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NEGATIVE AND POSITIVE ELEMENTS IN THE PROMOTER REGION OF THE HUMAN APOFERRITIN L GENE

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We have characterized the promoter of the human gene coding for the apoferritin L subunit. Transient transfections of 5' and 3' deletion mutants indicate that the efficiency of the L promoter depends on both negative and positive cis-elements, located upstream and downstream of the transcription start point. DNaseI footprinting analysis of this DNA region revealed the presence of five protected segments. The most upstream one (element 1) corresponds to the negative cis-element and is recognized by factor(s) sharing a GC-sequence specificity. Three positive elements are in the region upstream of the start of transcription; a fifth positive cis-element (element 5) is localized in the first exon of the L gene.

Two subunits, designated heavy (H) and light (L), constitute the iron storage protein Ferritin (1). The heavy and light subunits are encoded by two distinct genes (2-4), whose relative expression varies according to cell type (5). Modifications of the H and L gene expression have also been reported in cells undergoing differentiation (6) and neoplastic transformation (7, 8), or as a consequence of exposure of cultured cells to hormones (9), iron (10), or geneticin (11). The two genes are controlled at transcriptional (9-11) and post-transcriptional levels. Post-transcriptional controls act on the mRNA stability (12) and on the efficiency of translation (13).

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The promoter region of the ferritin H gene has been characterized; its efficiency depends on the interactions with two ubiquitous factors, Sp1 and Bbf (14). Bbf plays a key role in the transcriptional increase of the H gene in cells treated with geneticin (11). Although the sequence of human ferritin L gene has long been known (3), the promoter region has not been defined.

In this paper we present the structural and functional characterization of the promoter of the ferritin L subunit gene. Transient transfection experiments revealed that the activity of the L promoter depends on the DNA sequences included between nucleotides -320 and +60. A negative regulatory element spans nucleotides -268 to -245 and is most probably recognized by trans-acting factor(s) which binds to GC-rich sequences. Three other cis-elements are localized in the region upstream the transcription start point and they all act as positive regulatory elements; one of them (element 3) is also recognized by a GC-binding protein. An additional positive element is contained in the first exon of the L gene.

MATERIALS AND METHODS

Enzymes and chemicals: Restriction enzymes, DNA polymerase large fragment, T4 polynucleotide kinase, ^{32}P-labelled compounds and ^{14}C-labelled chloroamphenicol were supplied by Amersham. Bal 31 exonuclease was from Promega. Taq DNA polymerase was purchased from Perkin-Elmer Cetus; acrylamide and agarose were from Bio Rad Laboratories; oligodeoxynucleotides were supplied by MedProbe. The acetyl-coenzyme A was supplied by Sigma.

Plasmid construction: The recombinant clone LΔ1 was kindly provided by Dr. C. Santoro (Trieste, Italy). Clones LΔ2 and LΔ3 were derived by Bal31 exonuclease digestion of clone LΔ1. The LΔ4 construct was obtained by amplifying the region -228 to +60 from LΔ1 clone by PCR. PCR was carried out in 50 µl of PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin), 200 µM dNTPs, 50 pmoles of the oligonucleotide primers (forward 5'-CAGGGCCCCAACCC-3'; reverse 5'-TGTTCGTCAAACA-CTGTTGA-3') and 2 units of Taq polymerase. The amplification steps were performed with a Techne Thermal Cycler programmed as follows: 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, for 30 cycles. The LΔ5 construct was obtained by amplifyng the region -176 to +60 from LΔ2 clone by PCR as above with the following oligonucleotides: forward 5'-GCGCGTGACTTCCCCT-3'; reverse 5'-TGTTCGTCAAACACTGTTGA-3'. The amplified products were inserted in the correct 5' to 3' orientation in the SmaI site of the pEMBL8-CAT

vector. The LΔ6 construct was obtained by cutting LΔ2 clone with AvaI-HindIII. After filling-in, the 129-bp fragment was inserted into the SmaI site of the pEMBL8-CAT vector. Clone LΔ7 was obtained by amplifying the region -228 to +1 from the LΔ2 clone by PCR with the following oligonucleotides: forward 5'-CAGGGCCCCAACC-3'; reverse 5'-CCGAACTGCGAGGGGA-3'. The PCR program was as described above except for the annealing temperature (55°C instead of 52°C). All these constructs were checked by nucleotide sequencing.

DNA transfection, CAT and luciferase assays: HeLa cells were cultured as monolayers in DMEM medium supplemented with 10% (v/v) fetal calf serum. Transfections were carried out with the calcium-phosphate technique (15), using 10 pmoles of total transfected DNA/dish, i.e. LΔ-CAT constructs and CMV-luciferase construct (16). CAT assays were performed as described (17). The luciferase activity was assayed as described (16) with a luminometer (Berthold, model LB9501). Two different CAT plasmid preparations were assayed; the data reported in Fig. 1 are the mean of the results of four independent transfection experiments.

DNaseI footprinting analysis and electrophoretic mobility shift assays: DNaseI footprinting analysis and electrophoretic mobility shift assays (EMSA) were performed as previously described (11, 14). The competitor GC-oligonucleotide has the sequence: 5'-CGGGGCGGGGC-3', repeated 3 times. The unspecific competitor oligonucleotide has the sequence: 5'-GGGTTGACTCGAGCCTCCTGCCAGAGCCACTG-3'.
To analyze by EMSA the DNA-protein interactions on element 1, a region of the L promoter from position -293 to position -224 was amplified by PCR (forward 5'-TCTAGCCTCCCAGAGG-3'; reverse 5'-CCAGGCCCCAACC-3'); after purification from agarose gel, the fragment was labelled at the 5'-ends with T4 polynucleotide kinase. For the EMSA of the element 5, two double-stranded oligonucleotides were used. The El5 oligonucleotide has the sequence 5'-CGGGTCTGTCTCTTGCTTCAACAGTGTTTGGACGGAACCAGA-TCCGGGGA-3'; the El 5-mut oligonucleotide has the sequence 5'-CGGG-TCTGTCTCTTGCTTCAACAGAACCCACCAATTATCAGATCCGGGGA-3'.

RESULTS AND DISCUSSION

Identification of the apoferritin L gene promoter

To look for promoter activity in the genomic region flanking the transcription start site of the human apoferritin L gene, fragments of different length of this region were inserted upstream the CAT reporter gene in the correct 5' to 3' orientation. In the constructs from LΔ1 to LΔ6, the 3' boundary of the L gene is represented by nucleotide +60; the 60 bases of the first exon of the L gene were removed in construct LΔ7

which contains the promoter region from position -228 to +1. These plasmids were transiently transfected in HeLa cells, together with a CMV-luciferase construct, and their relative efficiency in promoting the expression of the CAT gene was normalized to the luciferase activity. The mean of the results of four independent transfection experiments is reported in Fig. 1, together with a schematic representation of the constructs used.

The removal of 180 bp from position -501 to position -320 does not affect significantly the CAT activity of the resulting LΔ1, LΔ2 and LΔ3 constructs. This suggests that this region is not essential for the promotion of transcription of the L gene, at least in HeLa cells. The deletion of a further 92 bp (clone LΔ4; -228+60) results in a 15-fold activation of the CAT activity. Construct LΔ5, which carries the -176+60 region shows a CAT activity comparable to that of LΔ4. The 69 bp upstream the transcription start site, contained in clone LΔ6 (-69+60), are unable to drive efficient transcription of the CAT gene. The deletion of the sequences included between positions +1 and +60 (clone LΔ7; -228+1) strongly reduces the activity of the construct (compare the activities of LΔ4 and LΔ7).

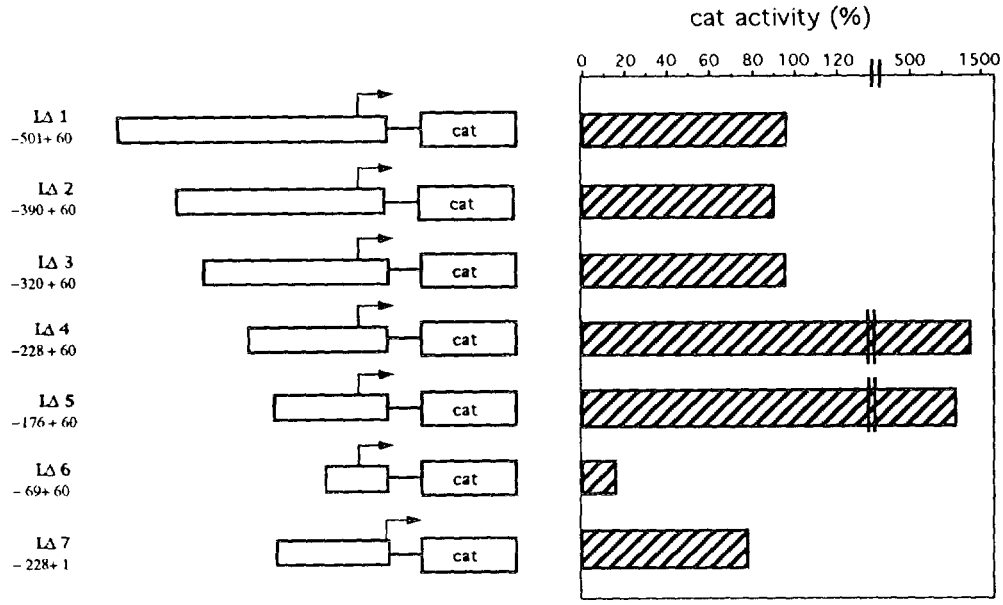

Fig. 1. In vivo analysis of the deletion mutants of ferritin L promoter. Left: Schematic representation of the constructs. Right: CAT activity in HeLa cells transfected with the L ferritin-CAT constructs. The values are the average of four independent transfection experiments, normalized to the luciferase activity of the CMV-luciferase construct.

The transient expression assays indicate: i) that the region -320 to +60 contains the sequence information required for the transcriptional activity of the human apoferritin L gene; and ii) that the promoter is constituted by negative and positive elements. The negative element is located between position -320 to -228; the positive element(s) are located upstream and downstream the transcription start point.

DNA-protein interactions within the L promoter

The cis-elements involved in the transcriptional control of the L gene have been further defined by DNaseI footprinting assays. The assays were performed on the region of the L promoter from -320 to -68 and on the region from -228 to +60 with crude nuclear extract from HeLa cells. To confirm the extension of the footprints, DNaseI assays were performed on both the DNA strands of the two promoter fragments. Figure 2 shows the DNaseI analysis of the lower strand of the -320-68 region and of the upper strand of the -228+60 region.

Five sequences are protected from DNaseI digestion by HeLa nuclear factors. The most upstream sequence, which we called element 1, spans from position -268 to -245. An inspection of the nucleotide sequence of this element revealed the presence of a canonical GC box. Lane 4 shows that the addition of a GC-competitor oligonucleotide to the binding reaction inhibits the formation of the DNA-protein complex on element 1, thus suggesting that element 1 is recognized by factor(s) sharing GC-sequence specificity. Recently, a 91-Kda GC-binding protein has been identified that is able to repress transcription of some house-keeping genes, e. g. those coding for calcium-dependent protease and b-actin (18). This 91 Kda factor could be responsible also for the repression of transcription of the L gene. Element 2 spans from position -207 to -184 and element 3 from position -132 to -105. It is likely that also element 3 arises from a trans-acting factor that recognizes GC-rich regions, because addition of the GC-oligo to the binding reaction acts as specific inhibitor (see lanes 4 and 9 of Fig. 2). Element 4 lies from position -94 to -68 and element 5 is located downstream the transcription start point, from position +20 to +41.

EMSAs of elements 1 and 5 of the ferritin L promoter

The putative negative element of the ferritin L promoter (element 1) lies between position -268 to -245 and is most probably due to the binding of factor(s) able to recognize GC-rich sequences. The DNA-

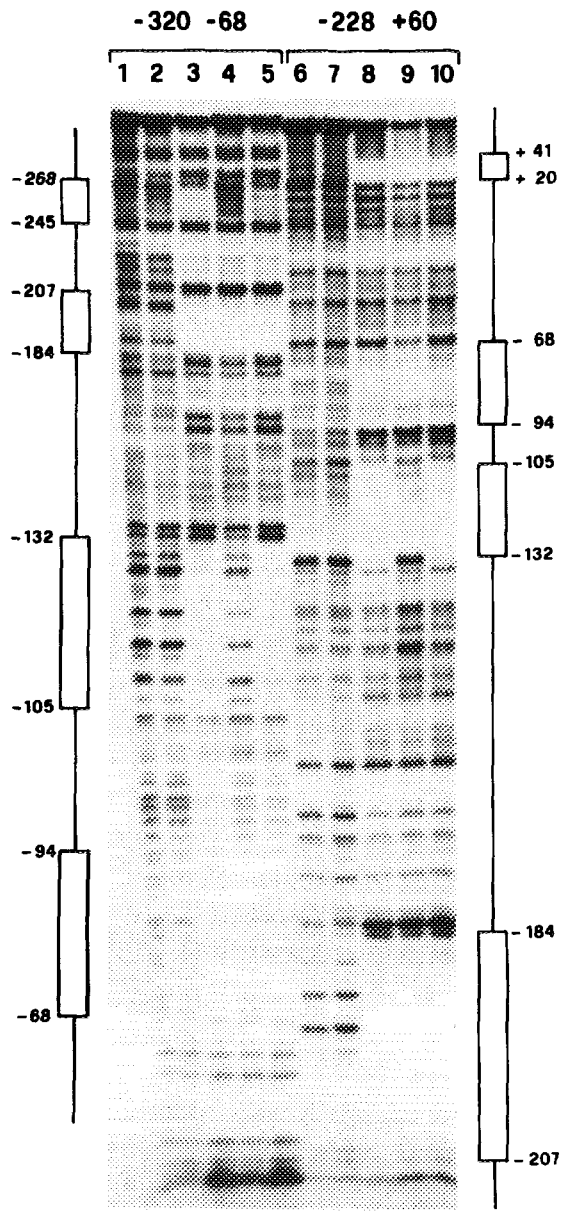

Fig. 2. DNaseI footprinting assay. Left: analysis of the -320-68 region, labelled on the lower strand, with HeLa nuclear extracts. Lanes 1 and 2: no nuclear extract, with 70 and 140 ng of DNaseI, respectively. Lane 3: 40 µg of nuclear extract. Lane 4: 40 µg of nuclear extract plus a 100-fold molar excess of a competitor GC-oligonucleotide. Lane 5: 40 µg of nuclear extract plus a 100-fold molar excess of an aspecific competitor oligo. Right: analysis of the -228+60 region, labelled on the upper strand, with HeLa nuclear extracts. Lanes 6 and 7: no nuclear extract, with 70 and 140 ng of DNaseI, respectively. Lane 8: 40 µg of nuclear extract. Lane 9: 40 µg of nuclear extract plus a 100-fold molar excess of a competitor GC-oligonucleotide. Lane 10: 40 µg of nuclear extract plus a 100-fold molar excess of an aspecific competitor oligo.

protein complex responsible for element 1 was further investigated using electrophoretic mobility shift assays (EMSA), with crude nuclear extracts from HeLa cells. As shown in panel A of Fig. 3, two specific complexes were detected (lane 2); the addition of a 100-fold molar excess of unlabelled element 1 fully competes for the faster migrating complex (lane 3), while a 200-fold molar excess of cold competitor (lane 4) is required to compete the slower migrating complex. This result suggests that the putative negative element of the L promoter can form two DNA-protein complexes with different affinity.

The element 5 of the L promoter lies between position +22 to +41. The first exon of the L gene contains a sequence that, in the encoded RNA, represents the Iron Responsive Element (IRE). The IRE, folded in a stem-loop structure, is the recognition element for the cytoplasmic factor IRE-binding protein (IRE-bp) (13). Element 5 overlaps the 5'

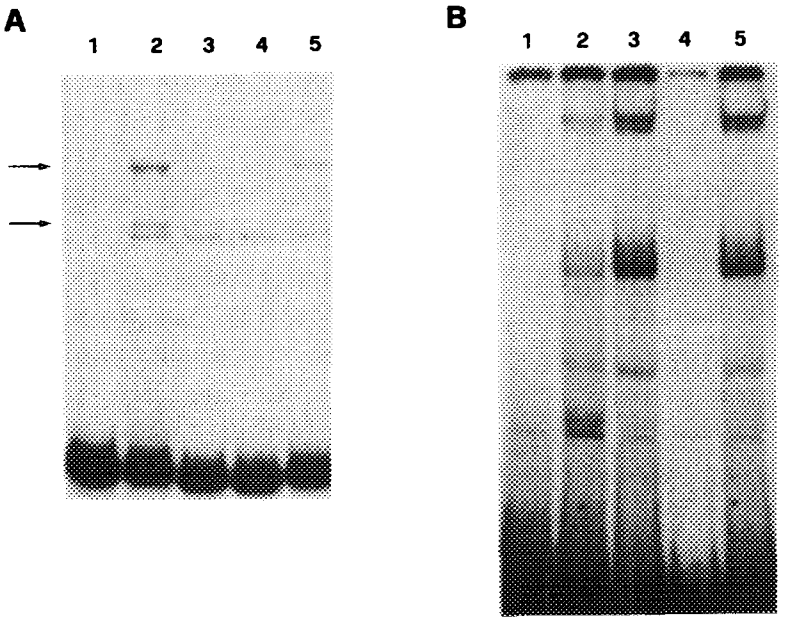

Fig. 3.EMSA of the elements 1 and 5 of the L promoter. Panel A: the region of the L promoter from -293 to -224, terminally labelled, was incubated with HeLa nuclear extracts. Lane 1: no extract. Lane 2: 4 μg of extract. Lanes 3 and 4: as lane 2 plus 50-fold and 100-fold molar excess of unlabelled DNA fragment. Lane 5: as lane 2 plus a 100-fold molar excess of unrelated competitor oligo. Panel B: the oligonucleotides El 5 and El 5 mut, terminally labelled, were incubated with HeLa nuclear extract. Lane 1: no extract. Lane 2: oligo El 5 challenged with 4 μg of extract. Lane 3: oligo El 5 mut challenged with 4 μg of extract. Lane 4: as lane 2 plus a 100-fold molar excess of unlabelled El 5. Lane 5: as lane 2 plus a plus a 100-fold molar excess of unrelated competitor oligo.

portion of the IRE sequence; three bases (UGU) at the 3'-end of the IRE loop are not included in this element. To ascertain whether the formation of the DNA-protein complex of element 5 requires a stem-loop structure, we challenged HeLa nuclear extracts with the El 5 oligonucleotide, whose sequence corresponds to that of the L promoter from position +17 to +64, and with oligonucleotide El 5 mut, that contains nucleotides +17 to +41 followed by an arbitrary sequence. The arbitrary sequence is 23 nucleotides long, and should prevents formation of the stem-loop structure. Figure 3 panel B shows the results of the EMSA: both the oligonucleotides are specifically recognized by nuclear factors, giving rise to two specific DNA-protein complexes. This result suggests that the DNA-protein interactions that occur on the element 5 are independent of a possible stem-loop structure.

In conclusion, we have characterized the promoter region of the apoferritin L gene as a first step towards understanding the mechanisms controlling the transcriptional efficiency of the H and L ferritin subunits. The ferritin L promoter appears to be much more complex than the H promoter, which essentially consists of 170 bp upstream the start of transcription, recognized by the transcription factors Sp1 and Bbf (14). Transcription of the L gene, instead, is driven by a longer promoter region with one negative and three positive elements upstream the transcription start point and a fifth positive element located in the first exon of the gene. Both ferritin promoters are GC-rich and both are recognized by positive nuclear factor(s) sharing a GC-sequence specificity; the H promoter on element A (14), and the L promoter on element 3. Apart from this, no other obvious similarities exist between the cis-acting regulatory sequences within the two promoters and possibly also the corresponding trans-acting factors. We are now investigating the nature of the nuclear factors binding the apoferritin L promoter.

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