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Structure of the gene for human uroporphyrinogen decarboxylase

Marc Romana, Anne Dubart, Denise Beaupain, Claude Chabret, Michel Goossens and Paul-Henri Romeo*

INSERM U 91, CNRS EA 607, Hôpital Henri Mondor, 94010 Créteil, France

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SUMMARY

Uroporphyrinogen decarboxylase, the fifth enzyme of the heme biosynthetic pathway, is an housekeeping enzyme whose activity is enhanced during erythropoietic differentiation. We have previously shown that this increased activity was in part accounted for by an enhanced transcription of the gene in erythropoietic tissues. To elucidate further the tissue specific regulation of an housekeeping gene we have isolated the human URO-D gene and determined its organization. The cloned gene comprises 10 exons spread over 3 Kb. Two transcriptional start sites were determined and analysis of 900 bp of the 5' flanking region suggests a very simple structural organization for the URO-D gene promoter. We also show that this gene is functional when transfected into mouse fibroblasts, and that its promoter is sensitive to a viral enhancer.

INTRODUCTION

The expression of housekeeping genes can be modulated from their basal level by different mechanisms. First, the production of different mRNAs by a family of genes could allow this modulation (1). Second, different mRNAs produced from a single gene by alternative transcription (2) or splicing (3) could be independantly regulated. Third, a transcriptional (4) or a post-transcriptional (5) control of a unique mRNA transcribed from a unique gene could be responsible for the regulation of this kind of genes.

The enzymes of the heme biosynthetic pathway are a good model of tissue specifically regulated housekeeping proteins : as heme is synthesized in all types of cells, the enzymes involved in its biosynthesis have an ubiquitous expression, but their activities are coordinately increased in the erythroid cell lineage (6). For these enzymes two types of gene organizations have been described thus far : the first enzyme of the pathway, δ aminolevulinic acid synthase, is encoded by two genes, one of them having an erythropoietic specific expression (7). In contrast, porphobilinogen deaminase, the third enzyme of the pathway, is encoded by a unique gene which is transcribed from two promoters, one of them being activated in the erythropoietic cell lineage

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(our unpublished data). We describe here the structural organization of the gene encoding another enzyme of this pathway.

Uroporphyrinogen decarboxylase (EC.4.1.1.37) (URO-D),is the fifth enzyme of the heme biosynthetic pathway. It is a polypeptide of 42 kilodaltons which is encoded at a single genetic locus on the short arm of human chromosome 1 (1 p 34) (8). URO-D activity is increased during erythropoietic cell differentiation and this has been correlated with an increase in the amount of the corresponding mRNA (9). We have recently shown that an enhanced transcriptional activity of the URO-D gene accounts in part for this mRNA accumulation (10).

Analysis of the human URO-D gene structure is a preliminary step for further studies of its regulated expression. We present here the isolation, structural analysis and functional studies of this gene. It is composed of 10 exons spread over 3 kb of DNA. Two transcription start points,a major one and a minor one, separated by 6 bases, were mapped by primer extension and RNAase mapping in several tissues or cell lines. The 900 bp located upstream from the initiation sites were sequenced and two obvious modules, a pseudo TATA box and a unique Sp1 box are clustered within 70 bp immediatly upstream from the start site. This observation suggests a very simple organization of this promoter. Transfections of this gene in mouse fibroblasts demonstrates that it is functional and sensitive to the SV40 enhancer, a feature which is not common to all housekeeping genes (11).

MATERIALS AND METHODS

Screening of the human library

A λ EMBL 4 human genomic library, constructed by inserting human DNA partially digested by Sau 3A into λ EMBL 4, was kindly provided by C. Besmond. Recombinant phages ($1.2\ 10^6$) were screened by plaque hybridization using a full length human URO-D cDNA (pUD 3) (10) as a probe.

Southern blot analysis and sequencing strategy.

Phage DNA was prepared from small liquid cultures. Restriction enzyme and Southern blot analysis were carried out as described by Maniatis et al. (12). ^{32}P nick translated pUD_3 was used as a specific hybridization probe. λ URO-D1 (Fig. 1) was digested by Bam HI or Hind III or both and restriction fragments were subcloned in pBR 322. DNA sequencing was carried out using both the chemical method of Maxam and Gilbert (13) and the dideoxy chain termination technique of Sanger et al. (14).

RNAase mapping analysis.

Total RNA was extracted from various tissues or cell lines by the LiCl technique (15) or the proteinase K-SDS procedure (16).

The plasmid used as template for synthesis of the URO-D mRNA complementary strand was constructed by inserting a Dde I - Dde I restriction fragment spanning the -51 to +77 (relative to the ATG codon) region and containing the URO-D first exon, into the p Gem 2 vector (Promega Biotec). This plasmid was then linearized by Hind III prior to transcription and a cRNA probe was prepared according to Zinn et al. (17) using α^{32}P UTP (Amersham 400Ci/mMol).

20 µg of total RNA were hybridized with labeled cRNA for 16h at 45° C in 80% formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl and 1 mM EDTA. Following hybridization, samples were diluted in RNAase buffer (Tris 10 mM (pH 7.5), NaCl 300 mM, EDTA 5mM) and treated with a mixture of RNAase A (45 µg/ml) and RNAase T_1, (700 U/ml) at 25° C for 60 min. The samples were further incubated for 15 min at 37° C after addition of 0,5 % SDS and 50 µg of proteinase K, extracted with phenol-chloroform, ethanol precipitated, and loaded on an 8 % sequencing gel.

S_1 mapping analysis

Single-stranded , end-labeled, RNA complementary fragments were mixed with 20 µg of total RNA in 80% formamide, 400 mM NaCl, 40 mM Pipes (pH 6.4), 1 mM EDTA, heated at 85° C for 5 min, immediately placed at 57° C and allowed to hybridize for up to 16 h. Ten volumes of ice-cold S_1 digestion buffer (NaAcetate 30 mM (pH 4.6), NaCl 300 mM, $ZnCl_2$ 4.5 mM, glycerol 2.5%) were added followed by 400 U of S_1 nuclease per ml of reaction volume. Incubation was carried out for 15 min. at 37° C then the reaction was quenched by the addition of EDTA, 12.5 mM final, and the samples were phenol extracted and ethanol precipitated. S_1 digested samples were electrophoresed in a 8% acrylamide denaturing gel.

Transfections in mouse fibroblasts

Cultures of L tk^- or 3T6 cells (5 x 10^5 cells per 10 cm dish) were treated for 16-18h with 0.8 ml of a calcium phosphate co-precipitate containing 10 µg of plasmid DNA and, only for stable transfections, 0.5 µg of a plasmid containing the HSV tk gene. The cells were then rinsed once and re-fed with fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum. For transient assays, cytoplasmic RNA was extracted 24 h later and analysed by the primer extension technique. For stable assays, HAT medium was added to the cells after 24 h and colonies were allowed to grow for an additional two weeks before harvesting for RNA preparation.

Primer extension analysis

Primer extension analysis was performed using an oligonucleotide complementary to 21 bases of the second exon of the URO-D gene. This oligonucleotide was end-labeled at is 5' end with $\gamma^{32}P$ ATP using T_4 polynucleotide kinase. 20 µg of total cytoplasmic RNA were denatured for 5 min at 95° C, then hybridized with 0.5 ng of the labeled oligonucleotide in 10 mM Pipes (pH 6.5), 400 mM NaCl and 1 mM EDTA for 16 h at 45° C. The hybrids were then ethanol precipitated and the primers were extended with reverse transcriptase as previously described (10). Extended primers were sized by electrophoresis in an 8% polyacrylamide, 7 M urea gel and detected by autoradiography.

RESULTS

Isolation and characterization of the human URO-D gene

Four human genome equivalents were screened using a ^{32}P labeled URO-D cDNA clone (10) as a probe. Eight independant recombinant phages were isolated, spanning 20 kb of genomic DNA, and their restriction maps showed that they were identical in their overlapping parts. One of them, λ URO-D_1, was further studied (Fig 1a).

Gene organization

Restriction fragments containing exons were identified with a full-length

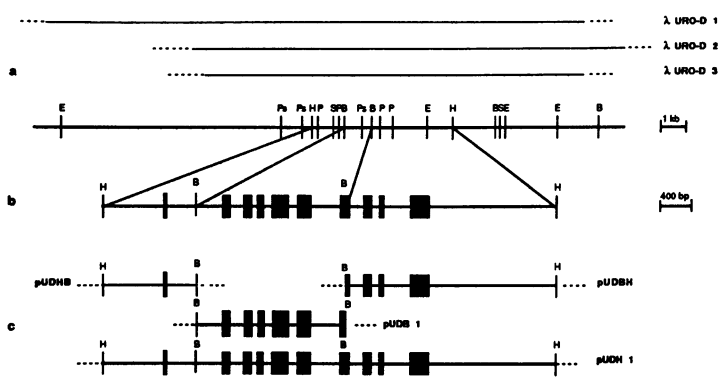

Fig. 1: Physical map of the URO-D gene region
a : the bottom line shows restriction sites in the URO-D gene region (P = Pvu II, Ps = Pst I, H = Hind III, S = Sac I, B = Bam HI, E = Eco RI). Some of the recombinant phages that we have isolated are represented above.
b : extended physical map showing the exon-intron organization of the URO-D gene. The exons appear as black boxes.
c : subclones that were constructed in pBR 322 for localization of intron-exon boundaries and functional studies of the gene.
The dotted lines indicate the vector sequences.

URO-D cDNA probe on Southern blots of λ URO-D 1 digested by several restriction enzymes. These blots revealed that the whole gene was contained in a 5.8 kb Hind III genomic DNA restriction fragment which was subcloned in pBR 322 and will be further referred to as $pUDH_1$. Three other fragments were subcloned and used to study the fine structure of the gene : pUDHB, $pUDB_1$ and pUDBH which are represented on Fig 1c. To define the boundaries of the exons,

TABLE 1 : Exon-Intron Organization of the URO-D gene

Intron Number	Sequence of Exon-Intron Junctions		Intron Size
	5' Boundary	3' Boundary	
1	GGG TTG GG gtgagt 38 Gly (13)	cacctgatcgccag A CCT CAG 39 Gly (13)	740 bp
2	TTA CCA G gtaaga 189 Glu (51)	cctcatgtatgcag AG TTT AGG 190 Glu (51)	150 bp
3	ACT CTG CAG gtgagg 269 Gln (77)	cctgtttcctacag CCA CTG CGT 270 Pro (78)	70 bp
4	GTA CCC CAG gtaccc 332 Gln (98)	ttctccccctccag GCA CTG GGC 333 Ala (99)	150 bp
5	GGT GCC CCA gtaatg 530 Pro (164)	ctatccttctctag TGG ACC CTG 531 Trp(165)	120 bp
6	GGT GCC CAG gtgagt 692 Gln (218)	cataccctaactag GCA TTG CAG 693 Ala (219)	430 bp
7	GTG CCC ATG gtgagg 830 Met (298)	ctgcttttttctag ATC ATC TTT 831 Ile (265)	180 bp
8	AAA GCC CG gtaagc 931 Arg (298)	ggctttgcttccag G GAG TGT 932 Arg (298)	110 bp
9	GCA TCT GAG gtaaca 998 Glu (320)	tgttggtcccctag GAG GAG ATC 999 Glu (321)	360 bp
Consensus from Literature	C a AG gt agt A g	ttttttttttt c n ag G cccccccccc t	

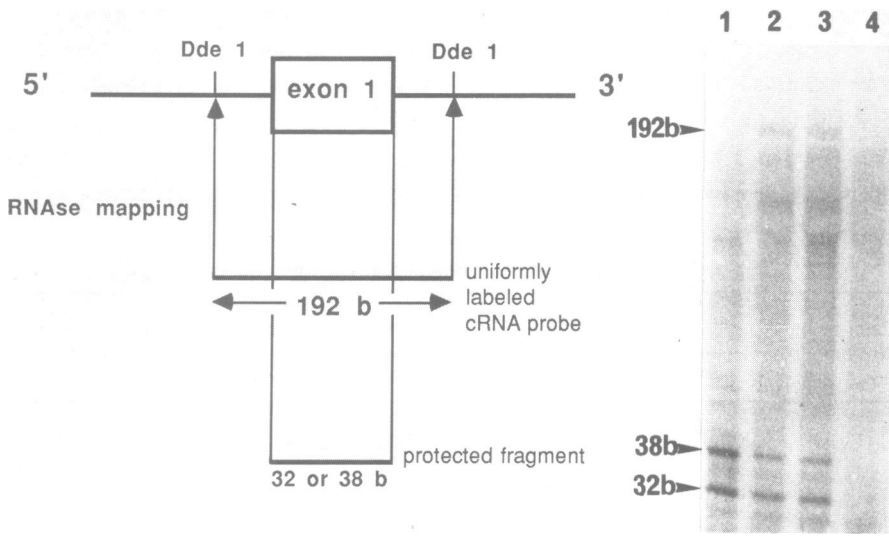

Fig. 2: RNAase mapping analysis of the 5' end of the URO-D mRNA
A 192 bases ^{32}P uniformly labeled cRNA was synthesized using a p Gemini 2 subclone containing a Dde I fragment derived from pUDHB (see Fig. 1). Hybridization and RNAase treatment were performed as described in the text using RNA extracted from an erythropoietic spleen removed from a patient having β thalassemia (lane 1), adult liver (lane 2), K562 cell line (lane 3) or wheat germ tRNA (lane 4).

we performed sequence analysis by the Maxam and Gilbert technique (13). This study resulted in the identification of 10 exons and 9 introns in the human URO-D gene as represented in Fig. 1b. The exon sequences were identical to the sequence of the human cDNA previously published (10). The first exon is located 600 bp apart from the others which are clustered in a 2.4 kb fragment. All sequences at the intron-exon boundaries are consistent with the consensus sequence proposed by Breathnach and Chambon (18) with the GT-AG rule applied to the splicing point (Table 1).

Determination of the site of transcription initiation.

The initiation site of transcription was determined by a combination of primer extension (10) and RNAase mapping assays using mRNAs extracted from erythroid and non erythroid tissues or cell lines (Fig. 2). In all tissues tested, the same two initiation sites were found in identical proportions. The major site is located 15 bases upstream from the ATG initiator codon and accounts for 90% of the initiations. The minor one is located 6 bases upstream from the other. Interestingly, the cDNA previously described (10)

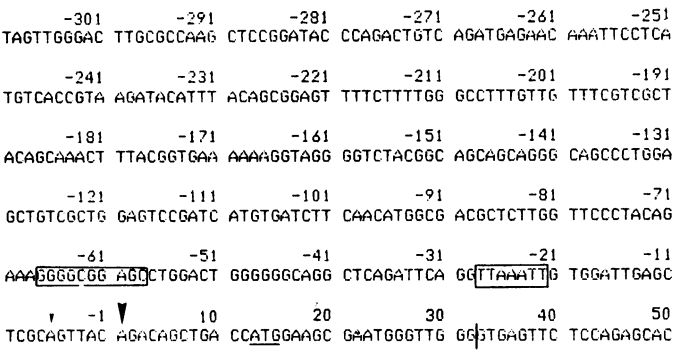

Fig. 3: URO-D gene promoter sequence
The nucleotides are numbered relative to the major mRNA start site. Sequences of interest are boxed. The two transcriptionnal start sites are indicated by arrows. The initiator methionine codon is underlined and the first exon-intron boundary is indicated by a vertical line.

corresponds to a mRNA initiated at the minor start site and demonstrates that this site is not an artefact of primer extension or RNAase mapping.

3' end of the URO-D gene

In order to look for any heterogeneity of the 3' end of the URO-D messenger RNA, an S_1 mapping experiment was performed using RNA isolated from various tissues or cell lines probed with an end-labeled DNA fragment spanning part of the last exon and 700 bases of the 3' flanking sequence (data not shown). Only one band was observed, showing the absence of heterogeneity of the 3' end of the URO-D transcript. The sequence of the 3' end of the gene shows that the translation stop codon, TGA, is located in the tenth exon, and precedes the AATAAA polyadenylation signal by 75 bp. We found 19 bp downstream this sequence a motif [5'TGTGTAGT 3'] which is homologous to the consensus sequence [5'YGTGTTYY 3'] (where Y is any pyrimidine) present 24 to 30 bp 3' from the polyadenylation site in numerous eucaryotic genes (19). The function of this sequence is unclear but it seems to be involved in the mRNA termination/cleavage/polyadenylation processes (19).

The 5' flanking region of the human URO-D gene

900 bp upstream from the cap site have been sequenced (Fig. 3)and the major features of this promoter region are the following : having only 57% GC over 150 bp upstream from the major transcription initiation site, it is not GC rich, as previously found for many housekeeping gene promoters. In these 150 bp, two elements are remarkable. First a TATA like sequence [5'TTAAATT 3'] is located at -21 relative to the major start site. This sequence is iden-

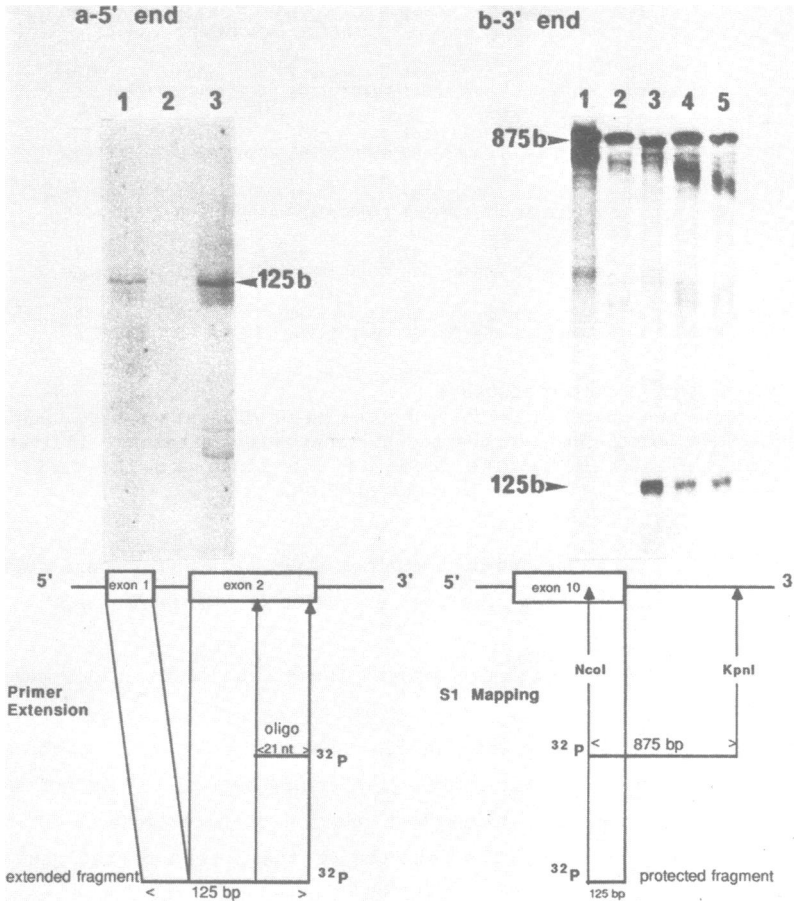

Fig. 4: Analysis of URO-D mRNA from transfected mouse fibroblasts

a : Primer extension assay for determination of the URO-D mRNA cap site. 20 µg of total RNA extracted from 3T6 cells transfected with pUDH1 (lane 1), 3T6 cells (lane 2) or spleen removed from a patient having β thalassemia (lane 3) were hybridized to an oligonucleotide complementary to the 5' end of the second exon of the URO-D gene. Hybrids were then treated as described in the text before loading on a denaturing gel.

b : S_1 mapping analysis for determination of the 3' end of the URO-D mRNA. The probe used was a NcoI - KpnI fragment of genomic DNA including the 3' end of the last exon of the URO-D gene and 600 bp of the 3' flanking sequence. This probe was either loaded alone (lane 1) or hybridized with 20 µg of total RNA extracted from 3T6 cells (lane 2), 3T6 cells transfected with pUDH1 (lanes 3 and 4) or spleen removed from a patient having β thalassemia (lane 5), before S_1 nuclease treatment.

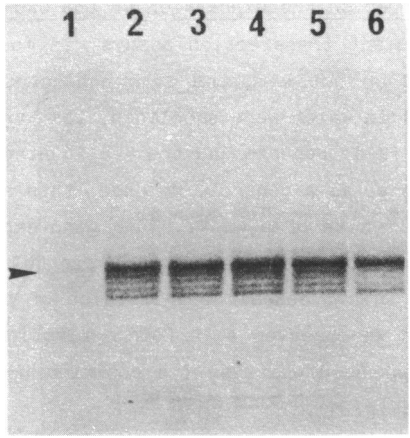

Fig. 5: Effect of SV40 enhancer on the URO-D gene transcription.
A primer extension was performed using the oligonucleotide shown in Fig. 4a and RNA extracted from 3T6 cells transfected with plasmids containing the URO-D gene but lacking SV40 sequences (lane 1), or having the SV40 enhancer 900 bp (lanes 2 and 3) or 3 kb (lanes 4 and 5) upstream from the start site of the URO-D gene. Lane 6 is also a primer extension assay using RNA extracted from a spleen with erythropoietic activity. The arrow indicates the extended products.

tical to the TATA box of the adenovirus 2 early (E_{IIa} E) promoter region (20). Second, a GC box [5'GGGGCGGAGC 3'] is located at position -60. Although this motif is frequently represented at multiple copies in numerous housekeeping gene promoters, there is only one in the URO-D gene promoter region. Another remarkable feature of this promoter sequence is the absence of any obvious CAAT box element, except if we consider that the sequence [5'CCAAG 3'] located at -80 on the non-coding strand could have this function.

URO-D gene expression

In order to test whether the genomic fragment we have cloned contains a functional URO-D gene, we have stably transfected the plasmid $pUDH_1$ in mouse tk^-fibroblasts. The expression of the URO-D gene was studied, either on individual clones or on pools of about 50 to 100 clones. Using RNAase mapping, we analysed the transcription start site, and S_1 mapping analysis was performed to study the termination of transcription. For single clones as for pools, our data demonstrate that the URO-D gene transfected in L tk^-cells is transcribed and processed exactly as in vivo (Fig. 4).

Effect of a viral enhancer on the expression of the URO-D gene

We also performed transient transfection assays, in mouse 3T6 cells, of the URO-D gene, associated or not with the SV40 enhancer. The vector used was pGem 4 (Promega Biotec) in which were subcloned, the Pvu II-Hind III fragment of SV40 containing the 72 bp repeats and the 5.8 kb Hind III genomic fragment containing the URO-D gene. As a control, we used this same Hind III fragment inserted at the Hind III site of pGem 4. URO-D gene expression was tested by primer extension analysis of the total cytoplasmic RNA extracted from mouse 3T6 transfected cells. In all cases the initiation of transcription remained the same as in vivo and we observed a 10 fold activation of transcription in the presence of SV40 enhancer whatever its position upstream from the start site (900 bp or 3 kb) (Fig. 5).

DISCUSSION

Using the human URO-D cDNA previously described (10), we have screened a genomic human DNA library and isolated eight independant overlapping recombinant phages spanning 20 kb of DNA. We have shown by Southern blotting that the whole human URO-D gene is contained in a 5.8 kb Hind III DNA restriction fragment. Its ten exons have been sequenced and are identical to the cDNA sequence, and all the intron-exon boundaries respect the splicing consensus sequences. RNAase mapping and primer extension assays of the 5' end of the gene have allowed us to determine precisely the initiation of transcription in various tissues. Only two start sites were found, the minor site corresponding to the 5' end of the cDNA previously described, and the major one lying 6 bases downstream. As this major initiation site lies 15 bases upstream from the initiator methionine, the corresponding mRNA has a very short 5' untranslated region. These two initiations are used in all tissues tested in identical proportions, no particular promoter being used in the red cell lineage.

Whatever tissue tested, no heterogeneity was found in the 3' end of the mRNA (see results) nor in the size of the mRNA (10), thus we deduce that this gene is transcribed into a unique mRNA species. Futhermore, analysing several cDNAs complementary to the URO-D mRNA extracted from lymphobastoid cells, de Verneuil et al. (21) have found the same sequence, except a few point substitutions. This result confirms the absence of an alternative splicing process of URO-D transcript in non erythropoietic tissues. We can thus conclude that this gene is expressed in a constitutive and in a tissue-specifically enhanced manner through the use of unique promoter and mRNA by means of an increased transcription.

As a first approach to the understanding of URO-D gene expression we have analysed the sequence of the promoter region. With only 57% GC over 150 bp upstream of the transcription initiation site, the URO-D gene promoter is not GC rich as compared to many housekeeping gene promoters (22). The 5' flanking region of the gene has a simple modular structure with only two obvious consensus sequences, a pseudo TATA box and a GC box. The pseudo TATA box sequence, found at position -26 relative to the major cap site is [5'TTAAATT 3'].Although this sequence is not homologous to the canonical TATA box sequence, it is identical to that of the E_{II} aE promoter of adenovirus type 2 which has been shown to be necessary (20) for transcription from the minor initiation site of the early promoter. The presence of a TATA box in this promoter is not surprising as it is part of many housekeeping gene promoter regions [G6PD (23), GAPDH (24) and tk (25)]. The GC box, found at -60, is perfectly homologous to the 10 bases consensus sequence described by Kadonaga (26) for the binding of Sp1, a factor known to activate transcription of many eucaryotic genes. Furthermore, assuming that the bases flanking the Sp1 consensus sequence could precisely regulate the affinity of the site for the Sp1 factor, we observe that the sequence found in the URO-D promoter is identical over 12 bases to that of two functional Sp1 binding sites of mouse DHFR promoter (27) and over 14 bases, to one of the Sp1 binding sites of the mouse HPRT gene promoter (28). In this latter promoter, the region demonstrated as critical for expression includes the GC box, and we can conclude that it plays an important functional role. The striking homologies with functional Sp1 binding sites described above, allow us to assume that the URO-D GC box binds Sp1, despite the absence of direct evidence. Finally, Sp1 binding sites are frequently found in clusters in the promoters of housekeeping genes and it can be noticed that, in the URO-D promoter, this motif is present only once. As it is known that binding of Sp1 is cooperative (29), the presence of a single binding site in this promoter could influence its expression. However, a recent report (30) has shown that the same level of transcription can be obtained with one or multiple Sp1 binding sites.

Those structural data suggest that the simple structure of the URO-D promoter sustains its ubiquitous expression and that its organization resembles that of the E_1B gene promoter of adenovirus 2 : it has the same two elements, a GC box and a TATA box (31) but the 5' ends of the two motifs are spaced by 18 nucleotides in the E1B promoter and by 39 nucleotides in the URO-D promoter. The spacing beetwen these two elements should be studied to determine whether or not it plays a role in the transcriptional regulation of these genes.

When regulated at a transcriptional level, the mechanisms of the tissue specific enhanced expression of housekeeping genes are unknown. We have previously shown that the increase in the amount of URO-D mRNA during erythropoietic differentiation is accounted for by a tissue-specific transcriptional activation of the gene. Assuming that the frequency of initiation and the block of the elongation are the main mechanisms that govern the amount of primary transcript, the enhanced transcription of the URO-D gene could be analysed as follows : first, we can hypothesize the existence of a "block in elongation" within the first intron, as it was described for the c-myc gene (32). Indeed the structure of the URO-D gene could favour such a regulation: the first exon which is only 32 bp long is located 700 bp apart from the others which are clustered in a 2.4 kb fragment of DNA. A stop in transcript elongation within the first intron, occuring in non-erythroid tissues and being suppressed during erythroid differentiation could explain the apparent increase in the transcription that we observed. The second possible explanation of the transcriptional increase of URO-D mRNA during erythroid differentiation is the effect of a tissue specific enhancer on the URO-D gene promoter. This implies two aspects : first the sensitivity of this promoter to the effect of an enhancer ; second, the existence of a nearby enhancer. The effect of enhancers has been tested on a few housekeeping promoters and some authors have made the hypothesis that insensitivity to viral enhancers was a characteristic of housekeeping gene promoters (11). In this work, we have tested the effect of the SV40 enhancer on the transcription of the URO-D gene from its promoter by transient transfection in mouse 3T6 cells. We have observed a 10 fold activation of transcription when the SV40 enhancer was located 900 bp or 3 kb upstream from the start site (Fig. 5). Thus, we have demonstrated that the URO-D gene promoter can be activated by a viral enhancer sequence, but whether or not the URO-D gene possesses a tissue specific enhancer remains to be proved by transfections experiments in cells having erythroid potentialities.

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*To whom correspondence should be addressed

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