-kb *Bam*HI fragment containing the previously mapped DHS [Razin et al., 1994] was recloned in pUC18 and the 4.1-kb area containing all constitutive and erythroid specific DHS was sequenced. This sequence is available from the GeneBank (accession number AF067138). Analysis of this sequence revealed a region that had a close homology (87%) to exon 4 of the human "−14" gene (Fig. 1A). The LCR of the human domain of α-globin genes resides within the intron 5 of the "−14" gene [Jarman et al., 1991; Vyas et al., 1995]; no apparent homology to the sequence of this regulatory element was observed within the 4.1-kb fragment of chicken DNA that included the DHS under study. Analysis of the DNA sequence containing the three DHS has shown a number of potential recognition sites for DNA-binding proteins, especially those for GATA-1. Six of them are clustered within a 250-bp region that contains the constitutive DHS (Fig. 1B). The same region also contains a CAC box that is thought to bind transcription activation factor TEF-2 and, surprisingly, three perfect copies of the polyadenylation signal AATAAA (two in the direction of globin gene transcription and one in the opposite direction, see Fig. 1B) and, in the 3′- terminal part, an AT-rich palindrome (Fig. 1C).

Identification of a Matrix Association Region Within the λFαG0 Insertion

The whole insertion of the λFαG0 clone (6.6 kb *Bam*HI-*Bam*HI fragment) was first treated with the *Bgl*II restriction enzyme. The resulting subfragments of 2.75, 2.50, 0.69, and 0.67 kb (see scheme A in Fig. 2) were labeled and assayed for the presence of MARs. The results presented in Figure 2 (see autoradiograph A′ in Fig. 2), indicated that the two smallest *Bgl*II fragments had the ability to bind isolated nuclear matrices in vitro in presence of a vast excess of competitor DNA. To localize the MAR element more precisely, a set of smaller restriction fragments (see scheme B in Fig. 2) was analyzed using the matrix binding assay. The 0.69-kb *Bgl*II fragment was digested with *Nco*I to obtain the fragments of 0.4 kb and 0.29 kb (fragments 3 and 4 in scheme B). The 0.67-kb *Bgl*II fragment (fragment 1 in scheme B) was

Fig. 1. Analysis of DNA sequences located within the cluster of DHS 11–15 kb upstream from the chicken embryonic α-type π gene. Upper: position of the area under study in relation to that of the π gene. Positions of constant (Const) and erythroid-specific (Ery) DHS are indicated by arrows above the detailed map of the 6.6-kb *Bam*HI fragment of the insertion of the λFαG0 recombinant phage. Hatched box, sequenced area; thick lines A,B,C, positions of the fragments (A,B,C) of the sequenced area. **A:** Fragment of the sequenced area with 87% homology to the exon 4 of the human "−14" gene described by Higgs and collaborators [Vyas et al., 1995]. **B:** Part of the sequenced area containing the constitutive DHS. Note the presence of numerous polyadenylation signals (outlined) and binding sites for GATA-1 factor (bold). The CCACCCC motive (bold) is supposed to bind the transcription activation factor TEF-2. **C:** The AT-rich palindrome present in the last 0.9 kbp of the 3′- end of the area under study.



A

836 AGGTTTTCAGATGTCATCCTGGCAACAATTTTGGCTACAAAGTCAGATAT
472 AGGTTTTCAGATGTTATTCTGGCAACAATTTTGGCAACCAAGTCTGAAAT

GTGTGGCAAAAAATTTGAACTGAAGATTGATAACGTTCGCTTTGTTGGTC
GTGTGGCCAAAAATTTGAACTGAAGATTGATAATGTGCGATTTGTTGGGC

ACCCTACGTTGCTTCAGCATGCCCTTGGGCAG 705 Chicken genomic DNA clone α0
ACCCAACACTGCTACAGCATGCTCTGGGGCAG 603 Human mRNA for -14 gene embl x90857

B

1531 ACAAAATAGG AAGGCAAG**TA TC**TACAAGGA GTCTGCCAAA TAAAATACAC
TCACAGCTGC AAAACTGGGT CAGAACTGCA ATGTGACATG GGAAGGATTC
TGCAACCACT TTCCGATGAC TTCACAACAT TGGCCTGGTC TTTAAAAATA
CTCCTTTTCC TCAGGGCATC CCCTGAGACA GCAGAGCTCA ATTCCATAGG
ACCTCAGTGA GCTGCCAGGG GAAGCTCATA GTTGCAGGAT GGGAGCAGTG
TGAGGTTT**GA TA**TTAGCAAT GTTCTTTCCT TGTCTGATTT CCCTGAGACT
CCCATTTTAC CAAATG**GATA TC**AAGTCAGC TTCTGAGGAC ACCACCTTTT
TTAATCATGG CAATATTTAT TTCAGTTTCT TTATTTACAT CCACGATGTG
TGCCTCCATC AGA**CCACCCC** CTCCACCATG CAGGTACCCC A**GATA**ACACA
G**TATC**ACTCA GAAAAAAGGG CAGACATGGG TGTGTTCCAC ACCCACCT**TA**
TCTCTCGGCC ACAAGCAGGC CCTCAGTGCC CACAGAAGTA GTCAATGTAC
CTCATTCCTG GTTCACTCTG AGTGAGTTAT GGTGCATTAC AGAAACTGAG
AC 2133

C

3793 5' AAAAATTCTTTATAATTTT
3830 3' TGTTTAAGAAACATTAAAC

Figure 1.

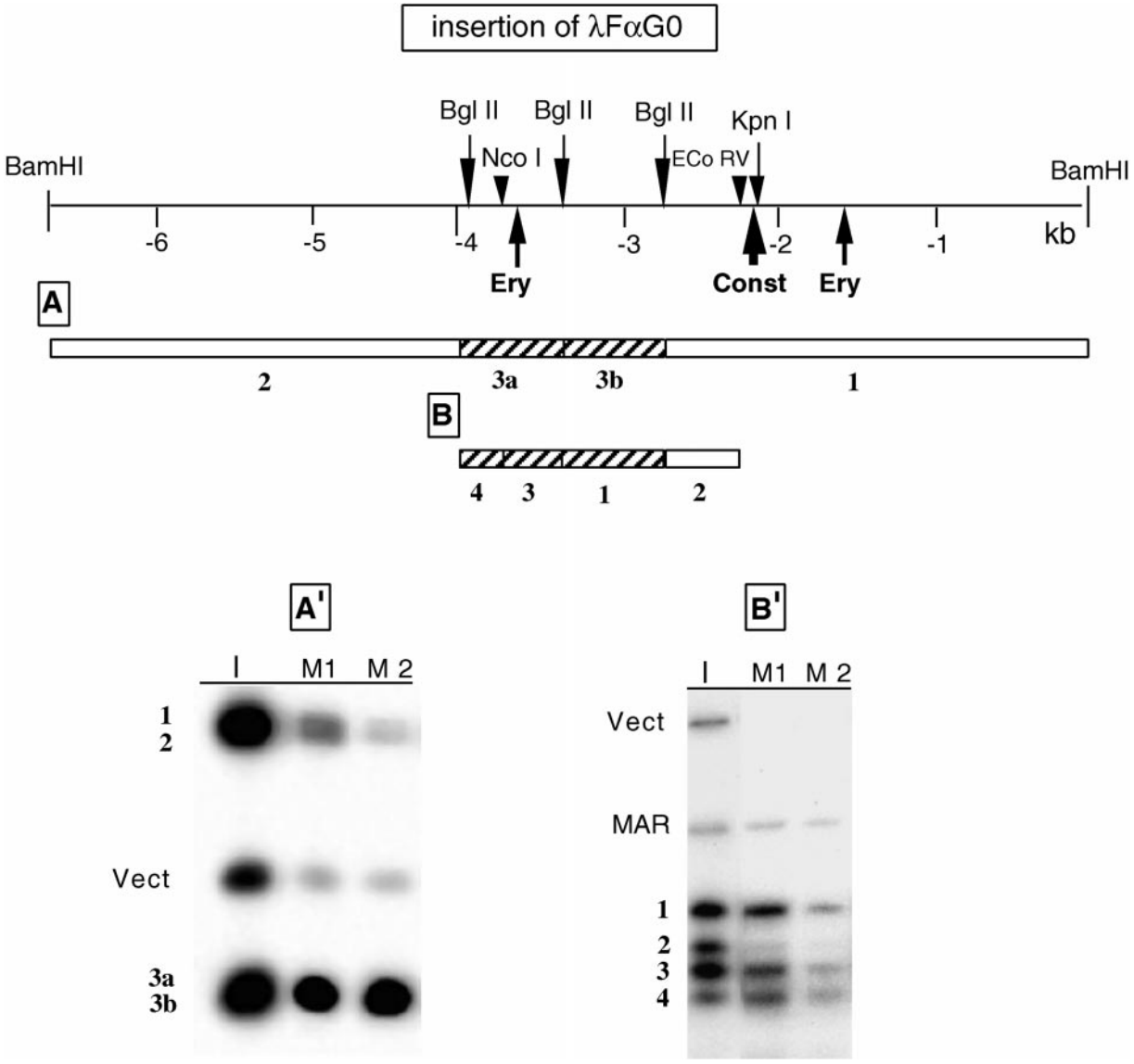


Fig. 2. Mapping of matrix association region (MAR). Upper: map of the insertion of λFαG0. The positions of recognition sites for the restriction enzymes used to prepare the fragments to be tested in matrix-binding assay are shown by arrows above the map of the λFαG0 insertion. Thick arrows below the same map, positions of DHS. Schemes A and B show two sets of restriction fragments tested in matrix-binding assay. Hatched fragments, found to possess properties of MARs. The individual fragments are labeled by numbers according to their sizes. The same numbers are used to indicate these fragments in autoradiographs shown below the schemes. Positions of prokaryotic vector (vect) and MAR from human histone gene cluster are marked correspondingly. I, slots loaded with input DNA; M1 and M2, the slots loaded with matrix-bound DNA obtained using 200 μg/ml (M1) and 400 μg/ml (M2) of a nonspecific competitor (*Escherichia coli* DNA).

as in the previous experiment. The mixture also contained the 0.5-kb *Bgl*II-*Eco*RV fragment flanking the above identified MAR to the right (fragment 2 in scheme B). As a positive control, the bone fide MAR isolated from the human histone gene cluster (1.4-kb *Hin*dIII-*Eco*RI fragment [Cockerill and Garrard, 1986; Mirkovitch et al., 1984] was used. The prokaryotic vector (pBR322; 4.363 kb) in which the above MAR was cloned constituted the negative control. The results of the matrix-binding experiments are shown in Figure 2 (autoradiograph B′). Four of the fragments present in the mixture were retained by the nuclear matrices: the bone fide MAR used as the positive control (1.4-kb fragment), the 0.67-kb *Bgl*II fragment and two subfragments (0.4 kb and 0.29 kb) of the 0.69-kb *Bgl*II fragment. The prokaryotic

vector and the 0.5-kb *Bgl*II-*Eco*RV fragment ("2" in scheme B of Fig. 2) located to the right of the 0.67-kb *Bgl*II fragment were not retained. We conclude thus that the MAR resides within the two *Bgl*II fragments of 0.69 kb and 0.67 kb (i.e., in position 1–1355 of the sequenced area; see Figs. 1 and 2). Interestingly, this region includes one of the erythroid-specific DHS (Fig. 2) and also the region of homology to the exon 4 of the human "−14" gene (see above).

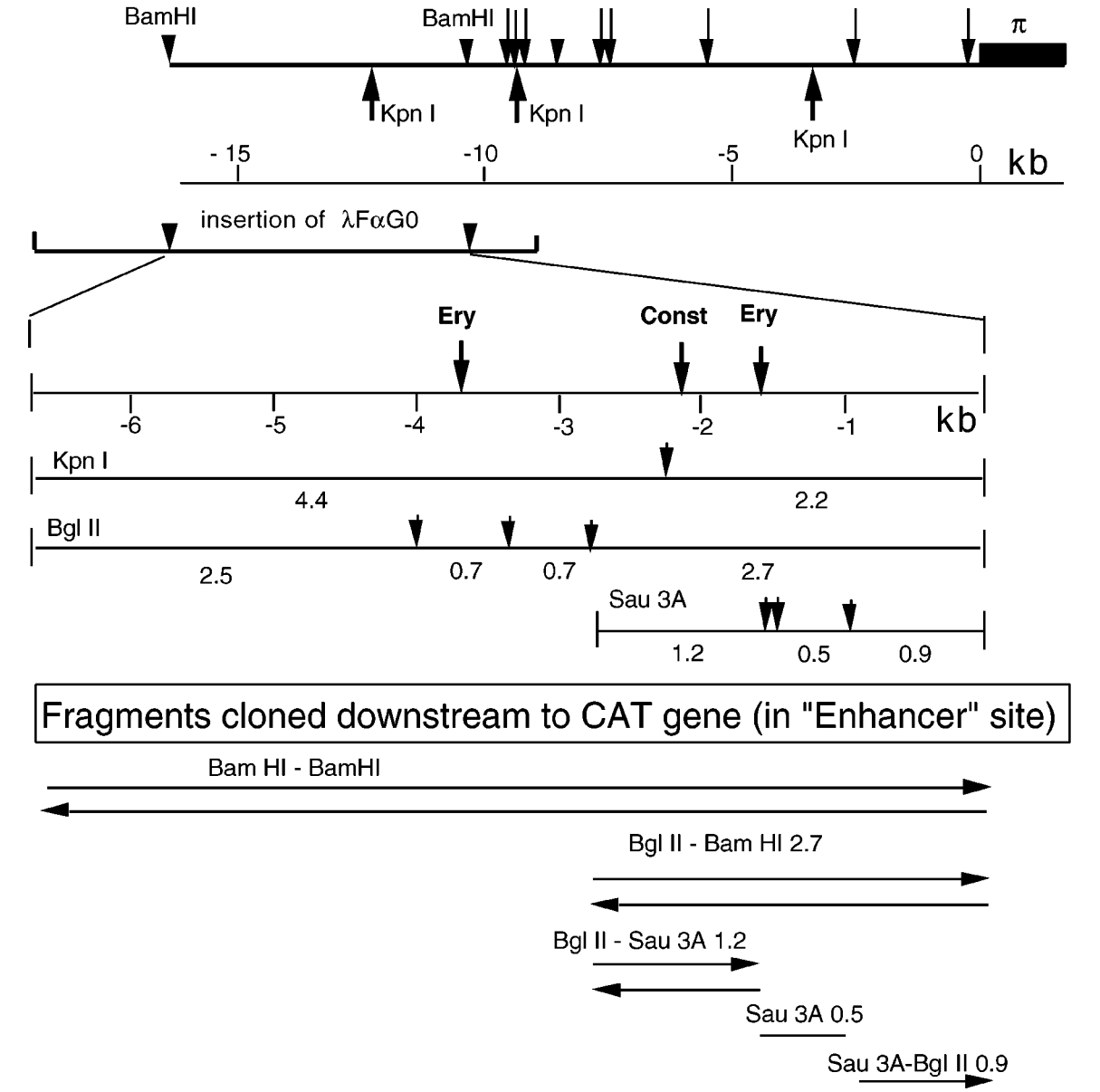
Analysis of the Ability of the DNA Sequences Containing the DHS Under Study to Influence CAT Gene Expression Driven by the Promoter of the Chicken α^D Gene

The activity of promoters of the chicken α-globin genes was studied previously [Knezetic and Felsenfeld, 1989]. The minimal promoter of the α^D gene was found not only to be the strongest one on its own, but also to cooperate with the authentic enhancer of the domain better than promoters of other α-globin genes [Knezetic and Felsenfeld, 1989]. Using polymerase chain reaction (PCR), we amplified this promoter element from chicken nuclear DNA and then cloned it into the *Sma*I site of the pCAT3 basic vector (Promega) to obtain the α^DpCAT3 vector containing the CAT gene placed under the control of the α^D gene promoter (see scheme in Fig. 3). The DNA fragments studied (the 6.6 *Bam*HI fragment described above, which contains the whole cluster of DHS, and several subfragments of this fragment obtained by digestion with *Bgl*II and *Sau*3A restriction nucleases) were cloned into the *Bam*HI site of the α^DpCAT3 vector, i.e., downstream from the CAT gene, as shown in Figure 3.

Equimolar amounts of each construct were transfected into cultured chicken AEV-transformed erythroblasts (line HD3) by means of the "lypofectin" transfection mixture. The construct containing the β-gal gene driven by the SV-40 promoter/enhancer (pSV-β-Galactosidase Control Vector, Promega) was co-transfected in all cases in order to monitor the efficiency of transfection. After cultivation for 60 h, the cells were lysed, and the activities of CAT and β-gal genes were determined by standard procedures, as described under Experimental Procedures. The results of these experiments are presented in Figure 3, which shows the activity of the CAT gene, normalized versus β-gal gene activity (in an average of three independent transfection experiments). One may make several immediate comments on these results. First, it is clear that the "leakage transcription" of the CAT gene in the pCAT3 basic vector is negligible compared with that driven by the α^D gene promoter in the α^DpCAT3 construct (see Fig. 4). Second, the bona fide enhancer (enhancer of the SV-40 virus) ensured a level of CAT gene expression twice that of the enhancerless vector with the same α^D gene promoter. Third, and most important, none of the chicken DNA fragments under study in either orientation activated the expression of the CAT gene driven by the α^D gene promoter. On the contrary, a silencing effect was observed with some of the fragments tested; the most prominent effect (2.5 times) was noted with the 0.9-kb *Bgl*II-*Sau*3A fragment inserted in the direct (globin) orientation. Surprisingly, the latter does not contain any DHS (see map in the upper part of Fig. 3). The only interesting feature of this fragment is the presence of an almost perfect AT-rich palindrome (position 3793–3830 bp on the sequenced area, an 18 bp stem with two mismatches and a loop (see Fig. 1C)). A similar silencing effect was observed when either the 1.2-kb *Sau*3A fragment or the 0.9 kb *Bgl*II-*Sau*3A fragment were inserted just upstream from the α^D promoter instead of downstream from the CAT gene (not shown).

Search for Promoter Activity in the DNA Fragments Under Study

Analysis of the DNA sequence discussed above has revealed a number of TATA motives and potential binding sites for erythroid-specific transcription factors. It was hence reasonable to check whether there is an erythroid-specific promoter within the area under study. A new set of constructs was therefore prepared with the fragments of interest inserted into the promoter site of the pCAT3 basic vector (Fig. 4). These constructs were transfected into HD3 cells, and the activity of the CAT gene product was assayed after 60 h of cultivation. The results of these experiments (Fig. 4) show that none of the fragments tested possesses a promoter active in erythroid cells. In all cases, the expression of the CAT gene product was even lower than that observed after transfection of the pCAT3 basic vector (Fig. 4).



Construct	% CAT activity
αDpCAT3 basic	100
αDpCAT3 -SV-40 enhancer	180
αDpCAT3 BamHI-BamHI 6.6 kb direct	44
αDpCAT3 BamHI-BamHI 6.6 kb reverse	79
αDpCAT3 Bgl II-BamHI 2.7 kb direct	74
αDpCAT3 Bgl II-BamHI 2.7 kb reverse	70
αDpCAT3 Bgl II-Sau 3A 1.2 kb direct	54
αDpCAT3 Bgl II-Sau 3A 1.2 kb reverse	67
αDpCAT3 Sau 3A 05 kb	72
αDpCAT3 Sau 3A 0.9 kb direct	39

Fig. 3. Analysis of the ability of DNA sequences containing the DHS under study to influence expression of the CAT gene driven by the promoter of the chicken α^D gene. Upper: map of the area under study. Positions of restriction sites used to subclone the area under study are shown below this map. Middle: map of the fragments cloned in the downstream "enhancer" site of this vector. Note that the orientation of the *Sau*3A 0.5-kb fragment was not determined. Lower left: α^DpCAT3 vector and the insertion sites. Lower right: CAT activities observed. Note that none of the constructs tested (except that containing the SV-40 enhancer) ensured higher levels of CAT expression than the α^DpCAT3 vector alone and that the strongest silencing activity resides within the 0.9-kb *Sau*3A-*Bam*HI fragment.

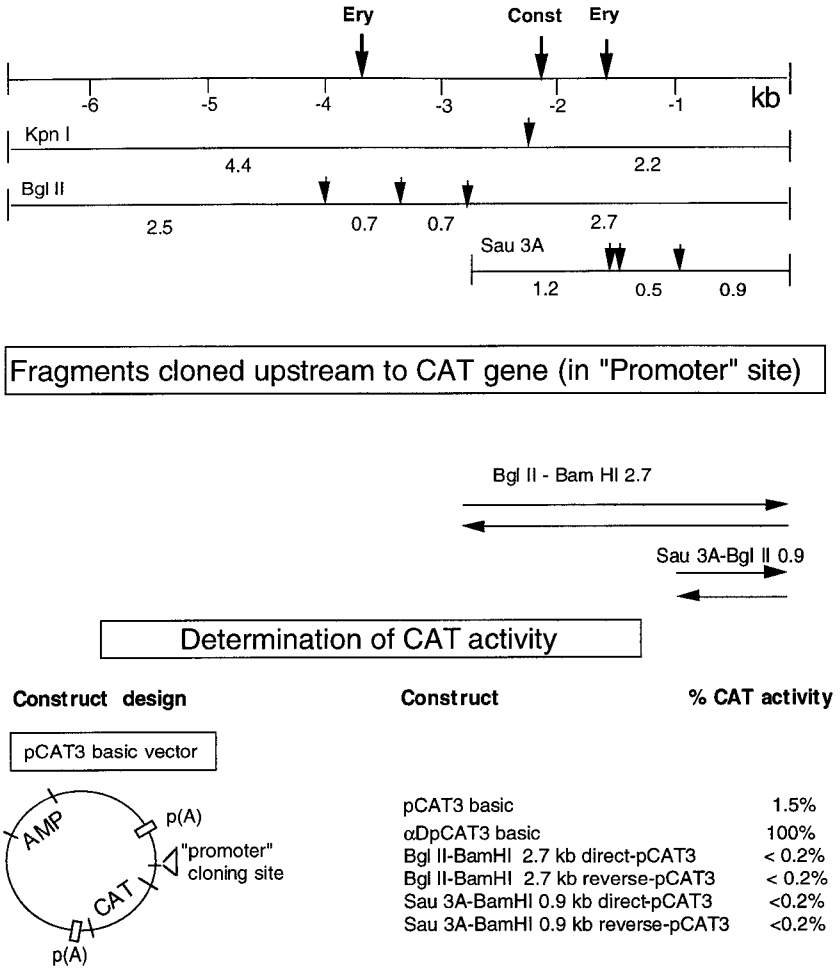


Fig. 4. Search for a promoter activity in the DNA fragments derived from the DNase I hypersensitive region. Map of the region under study as in Fig. 3. The promoterless pCAT3 basic vector was used to clone the fragments shown in the middle. Lower right: results of determination of CAT activity in cellular extracts. Note that none of the constructs tested ensured elevated levels of CAT gene expression as compared with the promoterless pCAT3 basic vector.

Effect of the Presence of Polyadenylation and Transcription Termination Signals Within the DNA Fragments Under Study

In this set of experiments, the DNA fragments containing the DHS were cloned in the α^DpCAT vector immediately downstream from the promoter of the α^D gene (i.e., between the promoter and the CAT gene) as shown in Figure 5. We tested the activity of the CAT gene using the transient transfection assay, as in all previously described experiments. Surprisingly, all fragments tested completely suppressed the activity of the CAT gene. Indeed, as mentioned above, the region under study contained numerous polyadenylation signals (AATAAA elements) that terminate mRNA (see Fig. 1B). It should be pointed out that the promoter of the chicken α^D gene used in our experiments contained the CAP site of the α^D mRNA; all the fragments under study were cloned downstream from this site. Hence the observed suppression effect could not be attributable to the separation of the promoter and the transcription start site by the inserted DNA. It is of interest that the 0.9-kb *Sau*3A-*Bam*HI fragment that contains the AT-rich palindrome mentioned above, but no AATAAA motif, suppressed expression of CAT gene as strongly as the fragments containing "classical" polyadenylation signals. This palindrome may thus correspond to a genuine transcription "terminator" with distinct properties, as compared to AATAAA polyadenylation signals or the putative function of insulators [Wolffe, 1994] as domain boundaries.

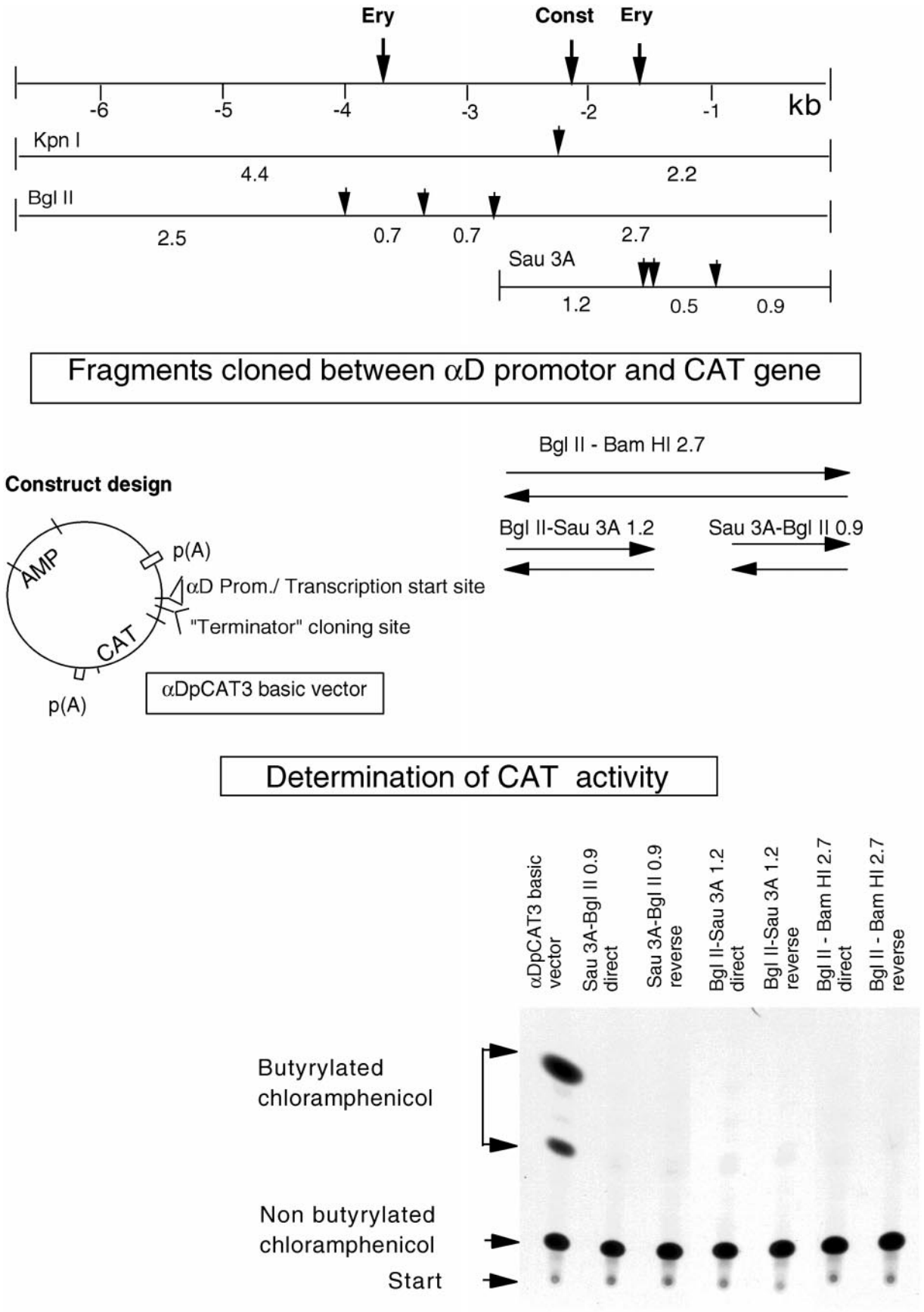


Fig. 5. Expression of the CAT gene by constructs with the fragments under study inserted between promoter (transcription start) site and the CAT gene. Upper: map of the area under study, map of the construct and positions of the fragments inserted in the "terminator cloning site" (i.e., between the promoter and the gene). Lower: results of determination of CAT activity in extracts of cells transfected with equimolar amounts of DNA of each construct are represented. Note that each of the fragments tested completely abolished the expression of the CAT gene product.

DISCUSSION

The domain hypothesis of eukaryotic genome organization has been in discussion for a long time [Scherrer, 1980; Scherrer and Marcaud, 1968]; it was originally based on polytene and lampbrush chromosome organization, the size of "puff" RNA [Edstrom and Daneholt, 1967] and of "giant" globin transcripts [Imaizumi et al., 1973]. The idea became especially attractive when it was demonstrated that DNase I-sensitive chromatin regions extend all over the transcription units and may comprise several genes [Lawson et al., 1982; Reeves, 1984]. Nevertheless, it is still unclear whether this hypothesis correctly describes the general principles of the functional organization of the eukaryotic genome [for reviews, see Bodnar, 1988; Dillon, 1993; Goldman, 1988].

Some of the best-studied genomic domains are probably those of the α- and β-globin genes in humans and chickens. The domains of β-globin genes are characterized by increased DNase I sensitivity in erythroid cells, but not in cells of other lineages. Regulation of expression of β-globin genes is achieved by a complex interaction of promoters of individual genes with erythroid-specific enhancers and also with the locus control region (LCR). The latter seems to regulate the transcriptional status of the human domain, as well as the chromatin conformation and the timing of replication [Forrester et al., 1990]. The domains of α-globin genes seem to be organized differently from those of the β-globins. Already in their initial studies, Weintraub and collaborators [1981] observed that in the chicken some noncoding areas of these domains were characterized by intermediate levels of DNase I sensitivity. More important, they are DNase I sensitive even in nonerythroid cells, in contrast to the domains of the β-globin genes [Craddock, 1995].

Recent observations suggest that continuous transcription of the LCR and intergenic regions of the human domain of β-globin genes may be essential to maintain the whole domain in an open (i.e., accessible to the transcription machinery) chromatin conformation [Ashe, 1997]. This supposition is in agreement with the observation that the upstream area of the chicken domain of α-globin genes is transcribed within a long full domain transcript (FDT) of 17 kb [Broders and Scherrer, 1987; Broders et al., 1990]. Following the above reasoning, one may propose that the borders of the domain-size transcription units are more or less identical to those of the domains. A failure to show at least basal levels of transcription can thus be regarded as a characteristic feature of the domain borders. Clearly, in this case the "terminators," i.e., the DNA sequences preventing the passage of RNA Pol II should be considered as important elements of domain borders. In the area studied in the present investigation we have found a "terminator" activity within two different fragments (see summary scheme in Fig. 6). The nature of the "terminator" signals present within these fragments may be quite different, as one contains numerous polyadenylation signals whereas the only interesting feature of the other is the presence of an AT-rich palindrome.

The present study demonstrates that the DNA sequences located within the cluster of hypersensitive sites 11−14 kb upstream from the π gene interfere with passing of the RNA polymerase II (Fig. 6). Indeed, expression of the CAT gene was abolished completely when the entire DNA fragment under study or smaller subfragments of this area were placed between the promoter/transcription start site and the CAT gene (see map of signals in Fig. 6). One may note in particular the coincidence of the strongest silencing activity with one of the "terminator" signals and the AT-rich palindrome on the terminal 0.9-kb-long fragment of the region under study. Assuming that continuous transcription is a hallmark of active genomic domains [Ashe, 1997; Broders et al., 1990], one may conclude that the cluster of DHS under study constitutes the functional border of the domain of the α-globin genes. The presence of a MAR in the same area corroborates the above supposition; indeed MARs frequently mark the borders of genomic domains [for a discussion, see Razin, 1996]. It is less easy to explain the apparent inability, in our constructs, of transcriptional complexes to pass the region under study in the opposite direction. Because the region is probably located in the intron 3 of the chicken analogue of the human "−14" gene, it hence should be transcribed; however the transcript of the chicken analogue of the "−14" gene has not been characterized as yet. Preliminary data from our laboratory suggest that the upstream area of the chicken domain of α-globin genes is indeed transcribed in both directions (Sjakste et al., in preparation). Nevertheless, we have not yet detected any "giant" transcripts

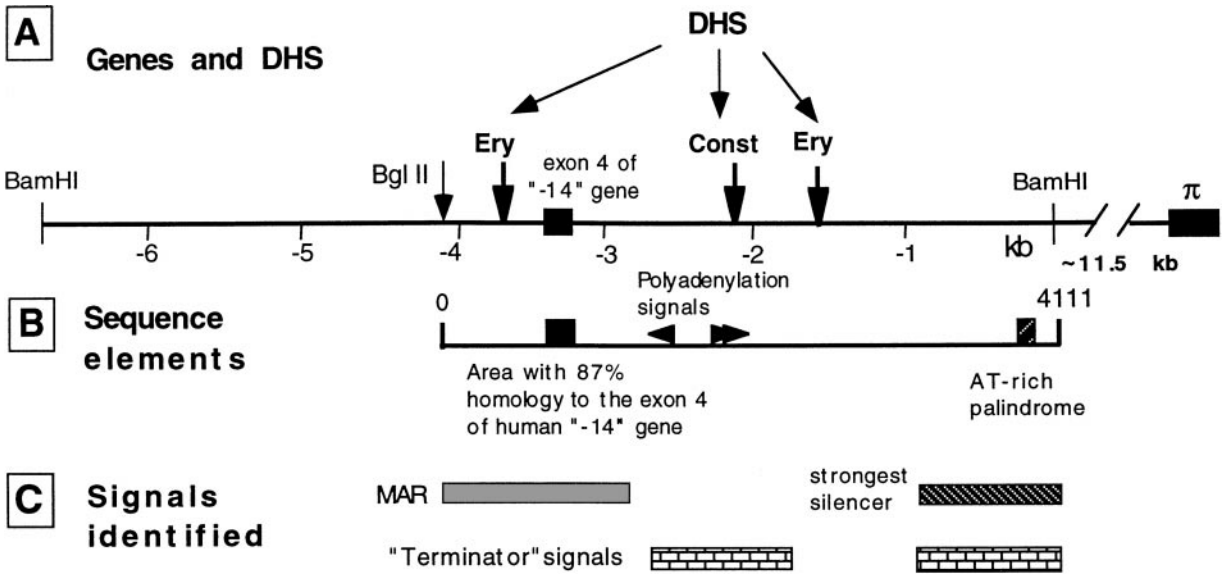


Fig. 6. Scheme illustrating mutual localization of various structural and functional DNA sequence elements placed at the upstream border of the domain of chicken α-globin genes.

going in the direction opposite to that of globin gene transcription. By contrast, several short mRNAs transcribed from the above region in both directions were found in the cytoplasm of chicken erythroid cells (Sjakste et al., in preparation). The pattern of transcription of the upstream area of the domain of the α-globin genes may therefore be different in chickens and humans.

It is tempting to assign the silencing activity found within the region under study to the suppression of the expression of the globin gene locus ("negative LCR") in the great majority of cells which do not express the globin genes. Interestingly, the α-globin genes seem to be present in active (i.e., DNase I sensitive) configuration even in nonerythroid cells [Craddock, 1995]. This idea is indirectly corroborated by the fact that the silencing effect of the full-size fragment containing all three DHS was higher in the globin direction than in the "−14 gene" direction. Although not very prominent, the above "directional" silencing effect was quite reproducible in several experiments. Interestingly, it was lost in shorter fragments lacking the MAR element. Of course, the direct approach to check the above supposition will be to compare the silencing activity of the DNA fragments described here in nonerythroid versus erythroid cells. These experiments are now in progress in our laboratory.

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