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ARTICLES

The 33 kb Transcript of the Chicken α-Globin Gene Domain Is Part of the Nuclear Matrix

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Abstract Giant nuclear transcripts, and in particular the RNAs of the globin gene domains which are much larger than their canonical pre-mRNAs, have been an enigma for many years. We show here that in avian erythroblastosis virus (AEV)-transformed chicken erythroleukaemic cells, where globin gene expression is abortive, the whole domain of α-globin genes is transcribed for about 33 kb in the globin direction and that this RNA is part of the nuclear matrix. Northern blot hybridisation with strand-specific riboprobes, recognising genes and intergenic sequences, and RT-PCR with downstream primers, show that the continuous full domain transcript (FDT) starts in the vicinity of a putative LCR and includes all the genes as well as known regulatory sites, the replication origin, and the DNA loop anchorage region in the upstream area. Absent in chicken fibroblasts, the globin FDT overlaps the major part of the *ggPRX* housekeeping gene that is transcribed in the opposite direction. RT-PCR and in situ hybridisation with genic and extra-genic globin probes demonstrated that the globin FDT is a component of the nuclear matrix. We suggest that the globin FDTs keep the domain in an active state, and the globin RNAs on the processing pathway are a component of the nuclear matrix. They may take part in the dynamic nuclear architecture when productively processed, or turn over slowly when globins are not synthesised. J. Cell. Biochem. 92: 445–457, 2004.

Key words: gene expression; globin; nuclear matrix; RNA processing; LCR; genomic domains; FDT

The domain of the chicken α-globin genes, analysed since 1966 [Scherrer et al., 1966] in our laboratory as well as by others, is at present one of the best known genomic areas [Recillas Targa et al., 1995; Recillas-Targa and Razin, 2001]. In recent years, we have been able to identify the extent of this domain by mapping erythroid-specific DNase I hypersensitive sites [Razin et al., 1994] and matrix attachment regions (MARs) [Razin et al., 1991b, 1999; De Moura Gallo et al., 1992]. Several controlling sites including a negative regulatory element placed at −12 kb [Razin et al., 1999] and a CpG island [Razin et al., 2000] just upstream of the cluster of the three α-type globin genes, π, α^D, and α^A, were found. Control of gene expression is particularly enigmatic in the case of this globin gene domain since the *ggPRX* housekeeping gene is transcribed on the opposite strand [Sjakste et al., 2000]; this implies that, in most cell types, chromatin is in an "open" configuration. Analysis of transcription of this domain, of which we have now sequenced over 29 kb (accession number AF 098919), was also the basis for the original proposition of the pre-messenger RNA concept [Scherrer et al., 1966; Imaizumi et al., 1973]. Later, we were able to demonstrate the existence of giant α-globin transcripts called full domain transcripts

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(FDTs) covering all of the chicken α-globin genes [Broders and Scherrer, 1987; Broders et al., 1990]. The significance of this type of globin transcripts, observed in addition to the conventional pre-mRNAs of each gene, 1.0, 1.3, and 1.5 kb long, respectively [Therwath and Scherrer, 1982], is not understood as yet. Recently, several reports on "inter-genic" globin transcripts have sparked renewed interest in such transcription [Ashe et al., 1997; Gribnau et al., 2000]. We, therefore, decided to investigate the nature of this globin FDT further in order to better define its structure and, possibly, elucidate its significance in relation to globin gene expression.

In the past, we had found that avian erythroblastosis virus transformed chicken red blood cells (AEV cells), where globin gene expression is abortive, have nuclear transcripts that are similar to those of normal erythroblasts, isolated from chicken bone marrow [Broders and Scherrer, 1987]. Furthermore, we were able to show that the amount of nuclear transcripts of α-globin genes in induced cells expressing haemoglobin is similar to that in the non-induced AEV cells [Therwath and Scherrer, 1978], where globin gene expression is blocked at post-transcriptional levels and the globin FDTs turn over, apparently idly. In view of the possibility to study these thermosensitive erythroleukaemic cells in both states, proliferating or induced and differentiating, we concentrated our recent studies on the AEV cell system [Iarovaia et al., 2001]. The latter study showed clearly that abortive globin gene expression in dividing AEV cells is characterised by modified processing patterns of the globin RNA but seemingly normal levels of transcription.

Here we show that the globin FDTs cover the entire chicken domain of α-globin genes. They are, thus, not "inter-genic" transcripts [Ashe et al., 1997; Gribnau et al., 2000] but rather supra-genic and, thus, apparently well described by the term "FDTs" [Broders et al., 1990]. These extend from the putative upstream LCR [Flint et al., 2001], across two controlling areas mentioned above [Razin et al., 1999, 2000], all three globin genes and the 3′-side enhancer/silencer area [Recillas Targa et al., 1995]. Interestingly, we found also that the FDTs are constituents of the nuclear matrix. The notion is entertained that globin FDTs serve in the nuclei of AEV cells at the level of the nuclear matrix, possibly representing a structural basis of the dynamic nuclear architecture, whether there is haemoglobin expression or not.

MATERIALS AND METHODS

Cell Culture

AEV cells of the line HD3 (clone A6 of line LSCC) were grown in suspension in Dulbecco's modified Eagles medium supplemented with 8% foetal bovine serum and 2% chicken serum. HD3 cells are chicken erythroid cells transformed by a thermosensitive mutant of avian erythroblastosis virus. They correspond to chicken haematopoietic cells of the red lineage arrested in early stages of differentiation [Therwath and Scherrer, 1974; Beug et al., 1982]. Primary chicken fibroblasts were prepared by conventional techniques from 9-day chicken embryos.

Isolation of Nuclear RNA

The cells were lysed and fractionated into nuclei and cytoplasm [Broders et al., 1990]. The nuclei were treated with DNase and proteinase K and RNA was isolated by hot-phenol extraction [Scherrer, 1969].

RNA Electrophoresis and Northern Hybridisation

RNA was fractionated by electrophoresis in 0.8% agarose gels in a TBE buffer-formaldehyde mixture according to the published protocol [Sambrook et al., 1989]. RNA (20 μg) was loaded in each slot after denaturation in formaldehyde-formamide-TBE mixture at 65°C, 5 min, in the presence of EtBr, followed by 5 min on ice. After electrophoresis, the RNA was transferred onto Hybond N^+ filters (Amersham Biosciences, UK) in a 20× SSC solution as described in the manufacturer's manual. Hybridisation of radioactive probes to RNA immobilised on Hybond N^+ filters was performed in Rapid Hyb Buffer (Amersham) according to the protocol of the manufacturer.

Synthesis of RNA Probes

The fragments of the chicken α-globin gene domain obtained by digestion of the original λCαG5 and λCαG6 clones of Engel and Dodgson [1980] with either *Hind*III or *Bam*HI restriction endonuclease (scheme in Fig. 1) were recloned in the vector pSP73 (Promega Life Science, Madison, WI), with promoters for SP6 and T7 RNA polymerases. After linearisation of plasmid DNA with the appropriate restriction

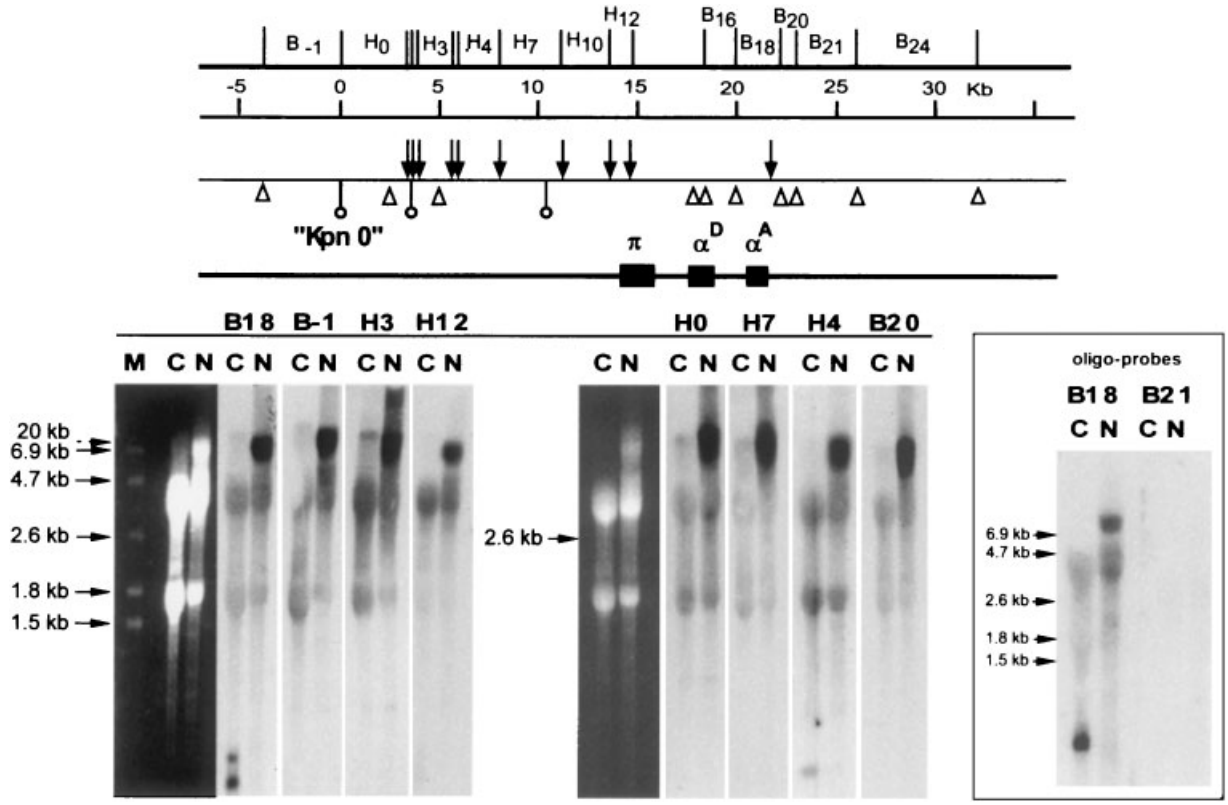

Fig. 1. Northern analysis of the transcription pattern of the α-globin gene domain in avian erythroblastosis virus (AEV) cells. The map of the domain is shown at the **top** of the figure. Filled arrows above the restriction map show the positions of *Hind*III sites. The tail-less open arrows below the map show the positions of *Bam*HI restriction sites. The names of the restriction fragments used to prepare the globin direction-specific probes are indicated above the distance scale. Positions of α-globin genes are given by rectangles below the restriction map. The results of hybridisation are shown at the **bottom** of the figure. Each probe was hybridised to a similarly prepared blot in lines with cytoplasmic RNA (designed by letter "C" at the top of blot) and nuclear RNA (designed by letter "N" at the top of the blot). Ethidium bromide staining of the gels used for preparation of blots is shown on the **left** side of two groups of radiographs of gels run in parallel. The insert on the **right** hand side of the figure shows the results of hybridisation of B18- and B21-specific oligonucleotide probes to similarly prepared blots containing cytoplasmic and nuclear RNA lines.

enzymes, the RNA probes were prepared using the SP6/T7 transcription kit (Roche, Basel, Switzerland). To make probes for Northern blot hybridisation, the reaction mixture included α^{32}P-UTP; for in situ hybridisation probes, biotinylated UTP was used. Oligonucleotides were labelled with [γ-^{32}P]ATP, as described [Sambrook et al., 1989], and purified on Spin-10 columns (Clontech, Palo Alto, CA).

Oligodeoxynucleotides

All oligonucleotides used in RT-PCR and Northern blot analysis were purchased from Genset. The following oligonucleotides were used for Northern blot hybridisation: B18 probes, 5′-AATGGCGAGGCTTGTGTGCTCT-CCC-3′, and 5′-TTC TGGAACCCGCTGCCA-CAGGAGG-3′; B21 probes, 5′-AGCTTGGAGA-GGGT GCCGGCGATGT-3′, and 5′-CCCAGC-TCTAGCCACCGTGCCGTCT-3′. The primers used to start RT reaction are listed in Table I and primers used to PCR-amplify different test fragments are listed in Table II.

RT-PCR Analysis

One microgram of total nuclear or cytoplasmic RNA treated with DNase I (PCR grade) (Invitrogen Life Technologies, Carlsbad, CA) was reverse transcribed into cDNA with the aid of the 1st strand cDNA synthesis kit for RT-PCR (AMV). Two or three α-globin domain fragment-specific primers, located close to one another and complementary to different subsequent fragments of the domain, were utilised in each set of RT reactions. The enzyme was omitted as a negative control (-RT). Synthesised cDNAs were treated with a mixture of RNase H and RNase A and amplified in 50 μl PCR reaction with an

TABLE I. Primers Used to Start RT Reaction

Position on the map	RT primers
Test position "A"	CTAGGAGTGTAGTGAAGATT
	AGGAGTGTAGTGAAGATTTG
Test position "B"	GAGAGTGTGCACTGAATGTC
	CTGTCAGGATGAGATGGAAG
	TCACTCAACCTAGAGCAAGC
Test position "C"	CCTATCTCTTCTCATAGTGC
	CCTTTTTATAACCCCGATGC
	GAAGAGTTTTCTTGGCTGTC
Test position "D"	GTCACTTTTATCGTAACTGC
	CCACATGTTCTACATTCTCT
Test position "E"	GTGAACCAGGAATGAGGTAC
	CAGAGAAGACTTGTCTGTGT
Test position "F"	CAGTTGAAGTCAGCAACAGG
	AAATGGACGGTTGTACCTCT
Test position "G"	AGAACTTGTCCAGGGAAGCA
	TACTTGGCGGTCAGCACAGT
H7/H10 set	GATGAATGCTCTGCTGTGATAAT
	ATCTCCCTTTCAGTCCCCTTCTC
	GAGCCTGGTGGGAGTAGTGTAAT
H12 (π gene) set	GACACCGAGTATGAATGCCTTTG
	GGAGGAGAACAAGACAACAAAGT
	CAGCCACAGAGCACAGGATACAG
B16 (α^D gene) set	CTTCTTGTCCTCGGCAGTCAGCA
	AGCCCC ATTCCGTCCCCCATTCT
	CAGACACGGCAGACAGGAACTTC
B18 (α^A gene) set	GGTAAGGCAGGGAGGGATAGG
	AGGCAAA AGGAGAGGATGAGC
	CAGGGCAGCAGGGTGGTGGAT
B18 set in globin direction	TCCTATCCCTCCCTGCCTTAC
	CCCCTCGCTCATCCTCTCCTT
	CCGACAGCGAGCAGCCAAATG
B20 set	GTCATAGCCCAAAGAGCAGGAA
	GTTTTACCCACCCGAGGCTATC
	TGAGGTGGAAGGGGAGATTGGA
B21 set	CAGCAGTGTCTCAGGTCTTTTC
	TGAGTGTCCTGAGAGCAAGTGA
	GTGTGATGATGGACTGGGAAGA

TABLE II. Primers Used to PCR-Amplify Different Test Fragments

Region to be amplified	PCR primers	Expected length of the product (bp)
Test region "A"	GCTGCTCTACCTTGTTCTCA	352
	GCCTTGTTATCTTCCCTACC	
Test region "B"	CCTGCTCAATTTACACTTCT	319
	ATTTTACCTGTTTACGTGGC	
Test region "C"	TGCTGTCAAATTAGCCGAGT	302
	TGTGGTACACTGTGCTGTTG	
Test region "D"	TGAAGAATTCAGAACATCAC	306
	CTAGTTTCCAGAATGTTCTG	
Test region "E"	CAGAGCTCAATTCCATAGG	265
	TTATCTGGGGTACCTGCAT	
Test region "F"	GCTCTTCTGGCTCATTTGT	218
	TCATCTCCCTTTCAGTCCC	
Test region "G"	TATCCCTCCCTGCCTTACCC	296
	GTCGCCCCTTCCCAGACACT	
π Gene	GCACTGACCCTTAGCGAAATGG	511
	ACACCGAGTATGAATGCCTTTG	
H4 fragment	AAGATGCGTTACCTCTGTTATGC	650
	TTGTTAGAAAGGCAGTGGTCGTC	
H3 fragment	AACAACTCAACTGCTCCACAAGA	740
	GTGAGTGGAATAGGCAAGGAAGG	
H3 fragment second set	GAAGCGCCAGTCTATCGCAGTAC	460
	GGAGTGAGCAGGTTTGTGAGAGC	
B-1 fragment	GGAACCAGTAATGAAGGGAGAA	790
	AAGGCATCCAGGGTTCAAGAGT	
B-1 fragment second set	GATAGCAGTCTTGGAGGTGTCT	1,184
	GCCATCTCTGCTGCTTCATTTG	

enzyme mix containing thermostable Taq DNA polymerase and a proof-reading polymerase (Expand Long Template PCR System, Roche) and at least two different sets of primers placed upstream of the cluster of α-globin genes, mainly in the B-1 and H3 fragments. PCR was performed for 35 cycles (94°C for 1 min, 48°C for 1 min, 72°C for 3 min in the case of Taq polymerase or 94°C for 1 min, 48°C for 1 min, 68°C for 3 min in the case of long expand enzyme mix), preceded by denaturation at 94°C for 3 min. Two microliters of the reaction were then reamplified with Taq polymerase; 10 μl aliquots of each reaction were electrophoresed on 1.5 % agarose/TAE gel and analysed by Southern hybridisation with the probes corresponding to the same fragments of the α-globin domain from which the primers for PCR reaction had been derived.

Isolation of Nuclear Matrices and In Situ Hybridisation

Nuclear matrices from isolated nuclei of AEV-transformed chicken cells were made, using the standard set of detergent extractions followed by DNase treatment and sequential extraction with different salt solutions, including 2 M NaCl solution (cf. [Rzeszowska-Wolny et al., 1988; Farache et al., 1990b]). The cells and nuclear matrices were spread on saline-coated microscopic slides using a "Cytospin" centrifuge. All samples were fixed with paraformaldehyde as described [De Conto et al., 1999]. Hybridisation in situ with biotin-labelled ribo-probes was carried out as described previously [De Conto et al., 1999; Iarovaia et al., 2001]. After hybridisation and washing out of the non-bound probe, the signal was visualised by sequential immunostaining with mouse anti-biotin monoclonal antibodies conjugated with Alexa 488 and goat anti-mouse polyclonal antibodies conjugated with Alexa 488 (Molecular Probes, Eugene, Oregon). The results were analysed using the TCS (Leica, Germany) confocal imaging system, equipped with a 63× objective (plan apo; NA 1.4). For Alexa-green excitation, an argon–krypton ion laser adjusted to 488 nm was used.

RESULTS

Northern Blot Analysis of the Transcription Pattern of the Chicken α-Globin Gene Domain in AEV Cells

When the approximately 17 kb long globin FDTs were found, it was not known that the α-globin gene cluster and the upstream sequences were also transcribed in the opposite direction, as part of the pre-mRNA of the *ggPRX* gene. Hence, the hybridisation signals from the globin and ggPRX direction transcripts could be confused. Therefore, in the present study, we used single strand-specific ribo-probes recognising the globin-direction transcripts. To map the transcribed regions, we carried out Northern blot analysis of nuclear RNA with local probes taken from the entire domain. The restriction map of the α-globin gene domain, showing the positions of *Hind*III and *Bam*HI fragments used to prepare the probes, is shown at the top of Figure 1. These fragments and probes are designated by a combination of a letter (H or B, respectively, for *Hind*III and *Bam*HI fragments) and a number indicating the approximate distance between such a fragment and a *Kpn*I site arbitrarily chosen as point zero.

The results are shown in Figure 1. Hybridisation signals above the rRNA bands were observed with probes B-1, H0, H4, H7, H12 (π gene), B18 (α^A gene), and B20. A strong signal in 7 to 30 kb-long molecules (according to MW markers) was observed on nuclear RNA only; it was absent from cytoplasmic RNA. Substantially weaker bands of lower molecular weight were slightly visible in both nuclear and cytoplasmic RNA. In nuclear RNA, they probably represent certain intermediate processing products of the FDTs [Imaizumi et al., 1973; Therwath and Scherrer, 1974; Bastos and Aviv, 1977; Shoul et al., 1981; Broders and Scherrer, 1987; Broders et al., 1990; Iarovaia et al., 2001]. Nevertheless, non-specific binding of the probe to the abundant rRNA bands (that were not blocked with an rRNA competitor) cannot be excluded. The hybridisation signal in the 7–30 kb-long RNA was effectively competed by a cold probe (not shown). In cytoplasmic RNA slots, the bands corresponding to the α^A gene mRNA (0.6 kb) and a relatively stable product of incomplete processing (0.7 kb) were clearly seen upon hybridisation with the B18 probe. The presence of globin mRNA is due to the spontaneous differentiation of 1–5% of AEV cells in non-induced cultures.

To map the end of the transcribed area, we carried out hybridisation with a probe derived from the B21 fragment. The latter contains a CR1-type repeat [Farache et al., 1990a] and, hence, cannot be used as such, for the preparation of specific probes. We, therefore, used an

oligonucleotide corresponding to the unique part of this fragment. As a positive control, hybridisation with an oligonucleotide derived from the α^A gene having the same length and overall nucleotide composition was carried out. The results of this experiment clearly show that the B21 fragment is not transcribed (Fig. 1, insertion to the right).

Extent of the Continuous Transcription Unit in the Chicken of α-Globin Gene Domain

The results of Northern blot analysis seemed to indicate that, in proliferating AEV cells, the entire domain of the α-globin genes is transcribed, giving rise to "giant" nuclear RNAs. However, it was not clear whether there was a single transcription unit covering the whole area studied or whether, alternatively, different sub-domains were independently transcribed, as reported for the domain of human β-globin genes [Gribnau et al., 2000]. In order to map continuous transcripts, RT-PCR was used. Several sets of oligonucleotide primers (three primers for each set) were designed to start cDNA synthesis from different regions further downstream in the domain (H7, H12/π gene, B16/α^D gene, B18/α^A gene, B20, B21). These primers were used to carry out reverse transcription on nuclear RNA. The reverse transcription products were probed with two sets of PCR primers, designed to amplify the possibly present cDNA sequences including the B-1 and H3 fragments. We considered that a positive signal in the PCR-amplification reaction would be proof of the existence of nuclear transcripts covering the area between the RT-transcription primers and the PCR amplification primers.

Figure 2 shows that the cDNAs synthesised starting from all fragments (H7 to B16) contained sequences from both the B-1 and H3 fragments. In control experiments, when the cDNA synthesised in the opposite direction, starting from the B18 fragment, was used as a template for PCR amplification, no signal was observed with any of the upstream primer sets (not shown). The cDNA copied from the globin RNA, starting from the B18 (α^A gene) fragment, contained the sequences present in H3, but not in B-1; this may reflect the inability of the RT enzyme to produce very long cDNAs. Indeed, the distance between the B18 and B-1 fragments is about 25 kb and the RT enzyme must pass an extremely GC-rich fragment present in H7 (70% GC). In a further experiment, we were

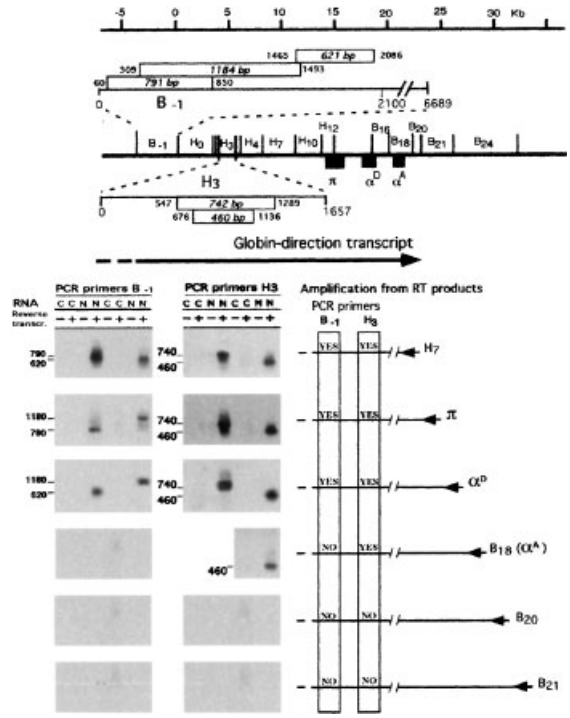

Fig. 2. RT-PCR mapping of the RNA of AEV cells representing the continuous transcription unit in the domain of the chicken α-globin genes. **Top**: Map of the domain with the positions of primers used for PCR amplification in the distal regions of RT products. The designations used in the map are essentially the same as those in Figure 1. **Bottom**: The results of the RT-PCR experiments and their interpretation. **Left**: Blots of PCR amplification with the two groups of primers in B-1 and H0. **Right**: Schematic representation of the position of the RT primers used and interpretation of the PCR results. Presence or absence of the RT enzyme in the reactions is indicated by (+) or (−), respectively. Note that the absence of PCR products in the (−) slots indicates the absence of DNA contamination.

able to PCR-amplify a part of the H12 (π gene) fragment using as a template the cDNAs made on nuclear RNA, starting from B18 and B20-specific primers but not the B21-specific primers (not shown). These results are in good agreement with the absence of a hybridisation signal on Northern blots probed with the B21-specific oligonucleotide, reported above.

Northern blot hybridisation and RT-PCR thus show that the FDTs end somewhere near the junction of the B20 and B21 fragments. The size of the transcripts identified by the B20 probe exceeded 20 kb (c.f. Fig. 1). The transcript must start way upstream from the beginning of the sequenced area, or beyond. One can thus conclude that the whole domain of α-globin genes, as well as several upstream regulatory elements, starting from at least 18 kb upstream

of the π gene and stretching down to about 1.5 kb downstream to the α^A gene, is covered by a single transcript.

Mapping of the Upstream Start of the FDT Transcription Unit

Experiments described in the previous section have demonstrated that in immature erythroid cells an extended upstream area of the α-globin gene domain is transcribed in the "globin" direction giving rise to the FDTs that also include coding sequences of the three α-type globin genes. It was not, however, clear where the domain-type transcription unit starts. We have thus selected additional pairs of PCR primers permitting us to study the transcriptional status of the area located beyond the limits of our recombinant clones. Primer design was based on a DNA sequence (accession number AY016020) published by Flint et al. [2001]. Positions of the test regions on the extended map of the chicken α-globin gene domain are indicated on the scheme represented in the upper part of Figure 3. The results of PCR amplifications made on cDNA synthesised in the globin direction transcript are represented below the map. Each section shows results of PCR amplification carried out on cDNA, with three dilutions to estimate the relative quantities of PCR products. Furthermore, in parallel experiments genomic DNA was used as a template to show the sizes of the "significant" PCR products, and to demonstrate that all pairs of primers permitted one to obtain comparable quantities of PCR products on genomic DNA templates. The results clearly show that the regions "A" and "B" are not transcribed in the globin direction. The regions "C" to "F" are, on the contrary, transcribed with about the same intensity. These results suggest that the

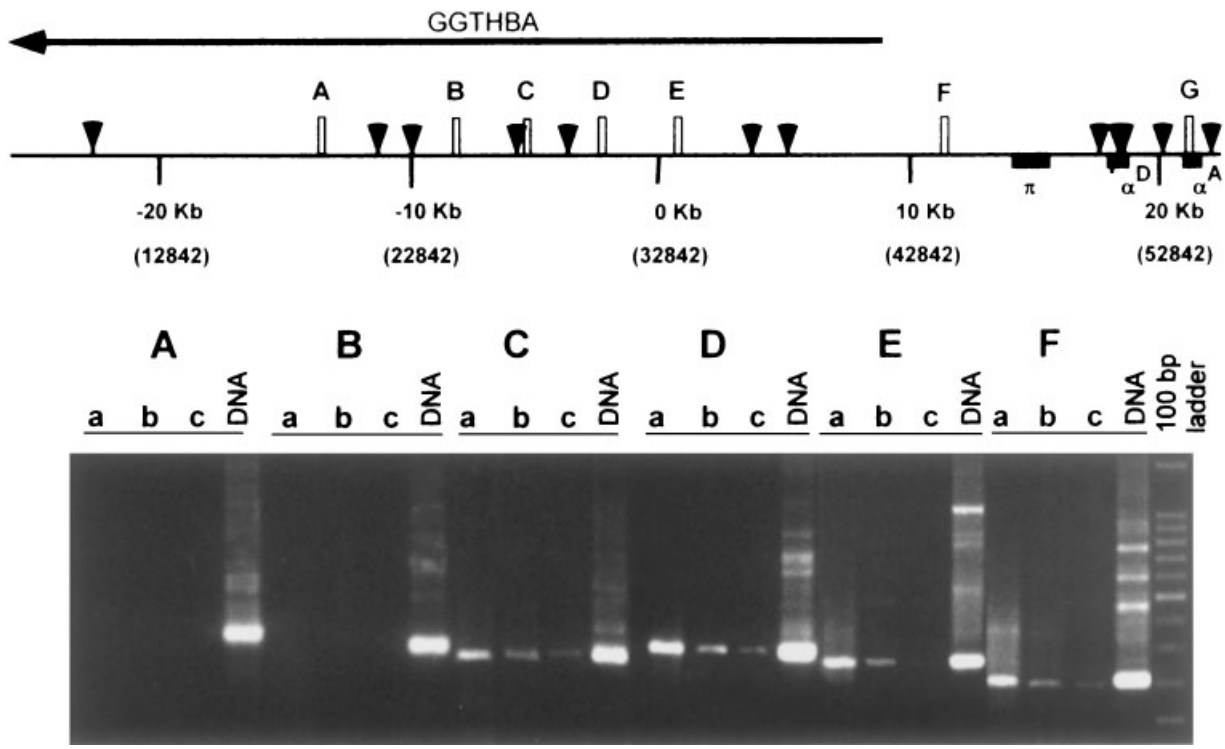

Fig. 3. Mapping of the upstream end of the full domain transcript (FDT) transcription unit. **Upper part**: The scheme showing positions of test fragments on the map of the domain. Numeration is presented in the same manner as in the scheme shown in Figure 1. The figures in parenthesis show the numeration used by [Flint et al., 2001] on a sequence deposited in GeneBank under the accession number AY016020. Globin genes are shown as closed rectangles. Dark arrowheads show the positions of *Bam*H1 sites. The test fragments indicated by the letters "A"–"G" are shown as open vertical rectangles. GGTHBA represents an open reading frame for the ubiquitous *ggPRX* gene transcribed opposite to the direction of globin gene transcription [Vyas et al., 1995; Sjakste et al., 2000; Flint et al., 2001]. **Lower part**: Results of amplification of test fragments A–F. For each set of amplification, three dilutions (1, 1:5, 1:25) of the RT product were used (slots a, b, c in each set). The first slot contained RT products made on 0.5 μg of nuclear RNA. Slots designated "DNA" show the results of amplification on 1 μg of chicken genomic DNA.

erythroid-specific domain-type transcription unit starts somewhere between the region "B" and the region "C". These two test-regions are separated by a 2.6-kb DNA fragment that, according to Flint et al. [2001], harbours a putative transcriptional regulator having some properties of LCR. It is also of interest that this particular region is located at the upstream border of a vast chromatin domain that, in erythroid cells, is characterised by a particular type of histone hyperacetylation [Anguita et al., 2001].

Comparison of the Transcriptional Status of the Upstream Area of the Chicken α-Globin Gene Domain in Erythroid Versus Non-Erythroid Cells

The data described in the previous section favour the supposition that FDTs of the α-globin domain play a certain role in maintaining the active status of this domain in erythroid cells. If true, the globin-direction FDTs should be present in erythroid cells only. Thus we have studied the transcriptional status of the upstream area of the α-globin gene domain in primary chicken fibroblasts. Two characteristic regions of the upstream part of the domain (test-fragments "D" and "F") and a part of the coding sequence of the α^A globin gene (test fragment "G") were analysed. In separate experiments, the RT products transcribed in the opposite direction were used as templates for amplification of the test-fragment "D". This provided an important internal control as in both, fibroblasts and erythroblasts, this fragment is transcribed in the direction opposite to that of globin gene transcription, as part of the ubiquitous pre-mRNA of the housekeeping gene *ggPRX* [Sjakste et al., 2000]. The results of the experiments are represented in Figure 4. As already shown above (Fig. 3), in erythroblasts the test fragments "D" and "F" are transcribed in the globin direction. Transcription in the opposite direction was also detected (note the band of correct size in the slot designated "anti-D"). In all cases, the possibility to observe a signal (an amplified fragment) was dependent on the reverse-transcription enzyme (note the absence of amplified bands in all slots with designation "-RT"). Furthermore, this result clearly demonstrates that our preparations of nuclear RNA are virtually free of contamination with genomic DNA. In contrast to cultured erythroblasts, in primary fibroblasts only transcription in the direction opposite to that of

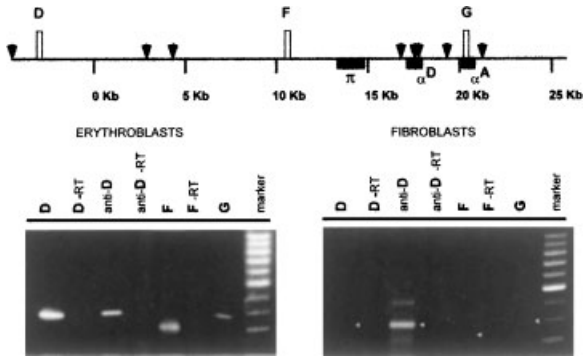

Fig. 4. Comparative analysis of transcription of the test regions ''D'', ''F'', ''G'' in erythroid cells (line HD3) and in primary chicken fibroblasts. The design of experiments was essentially the same as in Figure 3 except that a shorter genomic area was studied and RNA samples prepared from erythroblasts and primary fibroblasts were compared in parallel sets of experiments. The designations on the scheme of the domain are the same as in Figure 3. The slots designated ''-RT'' were loaded with products of PCR-amplification carried out directly on RNA (without RT). Note that the absence of signals in these slots demonstrates that our RNA samples were not contaminated (within the limits of resolution of the PCR approach) with genomic DNA. Slots designated ''anti-D'' were loaded with PCR products made on pre-mRNA of the *ggPRX* gene (i.e., the transcript synthesised in the direction opposite to that of globin gene transcription was used as a matrix for the RT reaction). Note that, in contrast to the globin direction transcripts present only in erythroid cells, this transcript is equally represented in fibroblasts and erythroblasts.

globin gene transcription was detected (slot "anti-D"). None of the regions tested ("D", "F", and "G") was transcribed in the globin direction in primary fibroblasts. This result suggested that the FDTs of the α-globin domain constitute a characteristic feature of the transcription pattern of this genomic region only in cells of the erythroid lineage.

Association of Globin FDTs With the Nuclear Matrix

The nuclear matrix is an internal nuclear structure that plays an important role in spatial organisation of the nuclear space [for review see Razin et al., 1995; Cremer et al., 2000]. Although there is no standard procedure for nuclear matrix isolation, most investigators use different variations of the original stepwise extraction protocol of Berezney and Coffey [1977]. In previous studies, we specially adapted this procedure for isolation of nuclear matrices from chicken erythroid cells, using as main criteria the preservation of the internal morphology of the nuclear matrix, as seen in

electron micrographs, and the possibility to obtain matrices characterised by a particular protein composition [Rzeszowska-Wolny et al., 1988].

In the present study, we have addressed the question of whether the FDTs of the α-globin gene domain are part of the nuclear matrix. To this aim, we first did in situ hybridisation on cells and isolated nuclear matrices, using biotinylated probes specific for the H7 and H12 (π gene) fragments. These probes were selected specifically in order to compare the distribution of signals representing "pure" FDT RNA (H7 probe) without coding sequences and the transcripts containing a globin gene. In situ hybridisation (Fig. 5) shows that, on intact AEV cells, the H8 probe (a shorter fragment of Hind 7) stained only nuclei, whereas the H12 (π gene) probe, in some of the cells, stained both the nuclei and the cytoplasm. The latter result may reflect a spontaneously induced cell or abortive π gene transcription; indeed some globin RNA is found in the cytoplasm of cells trying unsuccessfully to differentiate [De Conto et al., 1999; Iarovaia et al., 2001]. On nuclear matrices, both probes gave strong signals (except in the nucleoli) distributed in slightly different patterns; prior RNase treatment fully abolished both signals (not shown). These results indicate clearly that both inter/extragenic and globin gene transcripts are present in the nuclear matrix RNA.

In order to verify this conclusion, we carried out a set of RT-PCR experiments using the RNA isolated from nuclear matrices. One would not expect to find much non-degraded globin FDT in isolated nuclear matrices; hence in experiments with the nuclear matrix RNA we did not attempt to obtain a very long cDNA. Instead we tested whether the continuous transcript went through the "pure" FDT (exon-free) H7 fragment. We, therefore, used the H7/H10 primers for cDNA synthesis (these primers map close to the junction of the H7 and H10 fragments). PCR-amplification of the cDNA obtained was carried out with primers derived from the H3 and H4 fragments. As shown in Figure 6, both H3 and H4 primer pairs gave specific bands upon PCR amplification of the cDNA, initiated at the junction of H7 and H10 fragments. This experiment thus confirmed the presence of globin FDTs in the nuclear matrix.

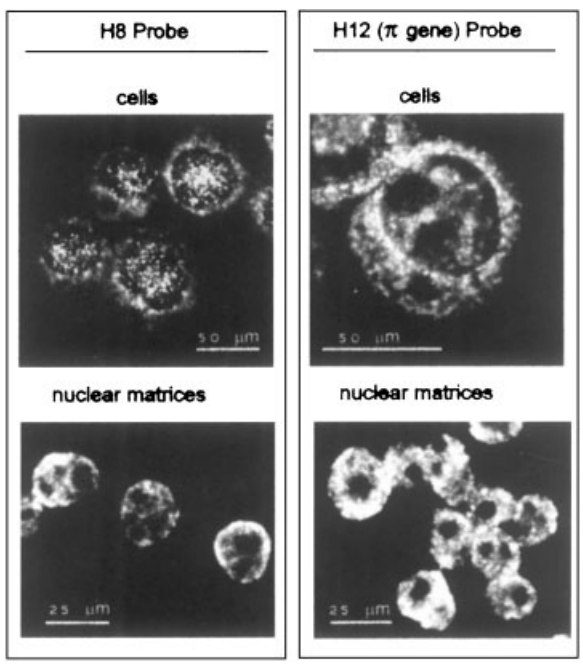

Fig. 5. Visualisation of the in situ pattern of globin transcripts on intact cells and nuclear matrices of AEV cells. To the **left** are shown the results of hybridisation with the H8 (part of H7) probe, which identifies the FDTs without coding RNA and, to the **right**, the H12 probe that identifies the transcripts of the π gene within the FDTs.

DISCUSSION

Since the first reports from our laboratory about the existence of "giant" globin gene transcripts in avian cells [Imaizumi et al., 1973], the significance of this type of nuclear RNA has been questioned. When the fragmentation of the genes in the genome and splicing of RNA were discovered, large transcripts could be easily identified as pre-mRNA, in general. Indeed, the combined intron–exon structures spread, in extreme cases such as the human dystrophin gene, over up to 2,500 kb [Burmeister et al., 1988]. When globin pre-mRNAs of distinct sizes were described, 1.0, 1.3, and 1.5 kb in the case of the three avian α-type genes [Therwath and Scherrer, 1982], these molecules did not account for the giant globin transcripts described by our laboratory [Therwath and Scherrer, 1978; Broders and Scherrer, 1987; Broders et al., 1990]. Reports on giant RNA termed "intergenic transcripts", observed in the human β-globin domain, have raised renewed interest in such RNA [Ashe et al., 1997; Gribnau et al., 2000].

Within this perspective, the important contribution of the present paper is the demonstra-

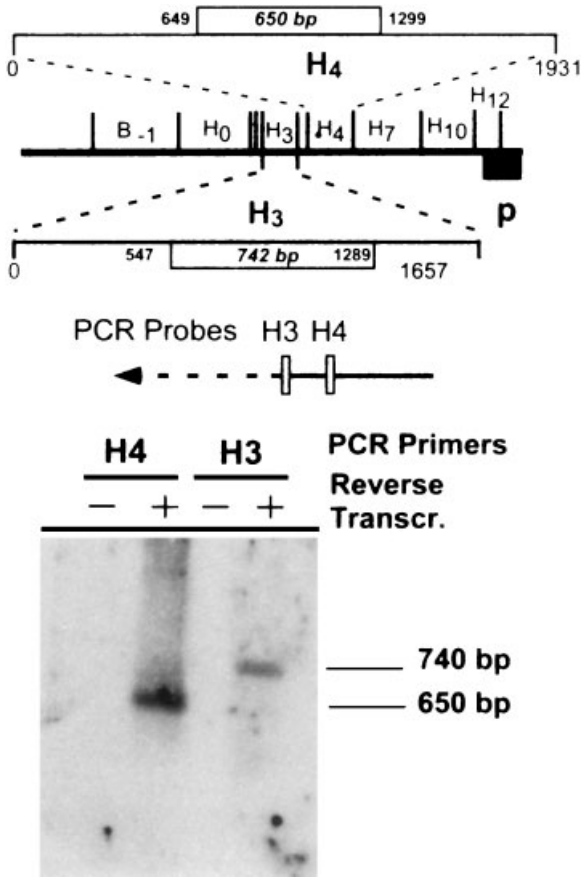

Fig. 6. RT-PCR analysis of RNA extracted from nuclear matrices of AEV cells (see Fig. 3, **bottom**) using the H7/H10 reverse transcription primers, defined in Materials and Methods. The experimental design is outlined at the **top** of the figure. The results of the PCR amplification of the distal regions of the RT products are shown at the bottom.

tion that in the chicken, the α-globin FDTs include all three α-type globin genes and all known regulatory upstream and downstream sequences. Even more interesting is the fact that the domain-type transcription apparently starts in the vicinity of a putative LCR of the α-globin gene domain [Flint et al., 2001] and at the border of the erythroid-specific domain of histone acetylation [Anguita et al., 2001]. These results suggest a role of FDTs in establishing an active chromatin domain. It has been shown that SWI/SNF and histone acetyltransferases (HATs) are associated with the RNA polymerase II complex [Wilson et al., 1975; Cho et al., 1998; Wittschieben et al., 1999]. Thus, initiation of transcription within the LCR would automatically recruit to the area enzymes necessary for the activation of the chromatin domain. Furthermore, it is possible that the elongating RNA polymerase II would act as a delivery vehicle that could bring important modifying factors from distant LCR elements to the gene promoters, by a processive RNA polymerase "piggyback" mechanism [Travers, 1999].

The presence of a housekeeping gene on the DNA strand opposite to the α-globin cluster [Sjakste et al., 2000] may account for the fact that the tissue specific domain of α-globin genes remains in an open chromatin configuration in both, erythroid and non-erythroid cells [Craddock et al., 1995]. This also implies that, in erythroid cells, there should be a way to prevent accidental formation of dsRNA with the subsequent destruction of both transcripts via the RNAi mechanism.

Turning to the association of FDTs with the nuclear matrix, we may recall old data that demonstrated for the first time the existence of two segments of matrix DNA, identified by DNase resistance in high-salt extracted nuclei, that framed the α-globin gene cluster in the transcriptionally silent erythrocytes. In contrast, in erythroblasts productively expressing globins, the whole domain (as it was then known) was found in the matrix DNA [Farache et al., 1990b]. It turned out that the upstream MAR identified in erythrocytes corresponded to a constitutive DNA loop anchorage region [Razin et al., 1991a; Recillas Targa et al., 1994], situated at the domain's origin of DNA replication [Razin et al., 1986; Verbovaia and Razin, 1995]. Here we show that in erythroid cells this loop anchorage region is transcribed in the globin direction as part of the α-globin FDT.

There are several reports about the presence of RNA in the nuclear matrix [Fey et al., 1986] but, to our knowledge, the exact nature of the RNA has never been clearly defined, except for the presence of transcripts of specific genes [Robinson et al., 1982; Ciejek et al., 1983; Jost and Seldran, 1984]. It was thus not clear whether a particular type of matrix or "architectural" RNA was the carrier of the RNA-dependent matrix. Indeed, the pre-mRNAs could possibly assume this function by themselves. The data reported here show that at least globin FDTs are an integral part of the nuclear matrix. Combining all data, one may, therefore, propose that these FDTs running through the full domain are transported apparently in a first step to the perinucleolar area, where they remain in the case of abortive transcription

[Iarovaia et al., 2001], prior to being carried over to the distinct globin RNA processing centres [Iarovaia et al., 2001]. Forming complexes of transcription and processing, they may eventually integrate into the nuclear matrix and, during processing and transport, condition the dynamic architecture of the nucleus. The fact that even the least stable parts of the FDT (e.g., Hind 7 RNA), without coding sequences, are part of the nuclear matrix indicates that the RNA in the matrix reflects a major fraction the globin domain.

In the case of large genomic domains having sometimes gigantic introns, it seems obvious that pre-mRNAs are likely to assume both roles, that of an architectural RNA and that of a carrier of the genomic information. This may not be the case for the relatively small globin immediate pre-mRNAs, which form relatively small ribonucleoprotein (RNP) complexes, possibly being unable to shape the dynamic nuclear architecture necessary for transport. Here, the globin FDT might assume the architectural function, whether the mechanism used to transport the genic message is identical or not. It seems premature to make specific models as yet. Nevertheless, the evidence we present here for the presence of globin FDTs as a part of the nuclear matrix may stir further interest in the possible need for a dynamic nuclear architecture, handling and controlling the transcripts individually. In the living cell, the transfer of information and the architectural structure may amount to the same physically: software becomes hardware, and hardware is software.

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