Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 416-423

PRELIMINARY CHARACTERIZATION OF A NUCLEAR FACTOR INTERACTING WITH THE SILENCER ELEMENT AT THE 3'-SIDE OF THE CHICKEN α-GLOBIN GENE DOMAIN

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Received September 4, 1992

SUMMARY: A silencer element has been identified previously on the 3'-side of the chicken α-globin genes placed next to the major enhancer in this domain (Recillas Targa et al., unpublished). Deletion fragments of this negative element show the requirement of the entire DNA segment for maximum silencing activity. Additionally, the sub-fragments including the previously defined SF1 (Silencer Factor 1) sequence seem to play an important role on the negative action of this silencer. Preliminary characterization of the nuclear factor interacting with the SF1 sequence was carried out. Methylation interference experiments show the contact points and, by electrophoretic retardation analysis with SF1 and SF1-mutated oligonucleotides, the critical role of these contacts points on the formation of the DNA-protein complex could be demonstrated. These results provide a basis for further experiments on the characterization and understanding of the mechanisms of action of the coupled positive and negative regulatory elements.

The chicken globin gene families are among the best known groups of developmentally regulated genes. The α-globin gene domain contains a single embryonic π gene, and the two adult α^D and α^A genes (1, Recillas Targa et al., unpublished). The two adult α-globin genes are expressed at low levels in primitive avian erythrocytes with an expression ratio of 3:1 (3). From day 1.5 to days 4 to 5 after fertilization, the blood of chicken embryos contains primitive cells that synthesize the embryonic π globin, as well as α^D and α^A. Beginning on day 5, a new class of erythrocytes (of definitive cell lineage), expressing only the adult genes (α^D and α^A) appears in the circulation. The structure and regulation of the chicken α-globin gene domain has been studied in detail in our and other laboratories and is actually well defined (Recillas Targa et al., unpublished, 4, 5). In a previous study we identified a silencer element in the 3'-flanking region of the α^A adult gene upstream of the 3'-side transcriptional enhancer composed of three GATA-1 recognition sites (see scheme in Fig. 1A), and a newly defined 4th homology box which contains a variety of putative regulatory elements (Recillas Targa et al., unpublished). In the present study, we report

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0006-291X/92 $4.00

preliminary characterization of the silencer element on the 3'-side of the chicken α-globin region. Deletion mutants of this negative element showed that the entire fragment is required for maximum silencing activity. A nuclear factor interacting with the SF1 DNA-binding sequence with estimated molecular weight of 50,000 Daltons was identified. Additionally, methylation interference experiments demonstrated that the interaction of this nuclear protein factor is centered in a direct tandem repeat of the protein binding DNA sequence. Electrophoretic retardation experiments with the SF1 motif, and SF1-mutated oligonucleotides revealed the importance of a specific G residue in the first direct repeat on the SF1 DNA-binding site.

Materials and Methods

Cells culture and Transfection experiments

AEV-transformed temperature-sensitive chicken erythroblasts of the LSCCHD3 (HD3) cell line (6) were grown at 37°C in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 8% foetal calf serum and 2% chicken serum. $3x10^6$ cells were transfected by 3-10 µg of plasmid DNA by the DEAE dextran method (250 µl Tris-buffered saline (8 mg/ml NaCl, 0.38 mg/ml KCl , 1 mg/ml Na_2HPO_4 -$7H_2O$, 1 mg/ml glucose, 3 mg/ml Tris-HCl (pH 7.2)) containing 10 mg/ml DEAE dextran). This method includes a 3 min treatment with 25% dimethyl sulfoxide in Tris-buffered saline (7). After transfection (48 hr) the cells were recovered, washed and resuspended in 100 µl Tris-HCl 0.25 M (pH 7.8) and lysed by three cycles of freeze-thawing.

CAT assay

This assay was carried out as in Recillas et al. 1992 (Recillas Targa et al. unpublished). The pTKCAT18 vector has been constructed by introducing the multiple cloning site of pUC18 in pTKCAT18 (8). This plasmid contains the Herpes simplex virus TK promoter (-105/+51) driving the transcription of the chloramphenicol acetyl transferase (CAT) gene. To generate the restriction deletion mutants we used Ban II and Alu I restriction sites located on the 370 bp Bam HI-Cfo I silencer fragment. To eliminate accidental fluctuations between samples, transfections were repeated 4-6 times; the given values of CAT activities are averages and the standard error is given.

"Southwestern" Blot Analysis

Nuclear extract (60 µg) were denaturated by the addition of an equal volume of sample buffer (5% SDS, 5 mM Tris-HCl (pH 6.8), 200 mM DTT, 20% v/v glycerol), heated at 90°C for 3 min, and size fractionated by electrophoresis in a 7.5% polyacrylamide gel that contained SDS. Proteins were electrophoretically transfered to nitrocellulose (Schleicher & Schuell) in 25 mM Tris-HCl (pH 8.8) and 190 mM glycine. Filter-bound proteins were renatured by incubation for 24 hrs at 4°C in 50 mM Tris (pH 7.5), 50 mM NaCl, 1mM EDTA, 1mM DTT, and 5% milk powder (Carnation no-fat). Following renaturation, the filters were rinsed twice for 15 min with binding buffer (same composition as renaturation buffer except that 0.25% milk powder was used). For DNA binding, the filters were transferred to a bath containing binding buffer supplemented with 1 µg/ml poly(dI-dC) and $1x\ 10^6$ cpm/ml of the ^{32}P-labeled SF1 oligonucleotide probe. The filters were gently shaken in this mixture at 4°C for 10 hr, and then washed three times over a period of 45 min in binding buffer (without DNA and poly(dI-dC)). Filters were air-dried and exposed to an X-ray film.

DMS Methylation Interference

DMS methylation analysis was performed using the procedure of Rigaud et al. (9). The double- stranded oligonucleotide used (corresponding to the SF1 sequence) was

labeled at either one of its 5' ends as follows: one oligonucleotide was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase prior to annealing with the complementary oligonucleotide. The resulting duplex was gel purified, methylated with DMS (10) and incubated with HD3 cell nuclear extracts (5 mg/ml) in a scaled-up version of the conditions for the retardation experiments (Recillas Targa et al. unpublished). The bound and free DNA bands were visualized by autoradiography and excised, and the recovered material was treated with piperidine and analyzed on a 20% polyacrylamide-8M urea sequencing gel.

Gel mobility shift assay and oligonucleotide probes
The nucleotide sequences of the oligonucleotides are presented in figure 4A, and were synthesized using a "Gene Assembler Plus" DNA synthetiser (Pharmacia) and purified on 15% acrylamide gels. Concentrations were determined spectrophotometrically. Oligonucleotides used as probes were labeled with polynucleotide Kinase using [γ-^{32}P]ATP. Oligonucleotides used as competitors were annealed in presence of 10 mM Tris-HCl (pH 7.9) and 10 mM $MgCl_2$, heated to 60°C for 30 min and cooled at 24°C for 15 min. The binding reactions and electrophoresis were carried out as described previously (Recillas Targa et al. unpublished).

Results and Discussion

Fragmentation of the silencer element

Several restriction deletion mutants were produced to further dissect the precise functional limits of the transcriptional silencer element (Fig. 1A). Using Ban II and Alu I restriction sites, we were able to separate every one of the three DNA binding-sequences SF1, SF2 and SF3 identified previously (Recillas Targa et al. unpublished) which were separately tested in a total of five recombinants. The results of transfection these constructs on transformed chicken erythroblasts (line HD3) and the results of the CAT assays are shown in the histogram in figure 1B. The reduction in CAT activity is largely due to the entire silencer element. This DNA fragment of 370 bp has maximum inhibitory action in its natural orientation (83% of inhibition of the TK promoter). When the opposite orientation is tested, the phenomenon is diminished but still present, reaching 71% of inhibition. Less significant degrees of inhibition were observed for any one the other sub-clones studied. In the constructs carrying the region including the SF1 DNA-binding sequence, the CAT activity is reduced, to between 57% and 49% of inhibition. Comparing these values of silencing activity with those presented by Gutman and coworkers for the human ε-globin silencer (11), we can consider 50% of inhibition is significant. These results suggest that the entire silencer region (370 bp) is required to give the maximum (83%) inhibitory activity; consequently, no increase in silencing activity was found with the sub-clones of the silencer element (Fig. 1B).

Molecular weight estimation of the factor interacting with the SF1 site

We found previously, by DNase I footprinting three protected DNA segments corresponding to different sites for DNA-protein interactions in the 370 bp Bam HI-Cfo I fragment silencer region (Recillas Targa et al. unpublished). The SF1 DNA-binding site showed highest protection and we analysed it in more detail. In an effort to identify

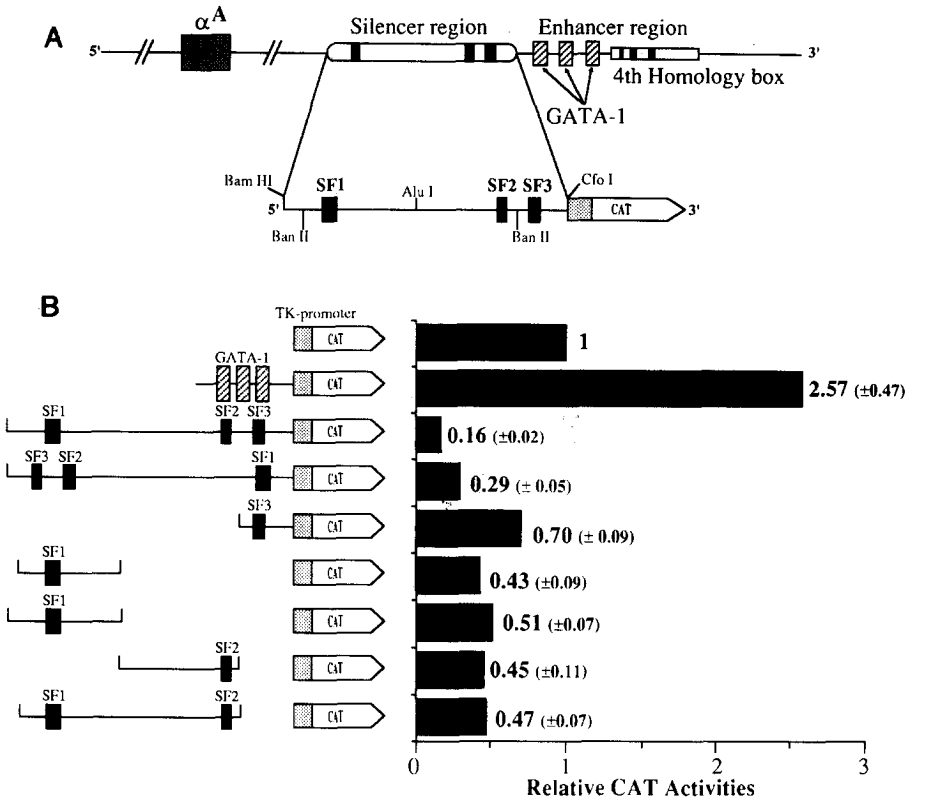

Fig. 1. CAT activities of the silencer deletion mutants
(**A**) Schematic representation of the DNA elements observed on the 3'-side of the chicken α-globin silencer-enhancer region (Recillas Targa et al., unpublished). The restriction enzymes which were used to generate fragments are indicated on the detailed scheme given for the silencer region. (**B**) Scheme of the constructs tested and the corresponding relative CAT activities. After transfection of constructs into HD3 cells, CAT activities were calculated relative to the pTKCAT18 plasmid. All the constructs were inserted using Bgl II linkers in the Bam HI site upstream of the TK-promoter of the pTKCAT18 vector. In the histogram the expression values represent an average of 4-6 transfections and the mean standard error is indicated in brackets. The black boxes represent the DNA-protein binding sites SF1, SF2 and SF3.

the nuclear factor interacting with the SF1 motif, the ^{32}P-labeled SF1 oligonucleotide was used to probe in a "Southwestern" blot assay the HD3 cell nuclear extract. As shown in figure 2, after electrophoretic separation, a single protein of approximately Mr 50 000 reacted with the SF1 oligonucleotide probe. These result suggest the existence of a polypeptide that interacts with the SF1 DNA-binding site. Based on these observations we are now trying to purify this nuclear factor and clone the corresponding cDNA.

Identification of the contact points of the nuclear protein factor recognizing the SF1 DNA-binding sequence

The SF1 recognition site includes a direct tandem repeat with the sequence: 5'-CGTCTGTC-3', this motif could possibly represent an interesting feature for the formation of a DNA-protein complex. To clarify this point we carried out a methylation

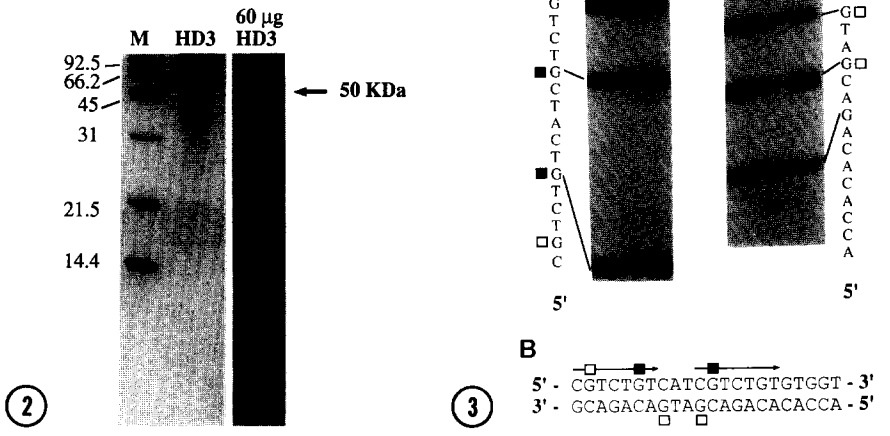

Fig. 2. "Southwestern" blot analysis of nuclear proteins from HD3 cells
HD3 nuclear extract (~ 60 µg of crude extract) was size-fractionated by SDS-PAGE, electro-blotted onto nitrocellulose, renatured, and subjected to a binding reaction with the ^{32}P-labeled SF1 oligonucleotide. Molecular weight markers that were run in parallel are indicated on the left in KDa. The estimated size of the SF1 binding protein (approximately ~50 KDa) is indicated on the right.

Fig. 3. Methylation interference analysis of protein binding to the SF1 site
(**A**) The SF1 binding oligonucleotide was end-labeled on the upper and the lower strand and annealed with the corresponding non-labeled complementary strand. Methylated G residues that interfere with protein binding are indicated on the left of the autoradiograph. The oligonucleotide sequence is presented with the methylated G residues. **B**, DNA-protein compex; **F**, unbound DNA as described in Materials and Methods (the penultimate G residue not included in the gel shown, was clearly visible in others, lacking, however, resolution at the top of the gel; data not shown). (**B**) Sequence of the SF1 oligonucleotide. Black squares represent strong methylation interference and the arrows delineate the direct repeats.

interference analysis using an oligonucleotide corresponding to the SF1 site. The end-labeled oligonucleotide was partially methylated at G residues by dimethyl sulfate and then used in retardation experiments with transformed chicken erythroblast (line HD3) nuclear extracts (see Materials and Methods). Free and bound DNA were eluted from the gel, cleaved by piperidine and analysed on a denaturing sequencing gel. Methylated G residues that interfere with protein binding are under-represented in the G-ladder of the bound DNA (Fig. 3A, lane B). The results of methylation interference analysis indicates the involvement of the direct tandem repeat for the SF1 binding within the protected area. As can be seen in the upper strand, the two methylated G residues that interfere with protein binding are situated within each one of the repeats (Fig. 3B). It was therefore to carry out of mutagenesis of this SF1 site to know if the DNA-protein interaction can be abolished.

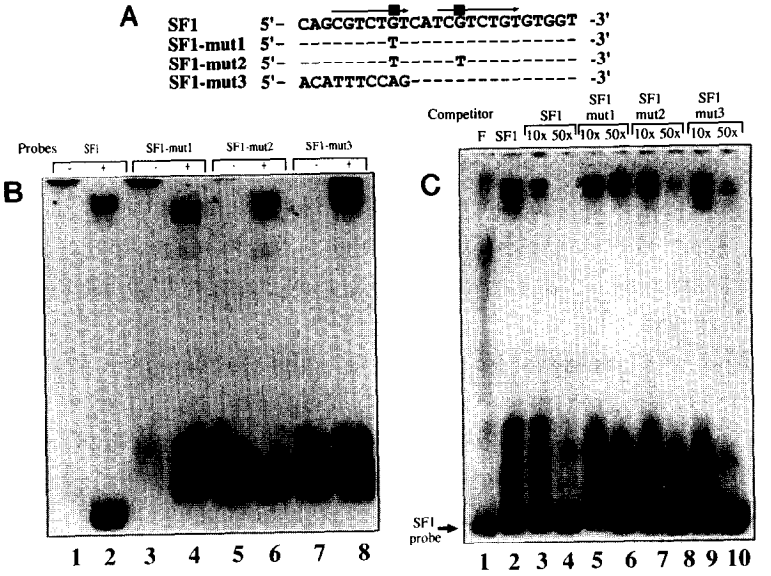

Fig. 4. Electrophoretic retardation analysis of SF1 and SF1-mutated oligonucleotides

(**A**) Sequence of the coding strand of the oligonucleotides used for electrophoretic retardation and competition analysis. The sequence of the wild type SF1 DNA-binding site is shown (SF1), G residues that possess strong methylation interference are indicated in black squares and the upper arrows delineate the direct tandem repeats. For the SF1 site-mutated oligonucleotides only the mutated nucleotide(s) are indicated. For SF1-mut3 the first repeat was deleted from the wild type sequence and replaced by the adjacent upstream DNA sequence. (**B**) Retardation experiment with SF1 and mutated oligonucleotides. Each probe was incubated in the absence (-) or presence (+) of 1 µl of HD3 nuclear extract (5mg/ml) for 30 min at 4°C. **F**, represents free probe. (**C**) Competition retardation experiments using the SF1 wild type probe and unlabeled competitor oligonucleotides. Nuclear extract was incubated with each unlabeled oligonucleotide at a 10- (10X) or 50- (50X) fold molar excess for 10 min, followed by a further 30 min incubation, at 4°C, with the labeled probe.

Electophoretic retardation experiments using SF1 and SF1-mutated oligonucleotides

To determine whether the methylation of guanine residues might interfere with the complex formation, electrophoretic retardation experiments using transformed chicken erythroblast (line HD3) nuclear extract and SF1 and SF1-mutated oligonucleotides were performed according to the sequences shown in figure 4A.

Incubation of the SF1 oligonucleotide containing the wild type sequence corresponding to the SF1-binding site, with HD3 nuclear extracts yielded three major retarded bands (Fig. 4B, lane 2). In the control slots 1 and 3 (incubation with out extract), for unknown technical reasons, the probe was maintained at the entry of the gel; this incident occasionally happens. However the free oligonucleotide bands are clearly visible in slots 2 and 4; furthermore as shown in slot 1 panel C, the oligonucleotide incubate without extract in general migrates normally, although leaving behind some smear, indicating its property to aggregate.

Mutation of one G residue (SF1-mut1) in the same experimental conditions, resulted in complete absence of the wild type pattern of retardation, suggesting the formation of a different DNA-protein complex (Fig. 4B, lane 4). For the double point-mutation (oligonucleotide SF1-mut2), the wild type pattern is maintained, but two additional retarded bands appear (Fig. 4B, lane 6). When the SF1 site is drastically mutated, eliminating one of the direct tandem repeats (oligonucleotide SF1-mut3), the SF1 retardation pattern is conserved (Fig. 4B, lane 8) but in addition to the three retarded bands, a new retarded band, with a slower electrophoretic migration in relation to the SF1 complex, is observed.

These observations suggest that there are some variations in the interaction of the protein factor recognizing the SF1 DNA-binding site, but when one G residue is mutated into a T residue, the formation of the complex appears to be drastically modified or even totally abolished. The interaction of SF1 with this sequence seem thus confirmed.

To fully demonstrate the sequence specificity of SF1-binding factor or factors, competition experiments using the wild type SF1 and mutated oligonucleotides were carried out (Fig. 4C). It was found that 10 and 50-fold molar excess of unlabeled SF1 oligonucleotide completely inhibit the complex formation (Fig. 4C, lanes 3 and 4). When SF1-mut1 oligonucleotide is used as a specific competitor in the same conditions (Fig. 4C, lanes 5 and 6), we observe that the complex is not disrupted, e.g., no competition occured. Such results demonstrate that the mutated G residue is responsible for the disruption of the DNA-protein complex. Interestingly, when SF1-mut2 and SF1-mut3 oligonucleotides are used as competitors, in the same experimental conditions, there is clear competition and inhibition of complex formation. These results suggest that for the SF1 recognition site, the first G residue that interferes with the protein factor (Fig. 3A) is extremely important for correct and stable formation of the complex. When a double mutation is performed (oligonucleotide SF1-mut2), there is a re-establishment of a symmetry and the elements interacting with these sequences are able to interact. With the deletion of one of the direct tandem repeats (oligonucleotide SF1-mut3), the complex still retains its competing ability, which shows that a single direct repeat can form a DNA-protein complex. It is important to mention that the mutated oligonucleotides, SF1-mut2 and SF1-mut3, could form new interactions with other nuclear protein factors. These observations need to be extended in future investigations.

In Conclusion

The silencer-enhancer elements situated on the 3'-side of the chicken α-globin gene domain (Recillas Targa et al. unpublished) represents a negative-positive control element that, probably, is activated in different developmental stages. We still do not know the mechanism of action of these elements, but one might speculate that they compete for the interactions (DNA-protein or protein-protein) with either positive or

negative elements. The silencer element investigated could have a role of repressor inhibiting interactions between regulatory regions of individual α-globin genes and could preferentially silence genes at different developmental stages. Another mode of action could be the blocking of the enhancer activity at specific developmental stages. This hypothesis might explain the proximity of the silencer and enhancer elements in the 3'-side of the chicken α-globin gene domain.

Acknowledgments

We are very grateful to Fayçal Bey and Sergey Razin for critical reading of the manuscript and to Richard Schwartzmann for his photography skills. Félix Recillas Targa is a recipient of a fellowship from the Consejo Nacional de Ciencia y Tecnología (CONACyT), México. This work was supported by the French CNRS, the Fondation pour la Recherche Médicale, the INSERM, the Association pour la Recherche sur le Cancer (ARC), and the Ligue Nationale Française contre le Cancer.

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