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The upstream area of the chicken α-globin gene domain is transcribed in both directions in the same cells

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Abstract It was demonstrated previously that in erythroid chicken cells an extended upstream area of the α-globin gene domain is transcribed in both directions as a part of ggPRX gene and a part of a full domain transcript of the α-globin gene domain. Here, we show that both DNA chains of the above-mentioned region are transcribed in the same cells and that the corresponding transcripts coexist in nuclei. The data obtained suggest that cells possess a molecular mechanism which in some cases prevents the formation of dsRNA and subsequent destruction of both transcripts in spite of the presence of complementary RNA chains in the cell nucleus.
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1. Introduction

It was long ago shown that tissue-specific vertebrate domains of α-globin genes reside in an open chromatin configuration in cells of different lineages perhaps because of the overlapping of the regulatory systems of these domains with housekeeping genes transcribed in the opposite direction [1–5]. More recently it was demonstrated that in erythroid cells an extended upstream area of the α-globin gene domain overlapping the above-mentioned housekeeping gene is also transcribed in the direction of globin gene transcription [6,7]. The role of these globin-direction domain-size transcripts starting close to the major regulatory element of this domain [3] is not yet well established. They might serve to maintain domain potentiation for transcription as proposed by Travers [8]. What seems, however, enigmatic is simultaneous transcription of both DNA chains within a 20 kb long DNA fragment, due to which a sufficient number of complementary RNA molecules capable of reannealing might be created. As a result, the RNA interference (RNAi) defense mechanisms might be triggered and destruction of both transcripts might finally be induced [9–13]. Yet this does not apparently happen in the case of vertebrate α-globin gene domains. One of simple explanations for this controversy is that only a portion of cells transcribes the region in the globin direction while the other portion of cells transcribes it in the opposite direction. Since RNA samples used in biochemical experiments are collected from a large number of cells they thus should contain both transcripts. To check if it is so, we have studied using in situ hybridization with strand-specific probes if the upstream area of the chicken α-globin gene domain is transcribed in both directions in the same cells. This was found to be the case. Thus, the cells possess a mechanism restricting the possibility of formation of dsRNA when it may be disadvantageous for the cell.

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2. Materials and methods

2.1. Cells

AEV cells of the HD3 line clone A6 of the line LSCC [14] were grown in suspension in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum and 2% chicken serum.

2.2. RT-PCR analysis

Total nuclear RNA (1 μg) treated with DNase I (PCR grade) (Invitrogen life technologies, CA, USA) was reversely transcribed into cDNA with the aid of the 1st strand cDNA synthesis kit for RT-PCR (AMV). Each RT reaction was started from either two "up" or two "down" primers (see Table 1). The pairs of RT primers located correspondingly upstream ("up") or downstream ("down") to the PCR primers were used to ensure specific priming of the RT reaction. The synthesized cDNAs were treated with a mixture of RNase H and RNase A and amplified with Taq DNA polymerase (Roche, Switzerland) using a combination of "rev" and "dir" PCR primers (Table 1) The products of PCR reactions were analyzed by agarose gel electrophoresis.

2.3. Preparation of labeled RNA probes

The fragments to be used as templates for synthesis of RNA probes were cloned in pSP73 vector (Promega Life Science, WI, USA) which has promoters for SP6 and T7 RNA polymerases flanking the polylinker on opposite sides. After linearization of plasmid DNA with appropriate restriction enzymes, biotin or digoxigenin-labeled RNA probes were prepared using a SP6/T7 Transcription kit (Roche).

2.4. In situ hybridization

The cells were spread on silane-coated microscopic slides using a "Cytospin" centrifuge. All samples were fixed with paraformaldehyde as described [15]. In situ hybridization with biotin-labeled and digoxigenin-labeled ribo-probes was carried out as described previously [16,17]. After hybridization and removing the non-bound probe, the biotinylated probe was visualized using anti-biotin monoclonal antibodies conjugated with Alexa 488 (Molecular Probes, Niderlands) with

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Table 1
Primers used to start RT reactions and to PCR-amplify fragments of the test regions 1 and 2

Region to be amplified	Type of primer	Sequence of the primer	Expected length of the product
Region 1	RT	up1 CGCTGAACAACGCACCACCTATGCT	421 bp
	RT	up2 CCCTCCTGAGCCACTATTTCACTTC	
	RT	down1 GTAAGTGTCTTGCTCCCATTTGTTG	
	RT	down2 TGAATTGTTCCCTGGTACTCGTCAG	
	PCR	dir CATGAGCTAAGGAAGAACACGAGGC	
	PCR	rev ACGGAGTCGATATGCGGTGAATAGC	
Region 2	RT	up1 CCTGGAGTACCCCAATTATGGAAAA	411 bp
	RT	up2 GTAGCCAAAATTGTTGCCAGGATGA	
	RT	down1 TTGTGAAGTCATCGGAAAGTGGTTG	
	RT	down2 GCAGCTCACTGAGGTCCTATGGAAT	
	PCR	dir TTAGCGGCAGGTTAATGAACTTACT	
	PCR	rev TTTGGCAGACTCCTTGTAGATACTT	

subsequent signal amplification using an Alexa 488 signal amplification kit for mouse antibodies (Molecular Probes). Digoxigenin-labeled probes were visualized using anti-DIG sheep IgG conjugated with rhodamine (Roche) and donkey anti-sheep IgG conjugated with Alexa 546 (Molecular Probes). In all cases the DNA was counterstained with DAPI (4′,6-diamidino-2-phenylindole). The results were examined under a DMR/HC5 fluorescence microscope (Leica) equipped with a HCX PZ Fluotar 100×/1.3 objective and recorded using a CCD DC 350 F camera (Leica).

3. Results

We showed previously that an extended upstream area of the chicken α-globin gene domain is transcribed in both directions as a part of the house-keeping ggPRX gene and as a part of a full domain transcript of the α-globin gene domain [5,6] (Fig. 1). This finding was based on the results of Northern blot analysis with strand-specific probes and the results of RT-PCR amplification of several test fragments using strand-specific primers. As stated in Introduction, this kind of analysis was not sufficient to conclude whether the region under study is transcribed simultaneously in the same cells. To clarify the question, we first carried out a set of in situ hybridizations using ribo-probes representing complementary DNA strands. To make probes, we selected a region located close to the downstream end (in the globin direction) of the symmetrically transcribed area. A ~800 bp fragment mapped in position 39340–40432 on the sequence AY016020 (region 1 in Fig. 1) was cloned in pSP73 vector (Promega) and transcribed in opposite directions using Sp6 and T7 RNA polymerases. The results of in situ hybridizations are shown in Fig. 2. It is clearly seen that both probes gave a strong signal in the nuclei of an absolute majority of the cells. Out of 200 arbitruary selected cells 183 gave positive signal with the probe recognizing

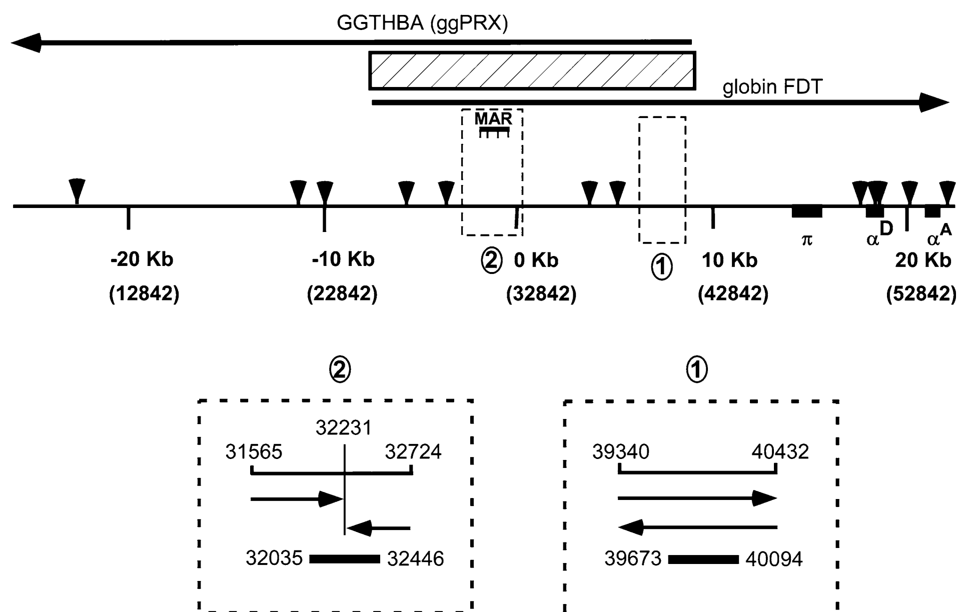

Fig. 1. A scheme of the region under study. The distances on the map are indicated starting from the arbitrarily chosen zero point [6]. The figures in the parentheses show distances on the DNA sequence deposited in the Gene Bank under Accession No. AY016020. Directions of transcription of ggPRX gene and of full domain transcript (FDT) of the α-globin gene domain are shown by horizontal arrows above the map. The area of overlapping is shown by a hatched box. Court filled arrows show the positions of Bam HI recognition sites. Two regions (1 and 2) studied in the present work are outlined by dashed boxes. Below the map the positions of hybridization probes (horizontal arrows) and of RT PCR products (thick bars) are shown. The numeration is according to AY016020.

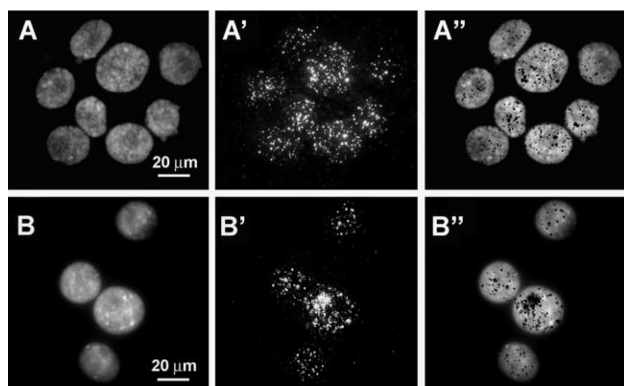

Fig. 2. In situ hybridization with HD3 cells of ribo-probes recognizing the globin-direction transcript of region 1 (A–A″) and the ggPRX-direction transcript of the same region (B–B″). (A,B) Staining of nuclei with DAPI. (A′,B′) Results of hybridization. (A″–B″) Superimposition of hybridization signals (black) and DAPI staining of the nuclei (light). Magnification is shown by bars.

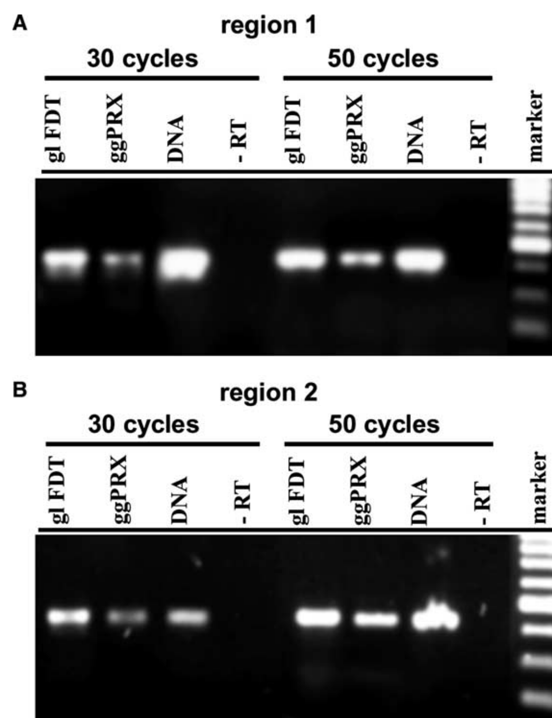

Fig. 3. RT-PCR analysis of RNA transcribed in opposite directions from DNA region 1 and DNA region 2. gl, PCR amplifications on RT products obtained in the direction of globin gene transcription. ggPRX, PCR amplifications on RT products obtained in the direction of ggPRX gene transcription. DNA, control PCR amplification using genomic chicken DNA as a template. RT, control PCR amplification on the products of mock-RT reaction a (reverse transcription enzyme was not added into the reaction mixture). In each case the PCR amplification was carried out for 30 and for 50 cycles to permit better estimation of relative quantities of the PCR products obtained.

ggPRX transcript and 167 gave positive signal with the probe recognizing globin-direction transcript. Taking into consideration the fact that efficacy of FISH with RNA probes never reaches 100%, one can really say that all cells expressed both transcripts. No signal was observed in control experiments, when the probe was omitted or a heterologous (prokaryotic) RNA probe was used for in situ hybridization (not shown). To estimate roughly the intensity of transcription in both directions we also carried out a RT-PCR analysis using strand-specific primers to initiate a RT reaction (Fig. 3). Although this approach is far from being quantitative, it is possible to see that the ggPRX gene transcript is more abundant than the globin-direction full domain transcript.

The fact that ribo-probes transcribed from the same area in opposite direction hybridize with the majority of cells already shows that in a significant fraction of cells the area should be transcribed symmetrically in both directions and hence complementary transcripts should be present in the same cell. To make this conclusion even more clear, an additional experiment was done. Two neighboring fragments of ∼700 and ∼500 bp were selected in the area with coordinates 31565–32724 (region 2 in Fig. 1). The first fragment was transcribed in a direction opposite to the direction of globin gene transcription and labeled with biotin. The second fragment was transcribed in the direction of globin gene transcription and labeled with digoxigenin. Then the mixture of these fragments was hybridized to immobilized HD3 cells and the biotinilated probe (recognizing globin full domain transcripts) was revealed in green color while the digoxigenylated probe (recognizing ggPRX gene transcripts) was revealed in red color. The results of the analysis (Fig. 4) clearly show that virtually all cells contain transcripts made in both directions. It should be stressed that the distribution of both transcripts is strictly nuclear unless one of the probes contains an exonic sequence of an expressed ggPRX gene (Fig. 4D and E). In the latter case the double nuclear centers of RNA accumulation and intensive cytoplasmic staining are clearly seen in full accordance with the previously published data [18]. Double hybridizing spots observed in the most of inspected nuclei clearly indicate that ggPRG gene is transcribed from both chromosomes. To make sure that the oppositely directed probes used in the latter experiment represent parts of longer overlapping transcripts, a 400 bp DNA fragment overlapping the junction of the probes (see Fig. 1, region 2) was PCR amplified on RT products made either in the direction of globin gene transcription or in the opposite direction. In both cases the result was positive (Fig. 3).

4. Discussion

In this paper, two interesting findings are reported. First, it is shown that in the same cells an extended upstream area of the chicken α-globin gene domain is transcribed symmetrically in both directions. Thus the complementary RNA chains can be detected in the cell nuclei. Symmetrical transcription is typical for some viruses bearing overlapping genes and was also reported in yeast cells [19–21]. However, in higher eukaryotes anti-sense transcription is one of the regulatory mechanisms involved in RNA degradation [22–24]. Nevertheless, overlapping genes exist in vertebrate genomes and at least some of them may be quite functional [25]. Our results favor the idea that eukaryotic cells may possess some special mechanism preventing annealing of complementary RNA chains transcribed in opposite directions. This mechanism is very likely to involve fast organization of nascent transcripts in RNA particles and/or interaction of these transcripts with the nuclear matrix. As a matter of fact, it was shown previously that the globin-direc-

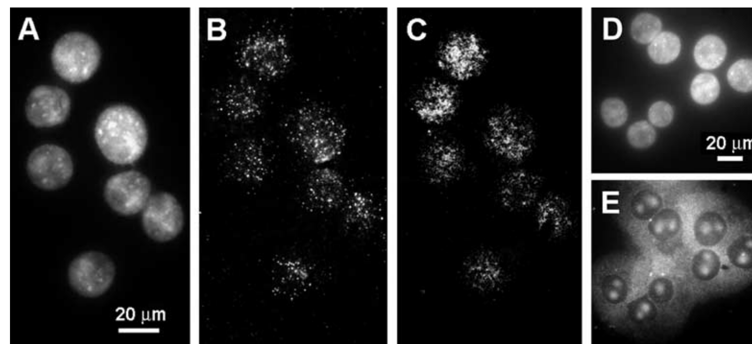

Fig. 4. Double immunostaining of HD3 cells hybridized with the ribo-probe recognizing the globin-direction transcript of region 2 (B) and with the ribo-probe recognizing the ggPRX direction transcript of region 2 (C). Counterstaining of nuclei with DAPI is shown in A. (D,E) In situ hybridization to HD3 cells of the globin-direction probe with coordinates 31565–32231 (i.e., the probe complementary to that used to visualize globin FDT in the experiment shown in section "C"). This probe recognizes exon 4 of the ggPRX gene and thus stains RNA present in cytoplasm (panel "E"). Counterstaining of the same group of nuclei with DAPI is shown in panel "D".

tion domain-size transcripts constitute a part of the nuclear matrix [6]. It is not clear how the same area may be simultaneously transcribed in opposite directions. One of the possibilities is that globin direction transcripts and ggPRX gene are transcribed from different chromosomes. However, our results clearly show that ggPRX genes is transcribed from both chromosomes (Fig. 4D and E). The situation with globin-direction transcripts is less clear. When nuclear distribution of these transcripts was studied in non-induced HD3 cells, no apparent spots corresponding to the sites of transcription were seen (this paper and also [6]). This may reflect relatively long life time of these transcripts that become integrated into the nuclear matrix [6].

The second interesting observation made in this study is that the strong matrix attachment region (MAR) located in the area 31500–32500 at AY016020 is transcribed in both directions. This result reinforces our previous conclusions that MARs do not present a barrier for transcription [16,17,26].

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