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TMEM8 – a non-globin gene entrapped in the globin web

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ABSTRACT

For more than 30 years it was believed that globin gene domains included only genes encoding globin chains. Here we show that in chickens, the domain of α-globin genes also harbor the non-globin gene *TMEM8*. It was relocated to the vicinity of the α-globin cluster due to inversion of an ~170-kb genomic fragment. Although in humans *TMEM8* is preferentially expressed in resting T-lymphocytes, in chickens it acquired an erythroid-specific expression profile and is upregulated upon terminal differentiation of erythroblasts. This correlates with the presence of erythroid-specific regulatory elements in the body of chicken *TMEM8*, which interact with regulatory elements of the α-globin genes. Surprisingly, *TMEM8* is not simply recruited to the α-globin gene domain active chromatin hub. An alternative chromatin hub is assembled, which includes some of the regulatory elements essential for the activation of globin gene expression. These regulatory elements should thus shuttle between two different chromatin hubs.

INTRODUCTION

Experiments on chromosome painting have demonstrated that chromosomes of different vertebrates are constructed from a number of more or less conserved fragments, which are arranged in different combinations in genomes of various species (1). This principle of chromosome organization results in the existence of long syntenic regions in the genomes of different organisms. One such region includes the cluster of α-globin genes and genes located upstream of this cluster in different vertebrates (2). This region of synteny and conserved gene order was initially thought to end right after the cluster of α-globin genes (2). Later it was found that at least one gene (LUC7L) located downstream of the α-globin gene cluster is also conserved in many vertebrates (3,4). In the chicken genome, the *LUC7L* gene is located ~170-kb downstream of the α-globin gene cluster, whereas the *TMEM8* gene is located directly downstream of the cluster of α-globin genes. Several orthologous genes present between *LUC7L* and *TMEM8* (*RGS11*, *AXIN1* and *MRPL28*) are located in inverse order in humans, as compared to chickens. It is thus likely that an ~170-kb genomic fragment located downstream of the α-globin cluster is inverted in the chicken genome as compared to the human genome (Figure 1).

The chicken domain of α-globin genes represents a typical example of functional genomic domains, also known as domains with loosely defined borders (5,6). It is not quite clear how the specificity of action of regulatory systems in these genomic domains, which frequently contain closely located or even overlapping tissue-specific and housekeeping genes, is achieved or what restricts the action of tissue-specific regulatory elements over long distances (6,7). If indeed the downstream area, including most probably the downstream border of the ancestral α-globin gene domain, was disturbed in the chicken genome due to the inversion of a genomic segment, the regulatory system of the domain might also have been disturbed and thus would need to be reestablished *de novo*. In this work, we have studied whether regulatory elements of the α-globin gene domain influence expression of the *TMEM8* gene that was brought into the vicinity of the α-globin gene domain by genomic inversion. In humans, the *TMEM8* gene is highly expressed in the placenta, pancreas and lymphocytes, but not in erythroblasts (8). We have demonstrated that in chickens this gene is preferentially expressed in erythroid cells and is not active in lymphocytes. Furthermore, in chickens, *TMEM8* is

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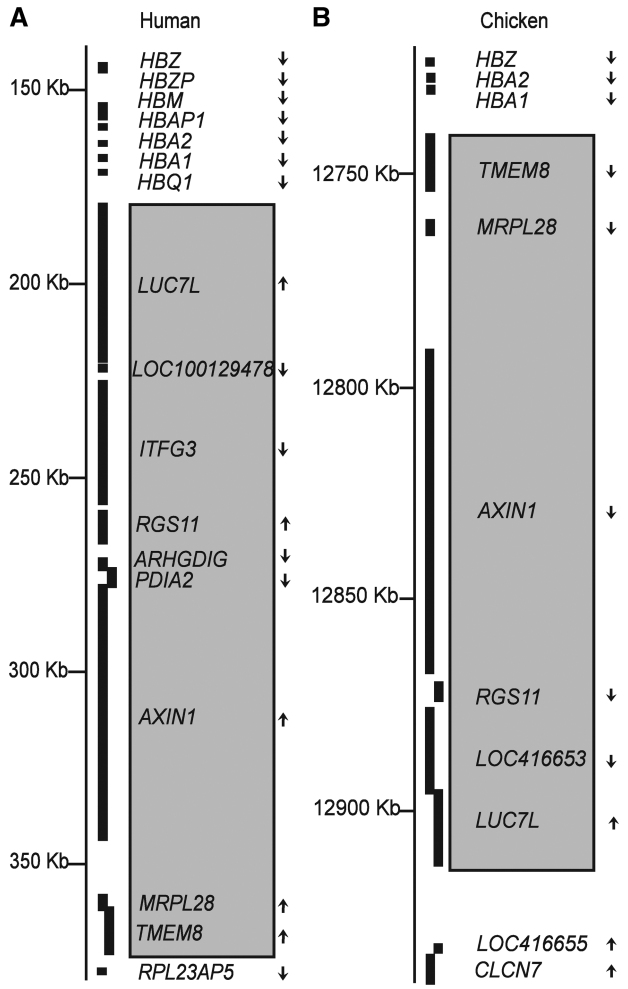

Figure 1. A scheme illustrating the position of an inverted genomic fragment in the downstream flanking area of the chicken α-globin gene domain. (**A**) Positions of globin genes and downstream genes flanking the α-globin gene domain in human chromosome 16. The scale shows actual distances along the chromosome. The genes are represented by filled rectangles. Arrows to the right of the gene names indicate the direction of transcription. The genes located in inverse order (in respect to the α-globin gene domain) in human chromosome 16 and chicken chromosome 14 are shown in a gray rectangle. The names of the genes are given according to the Ensembl Genome Browser. (**B**) Positions of globin genes and downstream genes flanking the α-globin gene domain in chicken chromosome 14. The scale shows actual distances along the chromosome. All designations are the same as in (A).

upregulated upon terminal differentiation of erythroblasts, which does not occur in humans. In accordance with these findings, we have demonstrated that in cultured chicken erythroblasts stimulated to terminal erythroid differentiation, the erythroid-specific downstream enhancer of the α-globin gene domain directly interacts with *TMEM8* and hence is involved in regulation of this gene's expression. Finally, an erythroid-specific enhancer co-localizing with a cluster of GATA1 binding sites was identified in one of the introns of the chicken *TMEM8* gene. These results suggest that in chickens, a non-globin gene, *TMEM8*, became incorporated into the functional domain of α-globin genes.

MATERIALS AND METHODS

Cell culture

The avian erythroblastosis virus-transformed chicken erythroblast cell line HD3 [clone A6 of the line LSCC (9)] and the DT40 lymphoid cell line (CRL-2111, ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% chicken serum and 8% fetal bovine serum (FBS) at 37°C with 5% CO_2. In the case of DT40 cells, the medium also contained 50 μM β-mercaptoethanol. K562 cells were grown in RPMI 1640 medium containing 10% FBS. Chicken embryonic fibroblasts (CEFs) were isolated from 9-day chicken embryos according to the standard protocol (10) and grown in DMEM supplemented with 8% FBS and 2% chicken serum.

Terminal erythroid differentiation of HD3 cells was induced by incubation at 42°C in a 100% air atmosphere in the presence of 20 μm of iso-H-7 (1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride (Sigma Aldrich, USA), as previously described (11). Terminal erythroid differentiation of K562 cells was induced by incubation in the presence of 10 mM sodium butyrate (12).

Source of biological tissues and cells

All experiments were conducted in accordance with Russian regulations for the protection of experimental animals. Stomach, muscle, spleen, brain, fat, lung, heart, liver and whole blood were taken from 5-month-old female chickens. To separate peripheral blood mononuclear cells and erythrocytes, 2 ml of heparinized blood were diluted with an equal volume of balanced salt solution (GE Healthcare, UK) and layered on 4 ml of Ficoll-Paque PLUS (GE Healthcare, UK). After centrifugation at 4°C for 30 min at 400 *g*, mononuclear cells remained at the interphase, while erythrocytes and granulocytes were in the pellet. Mononuclear cells were collected and washed three times with balanced salt solution. The pellet containing mainly erythrocytes also was collected and washed with balanced salt solution.

Extraction of RNA and real-time reverse transcriptase-polymerase chain reaction analysis

RNA was extracted from cells and tissues using 'Trizol' reagent (Invitrogen, Life Technologies, USA). All RNA samples were further treated with DNase I (Fermentas, Lithuania) to remove residual DNA. RNA (1 μg) was reverse transcribed in a total volume of 20 μl for 1 h at 42°C, using 0.4 μg random hexamer primers and 200 U reverse transcriptase (Fermentas, Lithuania) in the presence of 20 U of ribonuclease inhibitor (Fermentas, Lithuania). The cDNAs obtained were analyzed by real-time polymerase chain reaction (real-time PCR) using the CFX96 real-time PCR detection system (Bio-Rad). A PCR mixture in a volume of 20 μl contained 50 mM Tris–HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl2, 0.1% Tween-20, 0.5 μM of each primer, 0.25 μM of TaqMan probe (5′-FAM dye, inside -BHQ-1 quencher), 0.2 mM of each dNTP, 0.75 U of Hot Start Taq

Polymerase (Sibenzyme, Russia) and 25 ng of cDNA template. PCR reactions were performed as follows: initial denaturation for 5 min at 94°C; 60 cycles of 15 s at 94°C, 60 s at 60°C, and then the plate was read. Each PCR was performed in quadruplicate and the corresponding results were averaged. The sequences of the primers and TaqMan probes are available as Supplementary Data (Table S1)

3C analysis

3C analysis was performed as previously described (11,13). A random-ligation control was generated using DNA of a bacterial artificial chromosome containing the chicken α-globin gene domain along with the flanking areas (*Gallus gallus* BAC clone CH261-75C12, CHORI BACPAC Resources Center).

The ligation products were analyzed using real-time PCR with TaqMan probes as described in the previous section. Primers and TaqMan probes for PCR analysis were designed using the DNA sequence of the *Gallus gallus* BAC clone CH261-75C12 (AC172304, GenBank) and Primer Premier 5 computer software (PRIMER Biosoft International). The sequences of the primers and the TaqMan probes are available as Supplementary Data (Tables S2–S4).

To take into account the differences in the efficiency of cross-linking/restriction/ligation and in the quantity of DNA in a 3C templates obtained from cells of different types, an internal standard was used (13). The housekeeping gene *ERCC3* situated on another chicken chromosome was chosen as such standard.

Mapping of DNase I hypersensitive sites

DNase I hypersensitive sites (HSSs) were mapped as described (14). Genomic DNA from DNAse I-treated nuclei was digested with either HindIII or XbaI. After electrophoresis, Southern hybridization with the probe DHS 1 (for DNA digested with Hind III) or DHS 2 (for DNA digested with XbaI) was performed according to standard protocols (15). The probes were obtained using PCR amplification of chicken genomic DNA with the following pairs of primers: DHS 1 dir 5′ ATGGTGGAGAG TCTTGGTATTG 3′ and DHS 1 rev 5′ AAGCAGGAGA TTCACCACAA 3′ or DHS 2 dir 5′ TACGGATTGATG GCTGCTC 3′; DHS 2 rev 5′ TCAGGAGCACCACCTTT AGA 3′.

Isolation of Poly A+ RNA and northern hybridization

Poly A+ RNA was isolated from total RNA samples using 'The PolyATtract mRNA Isolation System' (Promega) and quantified using a Qubit fluorimeter (Invitrogen, USA). After electrophoretic separation in denaturing MOPS-formaldehyde agarose gel, RNA was transferred to a Hybond N+ nylon membrane (Amersham, USA) and hybridized with a ^{32}P-labeled DNA probes specific to the *cTMEM8* gene and to the *GAPDH* gene. The probe for *cTMEM8* was prepared by Random Prime labeling of a 1552-bp fragment of chicken genomic DNA, which was PCR-amplified using the following primers: 5′ CCGTAGATTGTGGGCAGTG 3′ and 5′ AATGTGGCCTTCCCTTGGT 3′. The probe for GAPDH was prepared by PCR amplification of RT product made on poly A+ chicken RNA. The following primers were used for the amplification: 5′ CTGTCAA GGCTGAGAACG 3′ and 5′ GCATCAAAGGTGGAG GAAT 3′.

Electrophoretic mobility shift assay

The Electrophoretic mobility shift assay (EMSA) assay was performed as previously described (7,16). The sequences of test-fragments harboring GATA1-binding sites 1–3 are presented in Figure 8A. A double-stranded DNA fragment (5′ AGTGGGGGCTGCAGGTGGCTG ATAAAGAGC 3′) harboring a known GATA1 binding site and a double-stranded DNA fragment (5′ CTCAGT TGATCTTAGGGCTCTCTCTGCCTG 3′), which does not bind GATA1 protein, were used as specific and nonspecific competitors, respectively (along with poly dI/dC, which was present in all cases).

Transient transfection experiments

The promoter of the *HBA2* gene was obtained using PCR amplification of chicken genomic DNA with the following pairs of primers: αD prom dir 5′ ttagaagcttATGCAGGCT CTTGGCGA 3′ and αD prom rev 5′ tactaagcttGGTGGC AGCTGGTGGG 3′. The DNA fragment containing a putative enhancer was PCR amplified from the same DNA using the following primers: Enh dir 3′ CATACTC GAGTGAGTTTATCCAGCCTT 5′ and Enh rev 5′ CAT ACTCGAGACACAGATGAATCACCAG 3′. The fragments were cloned in an appropriate order (see schemas in Figure 8) into the pGL3 vector (Promega, USA). The test fragment was also cloned into the pGL3 promoter vector (Promega, USA) upstream of the SV40 promoter. All manipulations with recombinant DNAs were performed according to standard protocols (15). Transfection of the luciferase constructs [1 μg of various test constructs + 1 μg of pRLCMV (Promega, USA)] into HD3 and CEF was performed using TurboFect® (Fermentas, Lithuania). The assays for luciferase activity were performed using the Dual-Luciferase® Reporter Assay System (Promega, USA), according to the manufacturer's instructions.

RESULTS

Chicken *TMEM8* gene is preferentially expressed in erythroid cells

The map of the chicken α-globin gene cluster and genes flanking this cluster both upstream and downstream is presented in Figure 2A. To delimit the upstream and downstream boundaries of the erythroid cell-specific gene domain, we have compared transcription profiles of the *C16orf35*, *TMEM8*, *MPRL28* and *AXIN1* genes in erythroid and non-erythroid chicken cells. With this aim, total RNA was isolated from primary chicken fibroblasts (CEFs), cultured chicken lymphoid cells [line DT40 (CRL-2111, ATCC)] and cultured chicken erythroblasts (line HD3, clone A6 of line LSCC) (9,17). The relative

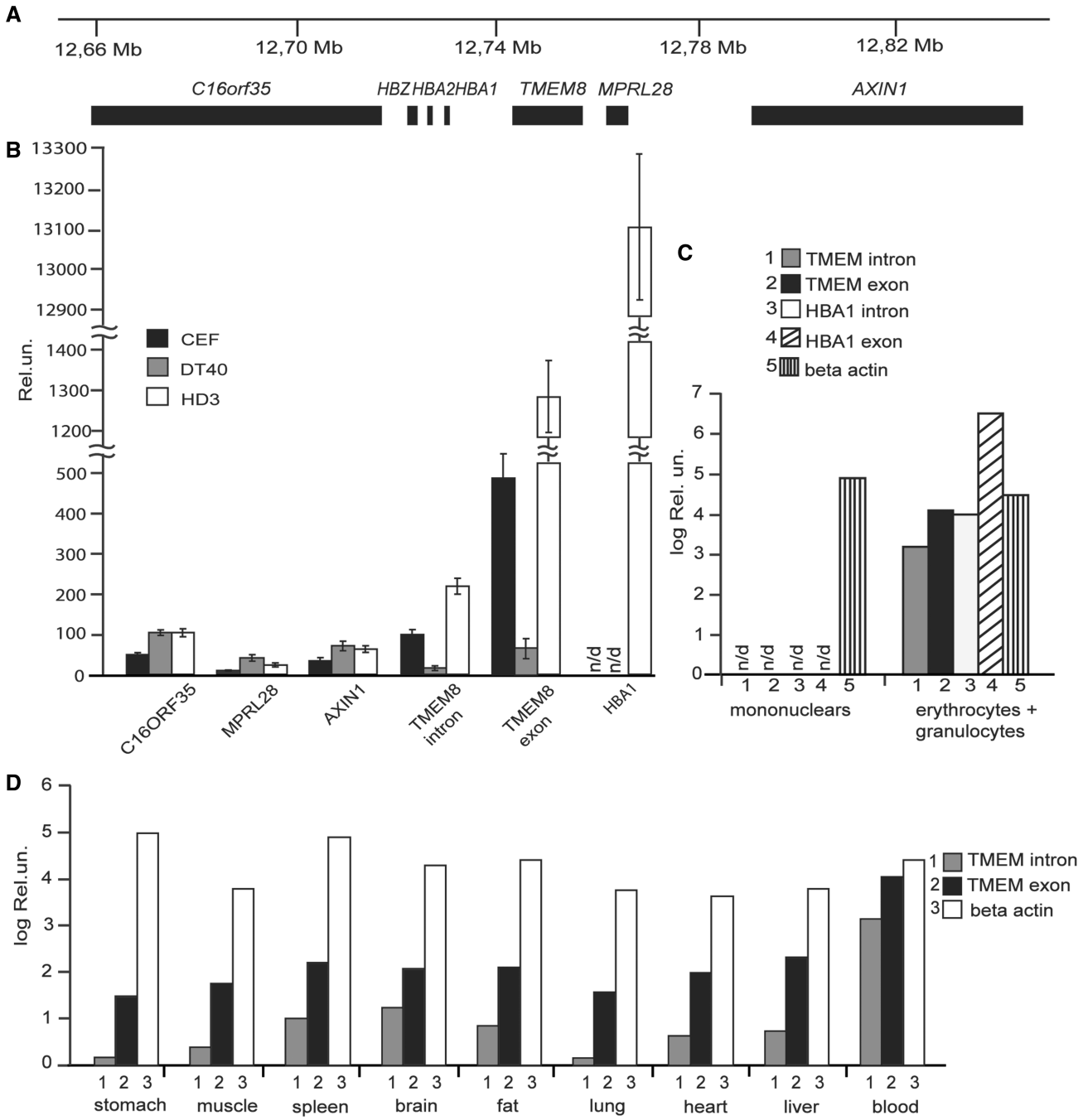

Figure 2. Expression profiles of *cTMEM8* and other genes flanking the chicken α-globin gene domain in cells of different lineages. (**A**) A scheme demonstrating genomic positions of the genes under study. The scale shows distances along chicken chromosome 14. (**B**) Expression of genes under study in cells of different lineages. The genes are indicated below the *x*-axis. The columns in the diagram show the level of mRNA of each gene normalized to the level of 18S rRNA. The steady state level of *HBA1* exonic sequences is shown for comparison. The relative unit (rel. un.) is 10^{-7} of the estimated number of 18S rRNA chains. Error bars represent the SEM of four independent experiments. (**C**) Relative levels of *cTMEM8* and *HBA1* intronic and exonic sequences in RNA isolated from peripheral blood mononuclear cells (mostly lymphocytes) and from a combined sample of erythrocytes and granulocytes (mostly erythrocytes) obtained from the blood of an adult chickens. The level of beta-actin exonic sequence is shown for comparison. The results were normalized as described above (B). 'nd' indicates that the test-sequence was not detected in the analyzed RNA sample. Note that in (C) and (**D**) common logarithms of relative units are shown on *y*-axis. (D) Expression of *cTMEM8* in different tissues of adult chickens. All designations are as in (B).

representation of the intronic regions from the above-mentioned genes in these RNA samples was determined using real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) with TaqMan probes. The results are summarized in Figure 2B. It is evident that, in contrast to the apparent housekeeping genes *C16orf35*, *MPRL28* and *AXIN1*, the *TMEM8* gene (further referred to as *cTMEM8* for chicken *TMEM8*) is

transcribed in proliferating HD3 cells more intensively than in CEF and much more intensively than in DT40 cells. Similar differences between the cell lines were also observed when the abundance of *cTMEM8* exonic regions in total RNA samples extracted from CEF, DT40 and HD3 cells was analyzed. The *HBA1* (a major 'adult' α-type globin gene also known as α^A) mRNA was not at all detected in CEF and DT40 cells. However, in proliferating HD3 cells the steady state level of this mRNA was 10 times higher than the steady-state level of *cTMEM8* mRNA (Figure 2B). It was reported previously that in humans the *TMEM8* gene (further referred to as *hTMEM8* for human *TMEM8*; also known as *M83*) is highly expressed in resting T-lymphocytes (8). To check whether this is also the case in chicken, we assayed RNA samples prepared from erythrocytes and peripheral blood mononuclear cells for the presence of *cTMEM8* intronic and exonic sequences. The levels of β-actin exonic sequences and *HBA1* gene exonic and intronic sequences were also determined. Peripheral blood mononuclear cells were separated from erythrocytes and granulocytes by using centrifugation through Ficoll-Paque PLUS (see 'Materials and Methods' section). The sample of peripheral blood mononuclear cells isolated by this procedure contained mainly peripheral blood lymphocytes (PBLs), contaminated with ~5% of erythrocytes, ~3% of granulocytes and <1% of platelets, as verified by microscopic analysis. The results of the analysis (Figure 2C) demonstrated that *cTMEM8* is either not transcribed or transcribed at very low levels (compared to the level of the β-actin gene transcription) in chicken lymphocytes. At the same time, in erythrocytes, the level of *cTMEM8* RNA was relatively high. Not surprisingly, the *HBA1* gene transcript (both intronic and exonic regions) was detected only in erythrocytes. In the next set of experiments, we studied expression of *cTMEM8* in different tissues of adult chickens. RNA samples were isolated from the stomach, muscle, spleen, brain, fat, lung, heart, liver and whole blood. The quantities of *cTMEM8* intronic and exonic sequences and β-actin exonic sequences in RNA samples prepared from these tissues were determined using real-time RT-PCR. The results presented in Figure 2D made it possible to conclude that the level of *cTMEM8* transcription (as follows from the comparison of the relative abundance of intronic sequences in RNA samples) was at least 350 times higher in whole blood than in any other tissue tested. The level of *cTMEM8* exonic sequences in the whole-blood RNA sample was at least 50 times higher than in RNA samples from other tissues (Figure 2D). As it was demonstrated in the previous experiment that *cTMEM8* was not expressed in lymphocytes, the present data strongly support a conclusion that *cTMEM8* is an erythroid-specific gene. It should be mentioned, however, that the erythroid-specificity of *cTMEM8* is not as strict as that of the globin genes. For example, a relatively high level of *cTMEM8* expression was observed in CEF (Figure 2A). This may reflect some special features of embryonic cells. Nevertheless, even in CEF, the level of cTMEM8 expression was at least two times lower than in proliferating HD3 cells. The difference becomes much more significant (more than 10 times) after induction of terminal differentiation in HD3 cells (see the next section).

Expression of *cTMEM8* is stimulated upon induction of proliferating HD3 cells to terminal erythroid differentiation

AEV-transformed chicken proerythroblasts (line HD3) correspond to chicken hemopoietic cells of the red lineage arrested at early stages of differentiation (9,17). They do not express globins, although the α-globin gene domain resides in an active chromatin configuration (18). After induction of differentiation, HD3 cells stop proliferation and start expression of 'adult' globin genes (11). To find out if transcription of *cTMEM8* is also stimulated upon differentiation of HD3 cells, we analyzed the relative representation of *cTMEM8* intronic and exonic sequences in total RNA samples isolated from proliferating and differentiated HD3 cells. The differentiated state of HD3 cells was confirmed by drastic increase of the steady state level of *HBA1* mRNA monitored by the analysis of abundance of *HBA1* exonic sequences in total RNA samples from proliferating cells and cells induced to differentiation (Figure 3A). The transcription of *cTMEM8* was also induced approximately five times upon differentiation of HD3 cells (Figure 3A). However, the levels of *MPRL28* and *AXIN1* expression are not changed significantly upon differentiation of HD3 cells. In the next experiment, we checked if expression of *hTMEM8* is similarly stimulated upon terminal differentiation of human erythroleukemia cells K562 (12). Analysis of RNA samples from proliferating and differentiated K562 cells (Figure 3B) demonstrated that transcription of *hTMEM8* was not induced upon differentiation of K562 cells, although an induction of *HBA2* (the human ortholog of the chicken *HBA1* gene) was quite obvious. Thus the expression profiles of *cTMEM8* and *hTMEM8* are basically different.

The *cTMEM8* primary transcript is spliced and poly A^+ RNA encoded by *cTMEM8* is accumulated in differentiated HD3 cells

In contrast to humans where both the mRNA and the protein product of the *hTMEM8* gene were characterized (8), *cTMEM8* was identified as an ORF by bioinformatics methods only (XM_425255, XP_425255). Thus we posed the question of whether the transcript of this gene (ORF) is correctly spliced and whether the mRNA encoded by this gene could be identified by northern blot analysis. Based on the predicted intron–exon structure of *cTMEM8* (Ensembl protein coding Gene: ENSGALG00000007471 (UniProtKB/TrEMBL: Q8UWG9_CHICK)), we have designed four pairs of PCR primers located in different exons (see Figure 4A–C). Using these primers for PCR amplification of RT products initiated from random primers on total RNA samples from differentiated HD3 cells, we demonstrated that introns 1, 3, 9 and 11 of *cTMEM8* were correctly spliced. We next performed a northern analysis of poly A+ RNA samples isolated from proliferating and differentiated HD3 cells. The *cTMEM8*-specific probe

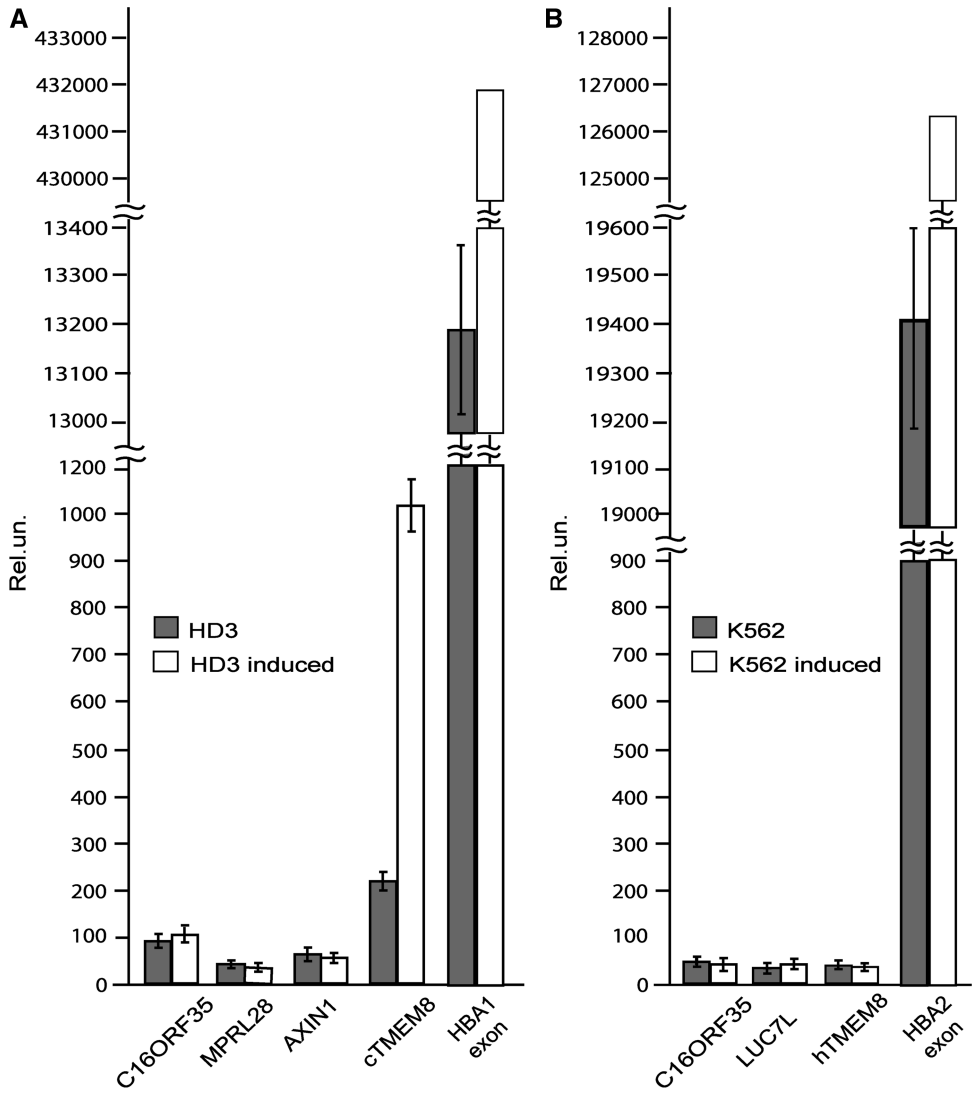

Figure 3. Induction of *cTMEM8* expression upon terminal differentiation of cultured chicken and human erythroblasts. (**A**) Changes in the expression levels of genes located downstream of the α-globin gene domain upon induction of cultured chicken erythroblasts (cells HD3) to terminal erythroid differentiation. The gray and white columns represent correspondingly the abundance of intronic sequences of the genes under study in total RNA samples isolated from proliferating and differentiated HD3 cells. The steady state levels of *HBA1* mRNA (exonic sequences) are presented for comparison. Error bars represent the SEM for four independent experiments. (**B**) Changes in the expression levels of *TMEM8*, and genes flanking the human α-globin gene domain upon induction of cultured human erythroblasts (cells K562) to terminal erythroid differentiation. The steady-state levels of *HBA2* mRNA (exonic sequences) are presented for comparison. Designations are the same as in (A).

identified a single band of ~5 kb present in both RNA samples (Figure 4D). The intensity of this band was, however, much more prominent in the slot loaded with poly A+ RNA from differentiated HD3 cells.

In cultured chicken erythroblasts (line HD3), the *cTMEM8* gene directly interacts with the downstream enhancer but not with the upstream LCR-like major regulatory element of the α-globin gene domain

In the next set of experiments, we checked whether the expression of *cTMEM8* is controlled by known regulatory elements of the α-globin gene domain. We have previously demonstrated that in differentiated HD3 cells, an activating complex [active chromatin hub (19)] is assembled that includes the upstream regulatory element (MRE), the −9-Kb erythroid-specific DNase I hypersensitive site (−9 DHS), the upstream CpG island of the α-globin gene domain, the *HBA2* gene promoter and the downstream enhancer of the α-globin gene domain (11). The simplest supposition was that *cTMEM8* was also recruited to this chromatin hub. To verify this supposition we performed quantitative 3C analysis (11,20). As in the previous study (11), the spatial organization of the analyzed genomic area in lymphoid (DT40) cells and erythroid cells (proliferating and differentiated HD3 cells) was compared. The BamHI and BglII restriction enzymes were used to cut the area of interest into fragments. Positions of PCR primers and TaqMan

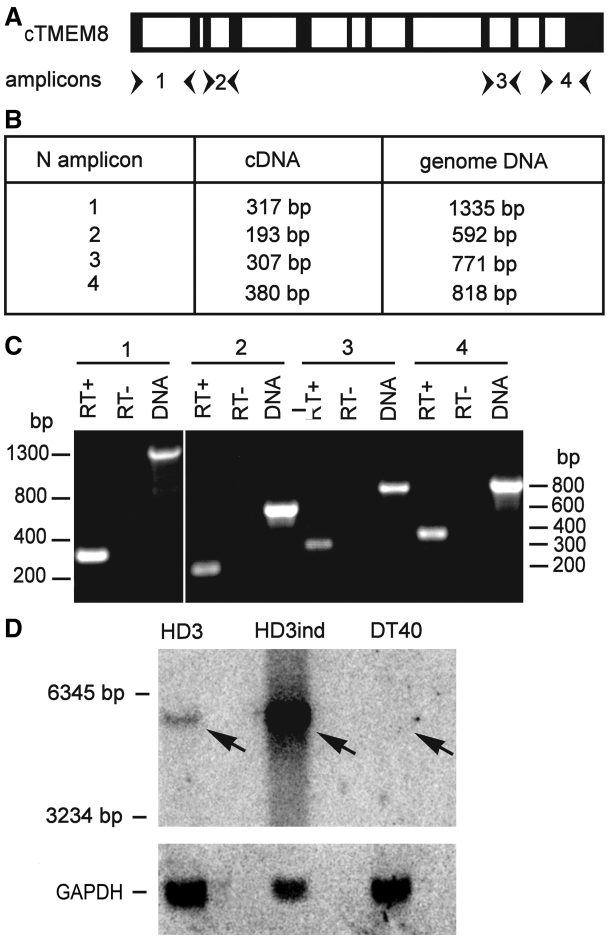

Figure 4. Analysis of *cTMEM8* transcript. (**A**) A scheme showing intron/exon structure of *cTMEM8* (according to the Ensembl Genome Browser) and positions of test-amplicons. (**B**) The expected sizes of PCR products upon amplification carried out on cDNA and on genomic DNA templates. (**C**) Results of PCR amplifications. The figures (1–4) above the picture show the amplicon numbers [as in (A)]. The following pairs of PCR primers were used: amplicon 1–5′ GGTATGGGAACGCCAAGGTC 3′ and 5′CAATTCTGCCAGCAG CCTCA 3′; amplicon 2 - 5′ CCTGTTCTGGAGCCTGGTG 3′ and 5′C CTCTGGAAGGACTGTGGC 3′; amplicon 3 - 5′ AGCTGCACAGAC GATACCA 3′ and 5′TTTCAGAATCTTCTTCACCC 3′; amplicon 4-5′ TCCCTGTCTGTTTGCCCTAA 3′ and 5′ AACCTTACCAGCCC TTCACC 3′. RT+: PCR reaction carried out on reverse-transcription template; RT–: control experiment in which PCR amplification was carried out after 'mock' reverse-transcription without the reverse transcription enzyme; DNA: PCR amplification on genomic DNA. Note that PCR products obtained on the cDNA template (slots 'RT+') have sizes expected for products obtained on a template with excised introns. (*D*) Northern hybridization of TMEM8 probe to poly A+ RNA isolated from proliferating HD3 cells, differentiated HD3 cells and DT40 cells. Position of TMEM8 RNA is indicated by arrows. Note the absence of signal in slot loaded with poly A+ RNA from DT40 cells. The results of hybridization of the same blot with a probe visualizing GAPDH mRNA are shown in a separate section below the results of hybridization of TMEM8 probe. The weaker signal in the slot loaded with poly A+ RNA from differentiated HD3 cells is likely to reflect the decrease of this gene expression upon induction of terminal erythroid differentiation of HD3 cells.

probes are shown in Figure 5A. We first used the DNA fragment containing the MRE as an anchor and analyzed the frequency of association of this fragment with all fragments located downstream of the α-globin gene cluster *per se*, starting from the downstream enhancer. Only association of the MRE with the downstream enhancer in differentiated HD3 cells was detected, which was expected, taking into account the previous data (11). Neither *cTMEM8* nor the DNA fragments flanking *cTMEM8* showed increased association frequency with the anchor fragment containing the MRE (Figure 5B). Quite different observations were made when the anchor was placed on the DNA fragment containing the −9 DHS. In HD3 cells, in addition to the downstream enhancer, this fragment was preferentially associated with two regions located downstream of the enhancer, namely with the DNA fragment containing the CpG island located upstream of *cTMEM8* and the DNA fragment derived from the middle part of *cTMEM8*. The association frequency of the −9 DHS with the middle part of *cTMEM8* was the same in proliferating and differentiated HD3 cells. In contrast, the association frequency of the −9 DHS with the CpG island was 3.5 times more prominent in differentiated HD3 cells than in proliferating HD3 cells. None of these associations was detected in DT40 cells (Figure 5C). It should be mentioned that association frequencies of the −9 DHS with the upstream CpG island of the α-globin gene domain and globin gene promoters were not analyzed in this experiment because this analysis was done previously (11).

When the anchor was placed on the downstream enhancer, a high frequency of association between the CpG island located upstream of *cTMEM8* and the middle portion of *cTMEM8* was observed in differentiated HD3 cells (Figure 5D). In addition, an elevated level of association between the enhancer and the −9 DHS was observed in these cells [in accordance with the previously published results (11)]. None of the above-mentioned associations was detected in proliferating HD3 and DT40 cells.

Taken together, the results presented in Figure 5C and D argue for a supposition that the −9 DHS is involved in the regulation of *cTMEM8* expression in proliferating HD3 cells and that both the −9 DHS and the downstream enhancer of the α-globin gene domain are involved in regulation of *cTMEM8* expression in differentiated HD3 cells. The targets of the above regulatory elements are likely located within the 6-kb BamHI–BglII fragment containing a CpG island and the 6-kb BamHI fragment representing the internal part of *cTMEM8*. To verify the above supposition we have placed the anchor sequentially on each of the two fragments. The results obtained (Figure 5E and F) demonstrate that in differentiated HD3 cells, these fragments interact with each other and also with the −9 DHS and the downstream enhancer. In accordance with the observations made with the anchor placed on the −9 DHS (Figure 5C), in reciprocal experiments we found that the frequency of association of the CpG island with the −9 DHS was much lower in proliferating HD3 cells as compared to the same cells after induction of differentiation (Figure 5E). The frequencies of associations of the middle part of the *cTMEM8* gene with the −9 DHS were confirmed to be similar in proliferating and differentiated HD3 cells (compare Figure 5C and F).

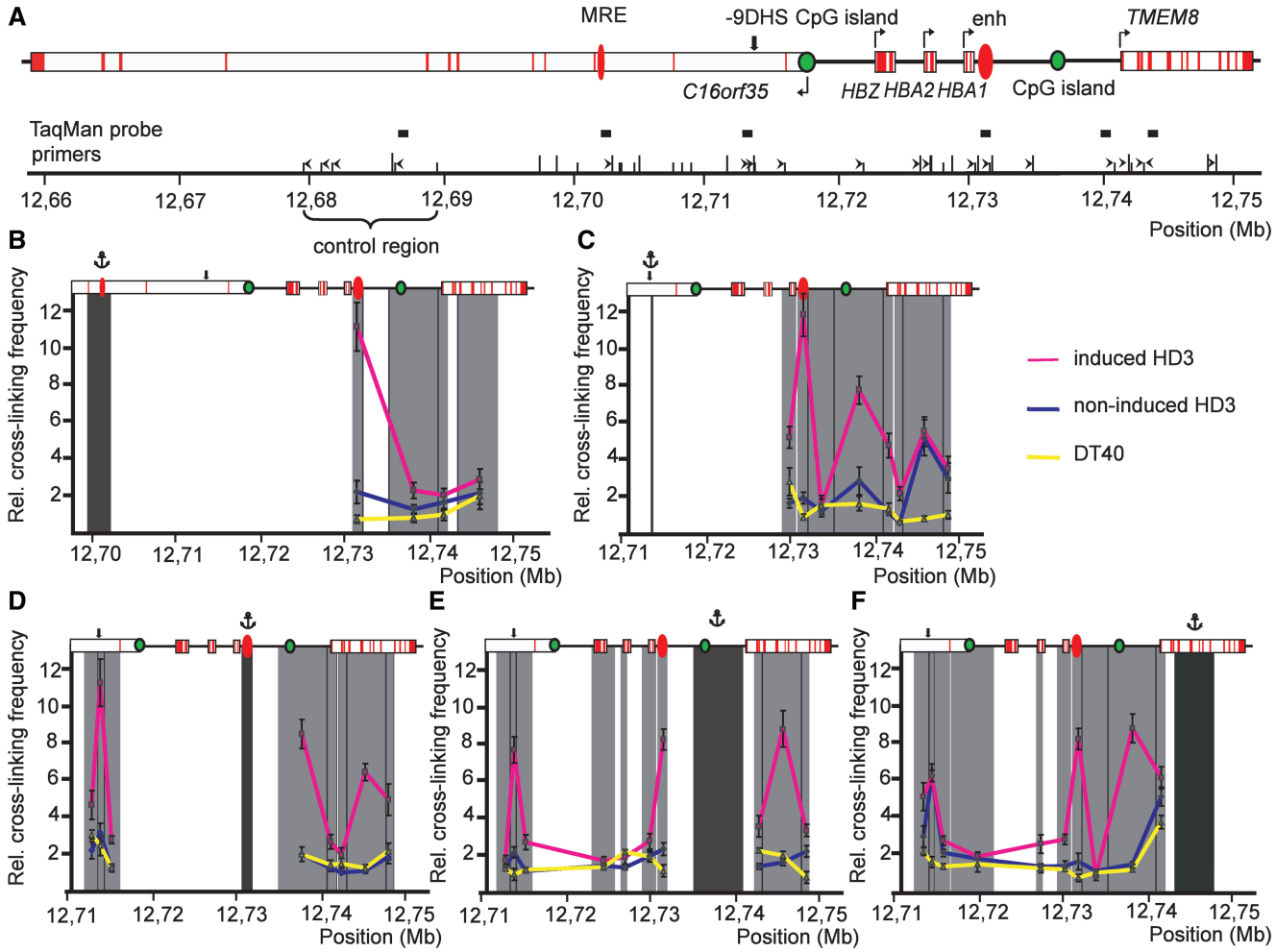

Figure 5. Analysis of the associations of *cTMEM8* with regulatory elements of the α-globin gene domain (BamHI/BglII 3C analysis) in non-erythroid and transformed erythroid cells. (**A**) BamHI/BglII restriction map of the area under study. The scale shows distances along chicken chromosome 14. Boxes represent genes (the red lines represent exons, the arrows show the directions of transcription); filled red ovals represent MRE and the downstream enhancer; the black bold arrow represents the −9-kb DHS; filled green ovals represent CpG islands. The long and short vertical lines above the scale show positions of BamHI and BglII restriction sites, respectively. Primers and TaqMan probes used for the 3C analysis are shown by black horizontal tailless arrows and rectangles, respectively. (**B–F**) Relative frequencies of cross-linking between the anchor fragments bearing (B) MRE, (C) the −9-kb DHS, (D) the downstream enhancer of the α-globin gene domain, (E) the CpG island located between the alpha-globin gene cluster and *cTMEM8* and (F) the middle part of *cTMEM8* and other fragments of the locus. The *x*-axis shows fragment positions according to the restriction map scale. On the top of each graph, a scheme of the domain with the same symbols as in (A) is shown. The results of 3C analysis for induced HD3, cycling HD3 and DT40 cells are shown by the red, blue and yellow lines, respectively. The dark grey rectangle in the background of each graph with the anchor drawn above indicates a fixed (anchor) DNA fragment, and the light gray rectangles indicate test fragments. The borders between the neighboring fragments are indicated by dark gray lines. The areas with white background were not analyzed. Error bars represent SEM for three independent experiments.

Neither of the two fragments in this study interacted with promoters of adult globin genes. This suggests that, due to the two alternative spatial configurations of the α-globin gene domain, the −9 DHS and the downstream enhancer may activate either globin genes or the *cTMEM8* gene. To increase the resolution of 3C analysis, experiments with the anchor fixed at the downstream enhancer were repeated after digestion of fixed chromatin with MboI. In these experiments, only differentiated HD3 cells were studied. Digestion with MboI allowed us to separate the CpG island from other parts of the *cTMEM8* upstream area and also cut the internal part of *cTMEM8* into several relatively short fragments (see the scheme in the Figure 6). The results of the 3C analysis (Figure 6) made it possible to conclude that the downstream enhancer of the α-globin gene domain interacts with the CpG island *per se*. As for the body of *cTMEM8*, the sites of preferential interaction with the downstream enhancer were mapped within introns 3 and 8 of this gene.

DNA fragments from the downstream flanking area of the α-globin gene cluster that interact with the erythroid cell-specific regulatory elements harbor erythroid-specific and permanent DNase I HSSs

To map DNase I HSSs in the downstream flanking region of the α-globin gene cluster, the indirect end-labeling

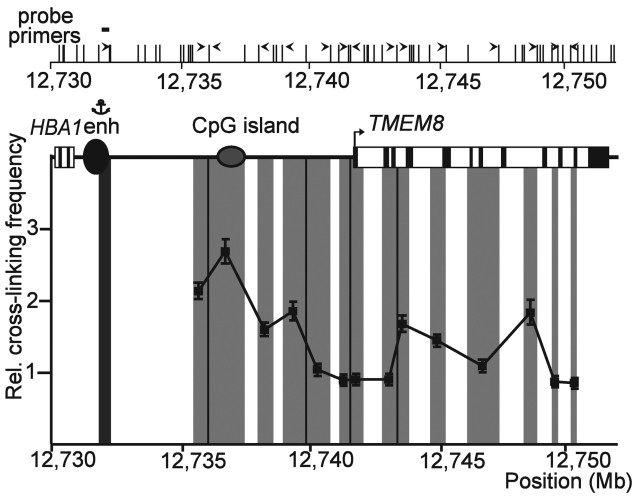

Figure 6. MboI 3C analysis of the associations between the enhancer of the chicken α-globin gene domain and the downstream regions (including *cTMEM8*) in differentiated HD3 cells. In the upper part of the figure, the MboI restriction map of the area under study is shown. The short vertical lines above the scale show the positions of MboI restriction sites. The other designations are as in Figure 5.

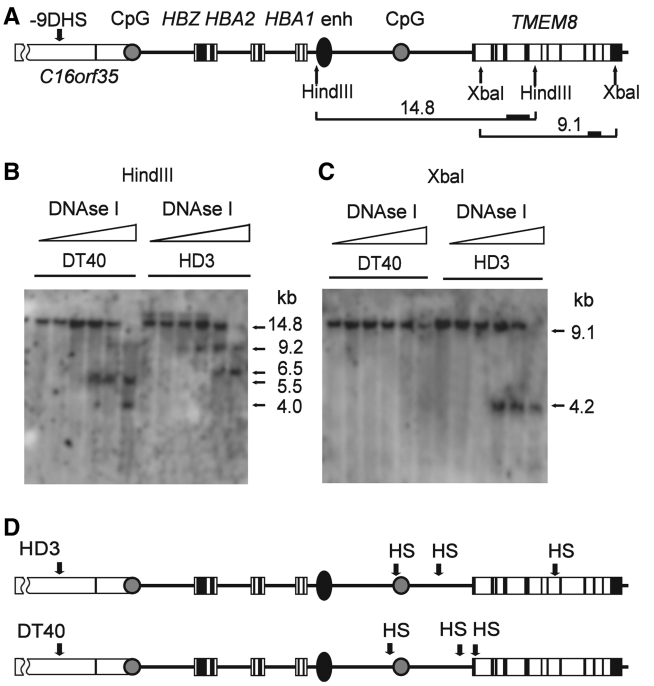

Figure 7. Mapping DNase I DHSs within cTMEM8 and the upstream flanking region of cTMEM8 in DT40 and HD3 cells. (**A**) A scheme of the area under study showing positions of genes (rectangles), CpG islands (filled circles) and the downstream enhancer of the α-globin gene domain (filled oval). The DHSs were mapped within HindIII and XbaI restriction fragments shown below the map. Figures indicate the sizes (in kb) of these restriction fragments. Positions of hybridization probes used for indirect end-labeling are shown by filled rectangles. (**B, C**) Results of hybridization with probes recognizing ends of selected fragments. DNA isolated from nuclei pretreated with increasing amounts of DNase I was additionally digested with (B) Hind III or (C) XbaI and hybridized with the probes shown in (A). Positions of full-sized restriction fragments and additional bands originating due to DNA cleavage by DNase I within DHSs are indicated by arrows at the right sides of the photos. Figures indicate the sizes of fragments (in kb). (**D**) Schemes showing positions of the identified DHSs (black arrows) in HD3 and DT40 cells.

approach (21) was employed. The design of the experiments and the results obtained are shown in Figure 7. Permeabilized cells (DT40 or proliferating HD3) were treated with increasing amounts of DNase I. After purification, DNA was digested with either the HindIII or the XbaI restriction enzyme and subjected to Southern analysis with probes recognizing the downstream ends of a 14.8-kb HindIII fragment harboring the upstream CpG island of *cTMEM8* and a 9.1-kb XbaI fragment harboring virtually all internal portions of *cTMEM8* (Figure 7A). Analysis of hybridization results (Figure 7B and C) has permitted us to localize three DHSs in DT40 cells and three DHSs in HD3 cells (Figure 7D); only one appeared common to both cell lines. A very strong erythroid-specific DHS was mapped to the middle of the *cTMEM8* ORF.

Identification of an erythroid-specific enhancer element in intron 7 of the *cTMEM8* gene

Direct interaction of the downstream enhancer of the α-globin gene domain with the CpG island and the body of the *cTMEM8* gene suggested that partner regulatory elements might be located within these regions. To investigate this possibility, we first searched for clustered GATA1 sites within the 20-kb genomic fragment that included both the CpG island and the body of the *cTMEM8* gene. GATA1 is a transcriptional activator that plays an important role in hematopoiesis (22). It has been reported previously that a cluster of three GATA1 binding sites present in the α-globin gene domain downstream enhancer is sufficient for activation of transcription driven by globin gene promoters (23). We performed *in silico* analysis of the genomic fragment with coordinates 12 735–12 755 (see the map in Figure 5) and found a cluster of three GATA1-binding sites (WGATAR) within intron 7 of the *cTMEM8* gene (Figure 8A). Using a gel retardation assay (16,24), we have demonstrated that each of the WGATAR sites present in the above-mentioned cluster indeed binds GATA1 (Figure 8B). The ~400-bp DNA fragment harboring DNase I HSS and clustered GATA1-binding sites was tested for enhancer activity in transient transfection experiments. With this aim, the fragment was cloned upstream of the firefly luciferase gene driven by the chicken *HBA2* gene promoter. This construct, as well as a similar construct without the tested fragment and a construct lacking both the tested fragment and the *HBA2* gene promoter, were transfected into HD3 cells and CEF. Subsequent analysis made it possible to conclude that the fragment under study ensured ~6-fold stimulation of the HBA2 promoter activity in HD3, but not in CEF (Figure 8C). In the next set of experiments, the DNA fragment under study was placed upstream of the firefly luciferase gene driven by the SV40 promoter. No stimulation of this promoter activity was observed

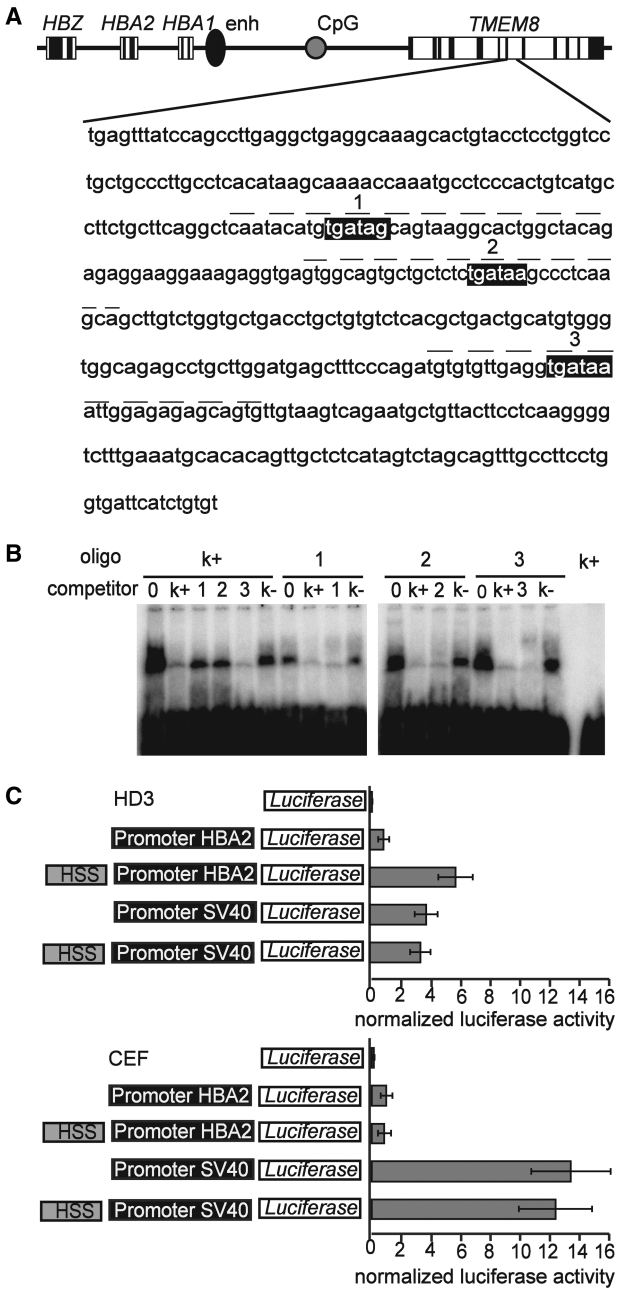

Figure 8. Identification of an enhancer within the *cTMEM8* gene. (**A**) A scheme showing the position and sequence of the DNA fragment under study. Oligonucleotides used in gel-retardation experiments are shown by broken lines. Putative GATA1 binding sites 1–3 are highlighted by black rectangles in the background. (**B**) The results of the gel-retardation experiments. The slots are designated in the following way: upper line. the name of fragment [k+: the control fragment harboring *bona fide* GATA 1-binding site, 1–3: test fragments shown in (A)]; lower line – the type of competitor (k–: fragment which does not bind GATA1; other names are as in upper line). Each sample also contained poly dI/dC (see 'Materials and Methods' section). The last slot was loaded with k+ fragment, which was not incubated with protein extract. (**C**) The results of transient transfection experiments. The constructs are schematically shown on the left side of the diagrams. The diagrams show normalized luciferase activity. The activity observed with the HBA2 promoter without the enhancer was arbitrarily considered as '1.0' and other data were normalized accordingly. Error bars represent SEM for three independent experiments.

in either HD3 cells or CEF (Figure 8C). Therefore, we concluded that the identified enhancer element is erythroid-specific and can activate only erythroid-specific promoters. It is important to mention that the cluster of GATA1 binding sites present in the intron 7 of *cTMEM8* is not conserved in evolution. It particular, this cluster is not present in the corresponding region of *hTMEM8* (Figure S1)

No GATA1 sites were found in the CpG island located upstream *to cTMEM8*, and the functional significance of this element presently remains unclear.

DISCUSSION

The chicken globin genes were among the first eukaryotic genes cloned and sequenced (25–29). Since this time, chicken globin gene domains have become popular models to study the regulation of gene expression (6). The same is true for other vertebrate globin gene domains (30–34). During the last 25 years, the models describing regulation of globin genes expression have become more and more complex. Yet one piece of knowledge about vertebrate globin gene domains had not changed during these years: it remained a common opinion that these erythroid-specific gene domains contained only genes encoding globin chains. Here, we show that, in chickens, the α-globin gene domain also contains *cTMEM8*, an erythroid cell-specific gene, inducible upon terminal erythroid differentiation, which does not encode a globin chain. This gene is likely to fall into the downstream region of the α-globin gene domain because of an inversion of a relatively small chromosomal segment (see the 'Introduction' section and Figure 1). In a prototypical α-globin cluster derived from multispecies comparisons, the place of *cTMEM8* is occupied by *LUC7L* (35), which is located in the downstream flanking region of the α-globin gene domain in genomes of many modern animals (3,4).

TMEM8 (aliases: *M83*, *TMEM6*) is a highly conserved gene that encodes a transmembrane protein with five membrane-spanning domains (8). The protein product of this gene has been characterized only in humans (UniProtKB: Q9HCN3). It was proposed that its biological role is related to the T-cell resting status, as it was highly expressed in resting T lymphocytes and down-regulated by cell activation (8). Although the level of *hTMEM8* transcription in whole-blood samples was found to be relatively high (GNF Symatlas data; http://symatlas.gnf.org), this is likely due to the intensive transcription of *hTMEM8* in resting T lymphocytes present in the whole blood samples (8). *hTMEM8* RNA was not detected in circulating human blood reticulocytes (36) (see also NIDDK Hembase at http://hembase.niddk.nih.gov). In accordance with these data, we have shown that *hTMEM8* is not upregulated upon terminal erythroid differentiation of human K562 cells. In contrast, expression of *cTMEM8* was upregulated upon terminal erythroid differentiation of chicken HD3 cells. Furthermore, the transcript of *cTMEM8* was present in circulating erythrocytes purified from the

blood of adult birds. The level of *cTMEM8* mRNA in circulating erythrocytes was comparable with β-actin mRNA levels and constituted about 0.4% of the *HBA1* mRNA level. On the other hand, *cTMEM8* was virtually not expressed in chicken lymphocytes. In cultured chicken B lymphocytes (cells DT40), the level of *cTMEM8* mRNA was also very low. Based on these observations, we concluded that, after relocation in close proximity to the α-globin gene cluster, *cTMEM8* came under the control of erythroid-specific regulatory elements and acquired an erythroid cell-specific expression profile. The genes *MPRL28* and *AXIN1*, which are located further downstream of the α-globin gene cluster, did not show any elevated expression in HD3 cells (as compared to DT40 and CEF) and were not upregulated upon terminal erythroid differentiation of HD3 cells. The relocation of *cTMEM8* to the proximity of the α-globin gene domain could hardly automatically make this gene erythroid-specific. Indeed, domains of vertebrate α-globin genes belong to the class of functionally determined gene domains (5,6). These tissue-specific gene domains are located in gene-rich areas that also harbor widely expressed housekeeping genes (5,6). One of characteristic features of α-globin gene domains of all vertebrates is that their profile of sensitivity to DNase I does not depend on cell lineage (18,37). In addition, in all vertebrates studied, the MRE of the α-globin gene domain is located in one of introns of the housekeeping gene *C16orf35,* located upstream of the α-globin gene cluster (2,38,39). Cell-linage-specific regulation of α-globin gene expression likely depends on the specific interactions of regulatory elements with the promoters of globin genes (11,40).

In humans, the α-globin gene domain is flanked downstream by the widely expressed (apparently housekeeping) gene *LUC7L* (3,41). This gene is located closer to the α-globin cluster in human chromosome 16 (8.7 kb between the *LUC7L* and the *HBQ1*) than the *cTMEM8* gene in chicken chromosome 14 (12 kb between *cTMEM8* and *HBA1*) and yet it does not seem to be controlled by erythroid-specific enhancers. Thus, location of a gene in close proximity to the α-globin gene cluster is not sufficient to put this gene under the control of globin gene regulatory elements. The presence of erythroid-specific DHSs within the body of *cTMEM8* and in the upstream flanking region of this gene suggests that during evolution, *cTMEM8* acquired erythroid-specific regulatory elements that might be functionally relevant themselves, but even more importantly, assure incorporation of *cTMEM8* into the regulatory network of the α-globin gene domain. One of these regulatory elements is an erythroid-specific enhancer, which we have found in the body of *cTMEM8*. Similar to the downstream enhancer of the α-globin gene domain (23), this additional enhancer likely depends on the GATA1 factor. Incorporation of the *cTMEM8* gene into the regulatory cascade of the α-globin gene domain constitutes an interesting example of the expansion of a functional genomic domain. Further studies of mechanisms regulating *cTMEM8* expression may provide new insights into how functional genomic domains are organized.

Using 3C analysis, we have demonstrated that two previously identified regulatory elements of the chicken α-globin gene domain interact with *cTMEM8* and the upstream flanking region of this gene in terminally differentiated HD3 cells. These regions are the downstream enhancer of the α-globin gene domain (see the scheme in Figure 5) and the as-yet uncharacterized regulatory element that co-localizes with the erythroid-specific −9 DHS.

Taking into account the stimulation of *cTMEM8* expression upon terminal erythroid differentiation of HD3 cells, one could expect that expression of this gene is controlled by regulatory elements of the α-globin gene domain. The unexpected finding was that the configurations of active chromatin hubs regulating expression of adult α-globin genes and *cTMEM8* were strictly different (Figure 9). The alternative chromatin hubs share two regulatory elements (the −9 DHS and the downstream enhancer) while the other players are different. To this end, it may be of interest that neither the −9 DHS nor the downstream enhancer of the chicken α-globin gene domain is present among the previously

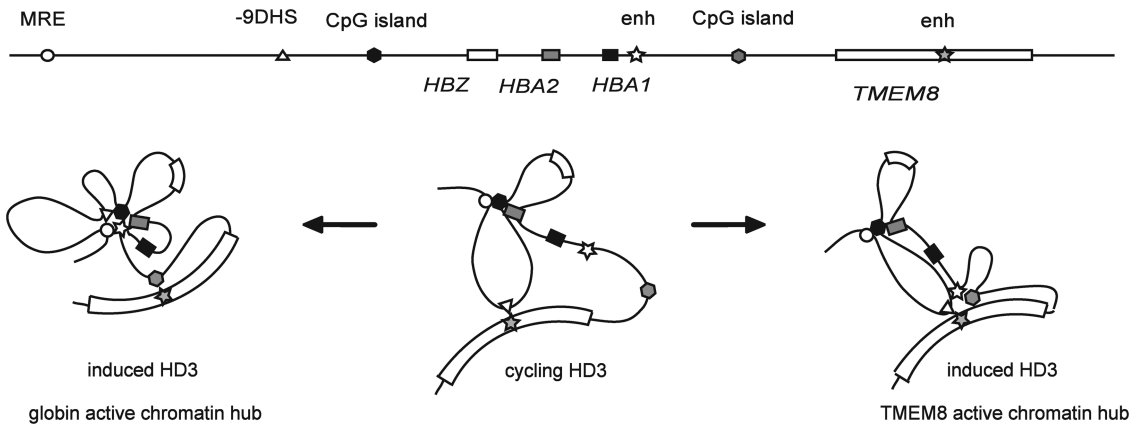

Figure 9. Alternative active chromatin hubs harboring the −9 DHS and the downstream enhancer of the α-globin gene domain. The 'globin' active chromatin hub is presented according to ref. 11.

identified multispecies conserved sequences (35). In other words, these regulatory elements are unique to chickens. It is possible that peculiar properties of the chicken α-globin gene domain regulatory system, especially the existence of the downstream enhancer element, made it possible for this system to redirect the expression profile of *cTMEM8*, making this gene erythroid-specific. It may be of interest that the −9 DHS and the downstream enhancer do not interact with the *HBA1* gene promoter in proliferating HD3 cells. They are recruited to the α-globin gene domain active chromatin hub only upon induction of HD3 cells to terminal erythroid differentiation. Here we show that the same elements participate in organization of the *cTMEM8*-activating complex. In this regard, it is important to note that chicken α-globin genes are not imprinted. They do not show the asynchronous replication pattern typical of imprinted genes (18), and transcription of both alleles can be detected by hybridization *in situ* (42,43). Thus, on each chromosome the −9 DHS and the downstream enhancer should shuttle between two chromatin hubs as predicted by the 'flip-flop' model (44,45). This model was proposed to explain the ability of the β-globin gene domain LCR to activate transcription of several genes that appeared to be transcribed simultaneously. Although this model was never disproved, it was almost forgotten after the discovery of active chromatin hubs (19,31,46–48), as the assembly of an enhancer and several promoters into a single active chromatin hub suggests a more simple explanation for the ability of an enhancer to simultaneously activate several promoters. Our data suggest that the two models are not mutually exclusive and that chromatin hubs should be regarded as dynamic rather than static.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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