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Multiple initiators and C/EBP binding sites are involved in transcription from the TATA-less rat XDH/XO basal promoter

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ABSTRACT

In the present study, we have explored further the organization of the TATA-less rat xanthine dehydrogenase/oxidase gene (XDH/XO). A DNase I hypersensitive site has been identified which it colocalizes with the basal promoter reported previously [Chow *et al.* (1994) *Nucleic Acids Res.*, 22, 1846–1854]. Gel mobility shift assays indicate the presence of multiple binding factors located in the promoter. At least six footprints were detected of which two have been shown to be C/EBP binding sites. Members of the C/EBP-α and C/EBP-β, but not C/EBP-δ, family are able to bind to these two sites. Deletional and mutational studies revealed that C/EBP binding is not essential for the basal level of transcription initiation of this promoter. Much of the transcriptional activity resides in the –102 to –7 DNA fragment, which contains all initator activity which acts unidirectionally. Within this fragment, four putative initiator elements could be identified; interestingly, the linear integrity of these initiators is important for efficient transcription of the XDH/XO gene. Separation of the initiators leads to a complete loss of transcription activity; however, this loss could be partially restored by the introduction of an Sp1 binding site upstream of the separated initiators. Despite a difference in usage/frequency of initiation at the various initiators, primer extension analyses reveal similar positions for transcription initiations in both XDH/XO reporter constructs and in the endogenous XDH/XO gene. The differential usage of initiators may imply a possible post-transcriptional regulation for the XDH/XO gene.

INTRODUCTION

Xanthine dehydrogenase (XDH) is an enzyme which plays a key role in the turnover and excretion of purines. Xanthine dehydrogenase catalyzes the oxidation of hypoxanthine to xanthine and subsequently to uric acid (37). However, under certain pathophysiological conditions, XDH can be covalently modified such that it is converted to xanthine oxidase (XO) (44). The XDH/XO conversion leads to a change in the substrate utilization. Xanthine oxidase utilizes oxygen instead of NAD as the electron acceptor, and as a consequence, superoxide radicals are produced (35). Subsequent generation of reactive oxygen species, such as hydroxyl radicals (33), can lead to severe cell damage. Such a process has been postulated to play a role in ischemia-reperfusion injury and in inflammation (7,20).

Due to the important role of XDH/XO in physiology and its clinical relevance (7,20,35), the biochemical characteristics of XDH/XO have been studied intensively (4,21,33,37). Conversion of XDH to XO is thought to take place either through a reversible oxidation and/or a proteolytic cleavage. In terms of tissue distribution, liver and intestine have been shown to have the highest XDH/XO activity (31,32), though it is now recognized that the enzyme is quite widely distributed in many tissues. Interferon has been reported to up-regulate the expresson of the gene in both rat (13) and mouse (16) species. Several other acute phase mediators have also been reported to affect the XDH/XO conversion (17). The cDNA sequences of XDH/XO have been identified in *Drosophila* (28), *Calliphora* (22), rat (2), mouse (42) and human (23). More than 90% sequence homology has been found among the vertebrate species (23).

Recently, our laboratory reported the sequence of the rat XDH/XO gene promoter (11). The identification of the promoter was complicated by the highly intronic structure of the 5′ end of the rat XDH/XO gene and the existence of multiple transcriptional start sites. The rat XDH/XO promoter contains neither TATA box nor Sp1 binding site. However, several putative upstream factor binding sites and initiator sequences were identified by computer analysis of the 5′ sequence of this gene.

In this report, we describe further our studies of the rat XDH/XO gene promoter. We have mapped the hypersensitive sites of the gene in rat liver nuclei and have found at least six footprints in the basal promoter involved in nuclear factor binding. Two C/EBP binding sites have been identified among these binding sites. Deletional analysis of the promoter and associated transfection studies have indicated a major role for the transcription initiators (Inrs) in maintaince of basal transcription. Further, the integrity of the DNA fragment containing all the initiators is essential to their function.

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MATERIALS AND METHODS

Hypersensitive site study

Isolation of rat liver nuclei was performed according to Gorski *et al.* with slight modifications (18). A freshly isolated rat liver was minced thoroughly with a razor blade and mixed with 30 ml homogenization buffer [10 mM Hepes (pH 7.9), 25 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 1 mM EDTA, 2 M sucrose and 10% glycerol] in a Teflon-glass homogenizer. The mixture was homogenized 10-fold with a motor-driven homogenizer. The homogenate was layered on the top of 5 ml homogenization buffer in a Beckman polyallomer tube and centrifuged at 24 000 r.p.m. for 45 min in an SW 27 rotor at 0°C. The nuclear pellets were collected together and washed 3× in 20 ml of buffer containing 10 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0.25 M sucrose, 0.5 mM spermidine and 0.15 mM spermine. Isolated nuclei were resuspended in the same buffer at a final concentration of 1.25 mg/ml DNA.

For the hypersensitive site study, increasing concentrations of DNase I were added to tubes containing 200 µl of liver nuclei (see above). After adjusting to a final concentration of 10 mM $MgCl_2$ and 10 mM $CaCl_2$, the sample was incubated at room temperature for 5 min. The reaction was stopped by adding 1/10th volume of 10% SDS, 0.25 M EDTA and 2 mg/ml proteinase K. The mixture was then incubated at 65°C overnight. After phenol-$CHCl_3$ extraction and ethanol precipitation, isolated DNA was digested with *Kpn*I and separated on a 0.8% 1× TAE agarose gel. Southern analysis was performed as described previously (11) using a 141 bp *Kpn*I–*Pst*I restriction fragment, which was located at approximately –6000 bp.

Gel mobility shift assays

Liver nuclei extract was isolated as described by Sears and Sealy (38). Isolated rat liver nuclei were resuspended in 1 ml of buffer B2 containing 10 mM Hepes (pH 8.0), 0.5 M NaCl, 0.125 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 25% glycerol, 1 mM PMSF, 10 mM benzamidine, 7 mM β-mercaptoethanol, 0.28 µg/ml pepstatin and 5 µg/ml leupeptin. The sample was kept on ice for 30 min with mixing every 5 min. The sample was clarified at 10 000 *g* for 10 min. Protein concentration was measured by the Bradford method.

DNA probes used for gel mobility shift assays were either 5′-end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase or by filling in with [α-^{32}P]dATP using the Klenow fragment. Conditions for the DNA–protein binding and electrophoresis conditions were as described by Cheyette *et al.* (9). The P1 DNA fragment is located from –118 to –19 while the P2 fragment is located from –273 to –114 in the XDH promoter. Sequences for the oligonucleotides used in gel mobility shift assays and footprinting were: CCAAT, 5′-CCGGTTGTTTCTCATTGGGTAACTTT-3′ (–118 to –92); ISRE, 5′-ACCGGGTAACTTTGTTTCATTTTGCTGGGAGG-3′ (–101 to –73); EF I, 5′-ACCGTGCATGCCGATTGGTGGAAGTA-3′ (14); CP2, 5′-AGCTCTTACCCCCATTGGGTGGCGCG-3′ (10); EF II, 5′-TCGAGATCTAATGTAGTCTTATGCAATACTCTTGTAGTCTTGCAACACCC-3′ (38); mEF II, 5′-TCGAGATCTAATGTAccggatccggaTACTCTTGccggatccggACACCC-3′ (38); IES, 5′-CAGCTGTTGGCTGCAATTGCGCCACCGCCACAG-3′ (27); mIES, 5′-CAGCTGTTGGCTtaccggGCGCCACCGCCACAG-3′ (27); PepA, 5′-TCCCACAGTCTCTGTAGCTCTAATCATTATCGATCCTGG-3′ (9); P1-C/EBP, 5′-CCGGTTGTTTCTCATTGGGTAACTTTGTTTCATTTTGCTGGGAGG-3′ (–118 to –73); mP1-C/EBP, 5′-GAGCCGGTTGTTTCTCATTcGcTAtCTTTGTTTCATTTTGCTGGG-3′; P2-C/EBP, 5′-GATCTCCCGTCCTTCCTGGATTGTGCAAACCTGTGACTCTTGCC-3′ (–178 to –139).

DNase I footprinting

A 252 bp *Eco*RI–*Sac*I fragment (XDH sequence –273 to –20) was labelled with [α-^{32}P]dATP by using Klenow polymerase. About 3–5 fmol of labelled probe was mixed with 5 µg rat liver nuclear extract under the binding condition used for gel mobility shift assays. The mixture was incubated at room temperature for 30 min. After the incubation, the mixture was transferred to an ice-water bath. DNase I was added to a final concentration of 50 U/ml and the mixture was further incubated at 4°C for 15 s. Digestion was stopped by adding 4 vol of DNase I stop solution (1% SDS, 10 mM EDTA, 10 µg/ml proteinase K and 10 µg/ml denatured salman sperm DNA) and incubating at 65°C for 20 min. After phenol-$CHCl_3$ extraction and ethanol precipitation, the product were resolved in a 6% denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Deletional and mutational analysis

Desired fragments for subcloning were generated from PCR reactions using appropriate oligonucleotides. Subsequent PCR products were subcloned into the luciferase reporter construct as described previously (11). Positive clones were identified by colony hybridization. For the constructs containing Sp1 binding sites, an Sp1 fragment was generated from a PCR reaction using a plasmid containing the Sp1 binding site as template. As a result, the additional DNA sequence between the initiator and the Sp1 binding site contains sequences of the multiple cloning sites of the plasmid. All constructs were amplified by liquid culture and sequenced to verify identity before use. Transient transfection assays were performed in HeLa cells as described (11).

Primer extension

Various XO-luciferase plasmid constructs (20 µg) were transfected into HeLa cells and total RNA was harvested 2 days after the transfection by using Tri-reagent (Molecular Research Center Inc.). Total RNA (20 µg) was used for primer extension analysis as described previously (11). An oligonucleotide (5′-GGCGTCTTCCATTTTACCAACAGTACCGG-3′) complementary to the coding sequences of the luciferase reporter gene was used as primer in the primer extension.

RESULTS

Determination of hypersensitive sites in the rat XDH/XO promoter

As a first step in the analysis of the organization of the rat XDH/XO promoter, we determined if any hypersensitive sites were present in the XDH/XO gene in rat liver nuclei. The presence of hypersensitive sites provides a good indication of regions involved in binding of transcription factors (6,19). As shown in Figure 1, a hypersensitive site was identified in a 9.0 kb *Kpn*I fragment, spanning from –7.5 kb upstream to +1.5 kb downstream in the XDH/XO promoter. The position of hyper-

sensitive site (HS) co-localizes with the previously identified rat XDH/XO promoter (11). This result suggests the rat XDH/XO promoter is indeed 'open' in the liver. Within this domain, we may expect that there are multiple binding sites for upstream regulatory factors. Furthermore, as we reported in the previous study, this result is consistent with the notion that the rat XDH/XO promoter is located immediately upstream of the first coding exon and that there are no additional introns. No other hypersensitive site could be detected within this *Kpn*I restriction digested fragment.

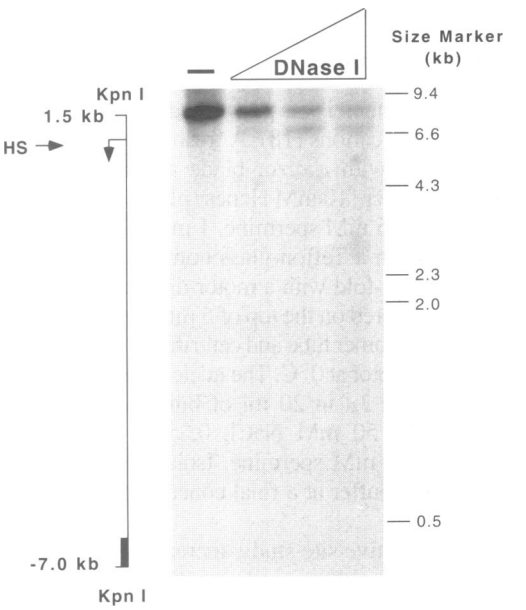

Figure 1. Hypersensitive sites in the rat XDH/XO gene. Various concentrations of DNase I (from 1 to 3 U/ml) were used to detect hypersensitive sites in the XDH/XO gene in isolated rat liver nuclei. The arrow head pointing vertically represents the promoter of the XDH/XO gene identified previously (11). Bold arrow represent the hypersensitive site (HS) found at –0.3 kb upstream of the ATG. Filled box indicates the probe used in the hybridization.

Identification of transcription factors binding to XDH/XO sequences

Previous work has shown that a 252 bp fragment upstream of, and including, the transcriptional start sites contains essentially all of the promoter activity (11). Computer analyses of this 252 bp fragment identified several putative factor binding sites including an interferon-α stimulatory responsive element (ISRE) and an inverted CCAAT motif at –91 and –100 respectively [because there are four regions for transcription start (see below), we have chosen to begin numbering at the translational start site; as a result, the transcriptional start sites are found at –10 to –22 (Inr 1), –30 to –35 (Inr 2), –48 to –60 (Inr 3) and –73 to –85 (Inr 4)]. To better understand the function of the rat XDH/XO promoter and the role of the putative factor binding sites, we focused our attention within the 252 bp fragment. Initial gel mobility shift experiments indicated this 252 bp fragment can form a range of specific DNA–protein complexes. Restriction enzyme digestion was then utilized to generate two XDH/XO promoter fragments [P1 (from –114 to –20 bp) and P2 (from –268 to –114 bp) fragments]. As shown in Figure 2A, multiple DNA–protein complexes can be observed when using either the P1 or the P2 fragment as a probe. Since putative ISRE and CCAAT motifs are seen in the P1 fragment, smaller oligonucleotides containing XDH/XO sequences encompassing these two motifs were synthesized and used as competitors in gel mobility shift experiments (Fig. 2A). Up to 100-fold molar excess of the ISRE oligonucleotide do not compete any DNA–protein complex formed in the P1 fragment. On the other hand, the majority of the DNA–protein complexes are competed by the CCAAT oligonucleotide effectively, even as low as a 5-fold molar excess (data not shown).

A similar competition experiment was performed with the P2 fragment as a probe (Fig. 2A). To our surprise, the majority of the DNA–protein complexes in the P2 fragment are also competed by the CCAAT oligonucleotide, and to a similar extent as seen for the P1 fragment, although no obvious CCAAT motif had been

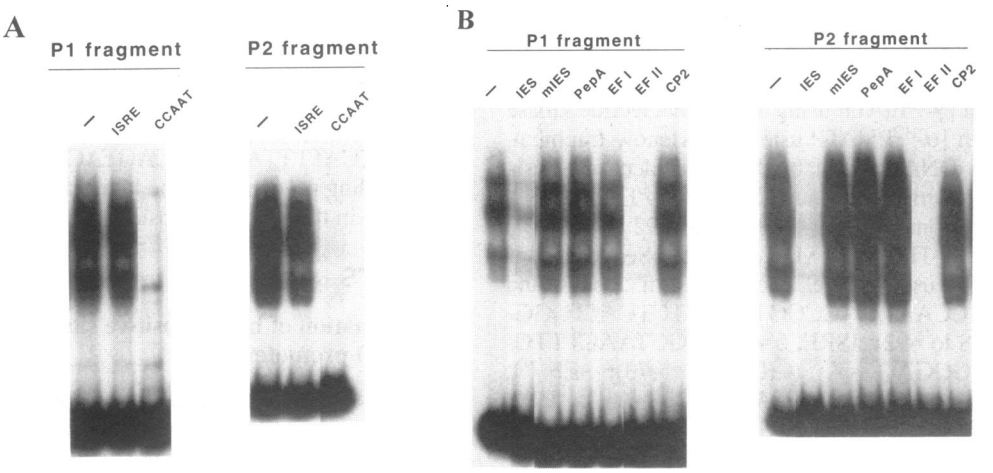

Figure 2. Gel mobility gel assays using the P1 and the P2 fragments of the XDH/XO promoter as probes. (**A**) Specific DNA–protein complexes formed when using the P1 or the P2 fragment in heated rat liver nuclear extract. A 100-fold molar excess of oligonucleotides encompassing the sequences of the ISRE and CCAAT motifs of the XDH/XO gene (sequence information is in the Materials and Methods section) were used as competitors. (**B**) Competition study of the P1 and P2 fragments. Oligonucleotides for known binding factors (IES, mIES, PepA, EF I, EF II and CP2) were used in 100-fold molar excess to compete factor binding to the labelled P1 and P2 fragments.

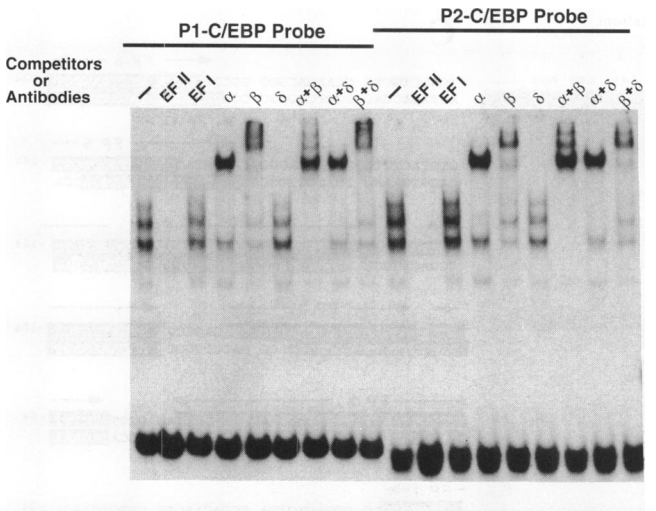

Figure 3. Gel mobility shift assay in the presence of different C/EBP antibodies. Oligonucleotides encompassing the P1-C/EBP and P2-C/EBP binding sites were synthesized and used as probes for the assay. Antibodies against C/EBP-α, C/EBP-β and C/EBP-δ were incubated with rat liver nuclear extract before adding the probe. EF I and EF II oligonucleotides were also used as competitors to assess the specificity of the probes.

identified in the P2 fragment. These results led us to search for any sequence similarity between the P1 and P2 fragments, with special attention focused upon the region encompassed by the CCAAT oligonucleotide. Direct sequence comparison between the P2 fragment and the CCAAT oligonucleotide suggested a motif that is similar to a C/EBP binding site. The putative C/EBP binding site is located at –103 and –162 in the rat XDH/XO promoter.

In order to test the notion that both P1 and P2 fragments bind C/EBP and not to a CCAAT factor, enhancer binding factor I (EF I) and enhancer binding factor II (EF II) from Rous sarcoma virus long terminal repeat were utilized as competitors in subsequent gel mobility shift assays. EF I oligonucleotides have been shown by Faber and Sealy to bind the CCAAT factor (14) while the EF II oligonucleotide avidly binds C/EBP factors (38). Besides EF I and EF II oligonucleotides, the CCAAT motif from mouse MHC H-2K gene promoter (CP2) (10) and the C/EBP motif from the internal enhancer sequence of Rous sarcoma virus (IES) (27) were also used as competitors. Mutated IES (mIES) (27) and an oligonucleotide derived from a liver-specific upstream binding element from the PEPCK gene (PepA) (9) were used as negative controls. As shown in Figure 2B, up to 50-fold molar excess of either EF I, CP2, PepA or mIES oligonucleotides does not compete any DNA–protein complex formed in both P1 and P2 fragments. However, EF II or IES oligonucleotides compete all the binding activity. This competition can be carried out in as low as 5-fold molar excess (data not shown). These competition experiments supported our supposition that C/EBP binding sites, instead of a CCAAT factor, are present in both P1 and P2 fragments.

Confirmation of the presence of C/EBP binding factors in both P1 and P2 fragments

The C/EBP family consists of a set of closely related transcription factors, though different members of the family seem to have different functions (3,5,8,26). Four members of the family can be distinguished using specific antibodies (8). Since multiple DNA–protein complexes that are formed in both P1 and P2 fragments can be competed by C/EBP specific oligonucleotides (EF II and IES oligonucleotides), this suggests more than one C/EBP member might bind to the P1 and P2 fragments. Gel mobility shift experiments were performed in the presence of specific C/EBP-subtype-antibodies in order to identify which member(s) of the C/EBP family bind to the P1 and P2 fragments. As shown in Figure 3, using oligonucleotides encompassing the putative P1-C/EBP and P2-C/EBP binding sites as probes, complexes formed can be competed by EF II but not by EF I oligonucleotides. In the presence of specific antibodies against C/EBP, both C/EBP-α and C/EBP-β specific antibodies can 'supershift' specific DNA–protein complexes in oligonucleotides containing either the putative P1-C/EBP binding site or the P2-C/EBP binding site. One of the DNA–protein complexes can be recognized by both C/EBP-α and C/EBP-β antibodies indicating the presence of heterodimers between C/EBP-α and C/EBP-β. Several other DNA–protein complexes are recognized exclusively by either C/EBP-α or C/EBP-β antibodies. Obviously one such band in each case could be a homodimer, however, multiple such bands might reflect the presence of different sub-members of the C/EBP-α and C/EBP-β family (for instance the presence of both LIP and LAP in a heteromeric complex). C/EBP-δ specific antibodies, however, do not recognize any of the DNA–protein complexes.

Mapping the C/EBP binding sites and other factor binding sites in the XDH/XO promoter

As mentioned above, two C/EBP binding domains have been identified in the XDH/XO promoter and most likely at –103 and –162. To confirm that these two sites are indeed responsible for C/EBP binding and to more precisely map their positions, we performed DNase I footprint analyses. As shown in Figure 4A and B, a total of six footprints (FP 1–FP 6) were identified on the the non-coding strand of the XDH/XO promoter. In the presence of EF II or IES oligonucleotides, two footprints, FP 2 (–114 to –76) and FP 4 (–174 to –147), become accessible to DNase I digestion. Furthermore, these two footprints are still detected in the presence of an excess of mutant EF II (mEF II), mIES, EF I or PepA oligonucleotides. These results confirmed that the two C/EBP binding sites previously identified (see Fig. 3) are indeed located at –103 and –162 upstream of the XDH/XO promoter. The other four footprints are not affected by either EF II/IES or mEF II/mIES/EF I/PepA oligonucleotides. Similar results can also be observed with the coding strand of the XDH/XO promoter (data not shown). A summary of the footprint analysis is shown in Figure 4C.

Deletion analysis within the basal promoter of the rat XDH/XO gene

Using transient transfection assays, we have previously shown that the majority of the promoter activity of the rat XDH/XO gene is located within the –116 to –19 fragment (11). In an initial series of experiments, we reduced the size of this fragment from the 3′ side (Fig. 5A). Removal to –46, which leaves Inr 3 and 4 intact, is accompanied by a 60% decrease in basal activity. Removal of sequences to –73, which leaves only Inr 4, abolishes all promoter activity. We also examined the basal activity of a construct which spanned from –116 to –7 bp. The presence of the additional

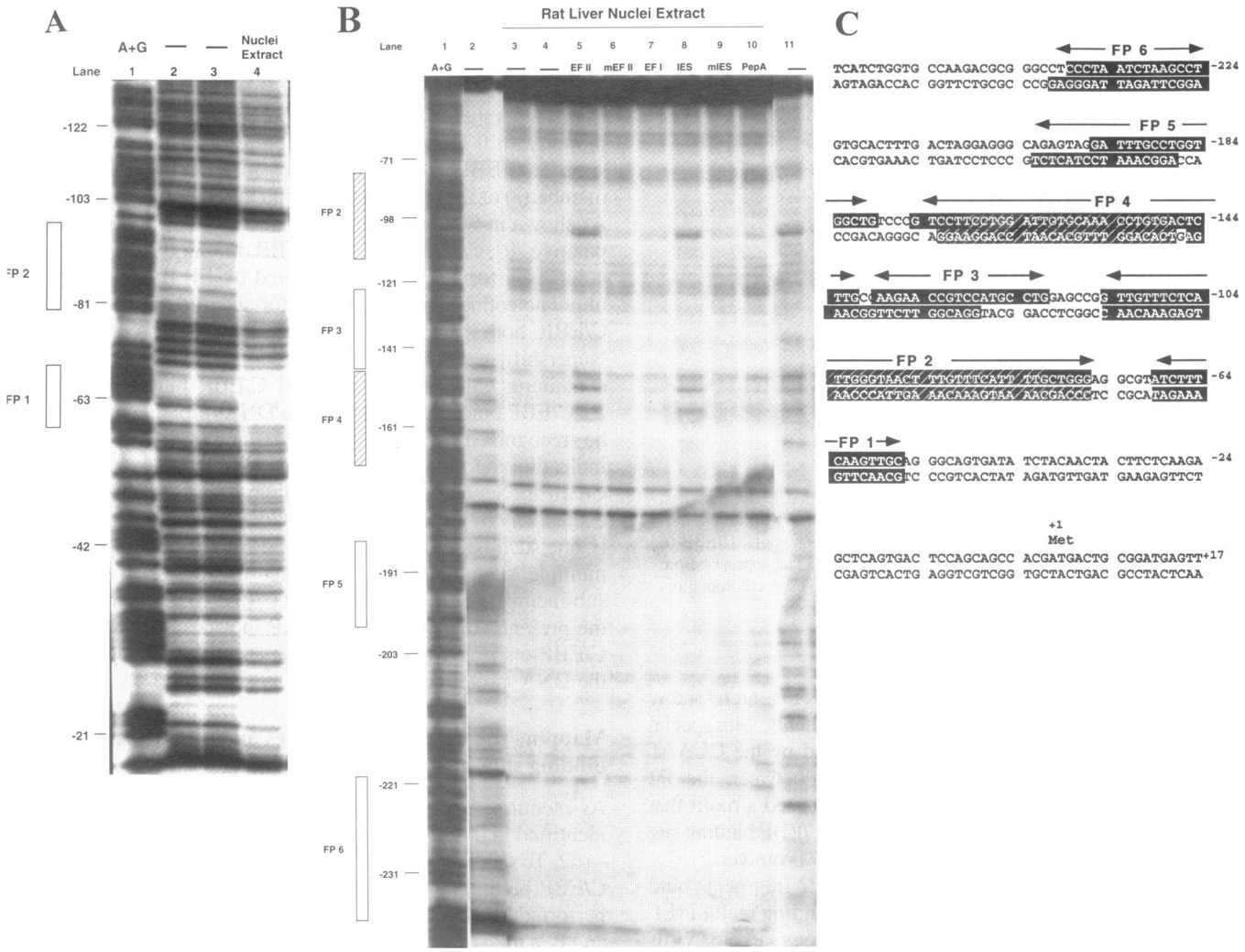

Figure 4. *In vitro* footprint analysis of the XDH/XO promoter. (**A**) DNA, encompassing sequences from –447 to +42 of the XDH/XO gene, was labelled with T_4 polynucleotide kinase on the non-coding strand of the DNA and used as probe for DNase I digestion following incubation as described below. Identified footprints are indicated as open boxes. Lane 1, size marker from Maxam and Gilbert reaction on adenine and guanosine nucleotides of the probe. Lanes 2 and 3, partial digestion of the probe in the absence of added nuclear extract. Lane 4, partial digestion of the probe in the presence of unheated rat liver nuclear extract. (**B**) DNA encompassing sequences from –273 to –20 of the XDH/XO gene was labelled with the Klenow fragment on the non-coding strand of the DNA and used as a probe (lanes 1–11). A 500-fold molar excess of different competitors were used in these experiments. Identified footprints were labelled from footprint 2 (FP 2) to FP 6. Open boxes represent the identified footprints. Hatched boxes represent the C/EBP binding sites. A+G is a Maxam and Gilbert reaction used as a size marker. Lane 1, A+G reaction of the non-coding strand. Lanes 2 and 11, partial digestion of the non-coding strand probe in the absence of nuclear extract. Lanes 3–10, partial digestion of the non-coding strand probe in the presence of nuclear extract, plus different competitors as shown. (**C**) Summary of the footprint analysis of the coding and non-coding strands of the XDH/XO promoter. Nucleotide sequences encompassing the six footprints are marked with filled boxes. The hatched area represents nucleotide sequences protected by the C/EBP binding factors.

sequences at the 3′ end led to a significant reduction in basal activity (Fig. 5A). As an control, we showed that the reverse orientation of the –102 to –7 construct does not support any promoter activity. We then removed sequences from the 5′ side of the –116 to –7 fragment. Deletion of 14 bp (to –102), which removes the P1-C/EBP, has no effect on the level of basal activity. However, further deletion of sequences to –73 bp totally inactivates the residual construct containing Inrs 1, 2 and 3. Since the 14 bp sequence at the 5′ terminal of the –116 to –7 fragment appears to have no net effect on basal activity, we assayed for the effect of the C/EBP site specifically on the basal promoter activity of the rat XDH/XO gene.

C/EBP binds the basal promoter but does not play a role in basal transcription

The P1-C/EBP binding site (TTGGGTAAC) was mutated to TTcGcTAtC and subsequently, the mutated fragment was subcloned upstream of a luciferase reporter gene as described in Materials and Methods. As shown in Figure 5A, this experiment demonstrated that mutation of the P1-C/EBP site did not decrease the basal promoter activity. Since a gel mobility shift assay confirmed that this C/EBP mutant cannot bind C/EBP proteins (data not shown), this result, along with the deletional analysis,

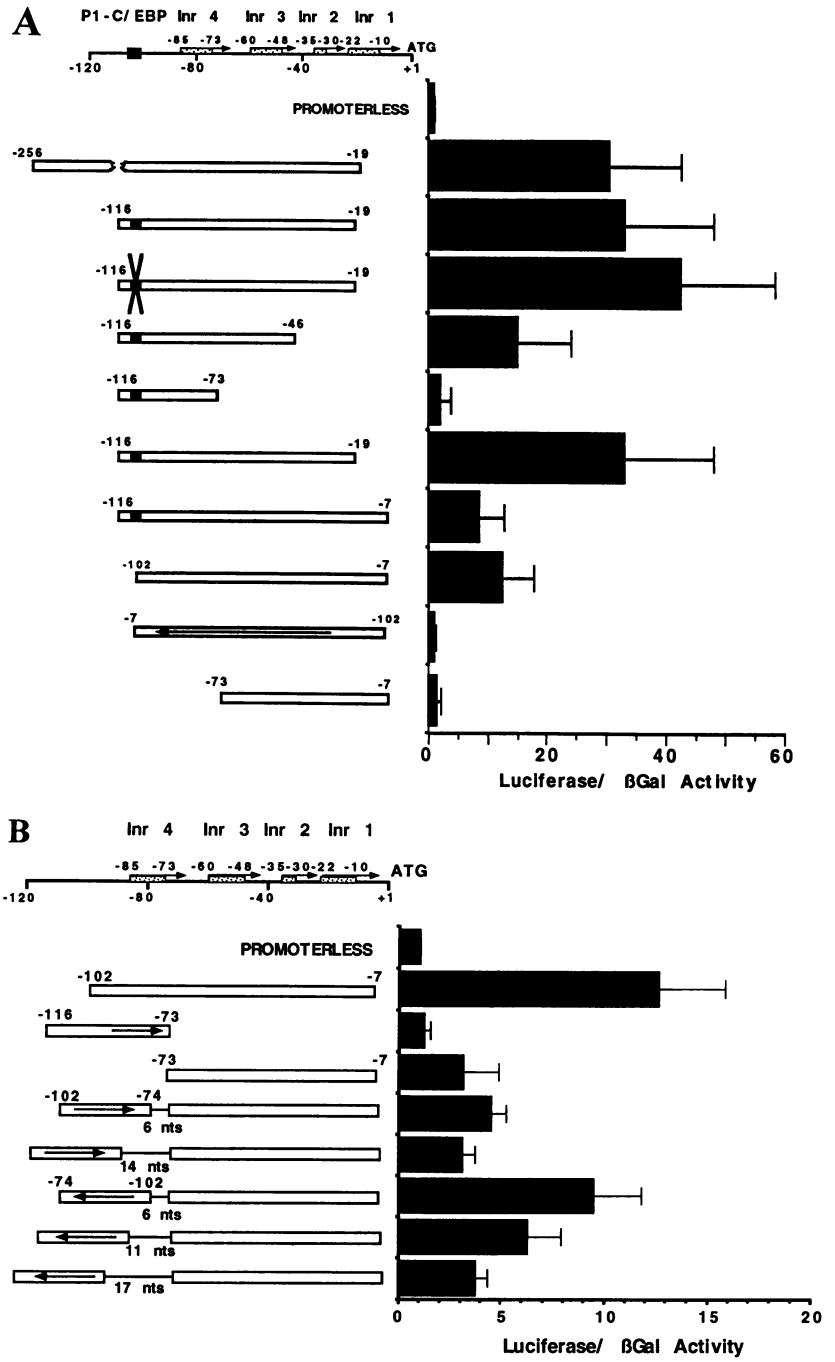

Figure 5. (**A**) Deletional and mutational analysis of the XDH/XO basal promoter. Constructs containing various XDH/XO basal promoter sequences were transiently transfected into HeLa cells, along with a RSV-ß-galactosidase reporter construct as an internal control. (**B**) Rejoining of the upstream element (–102 to –7 fragment) and the downstream initiators (–73 to –7). As indicated, 6, 11, 14 and 17 bp of DNA were introduced between the two fragments. The upstream fragment was introduced in either orientation relative to the downstream initiator fragment. At least three independent experiments were performed in duplicate and the results are normalized to a value of 1.00 for the promoterless construct. Filled boxes represent the P1-C/EBP site. Dotted boxes represent the different Inr regions. An arrow indicates the orientation of the Inr.

suggests that P1-C/EBP does not play an important role in the basal promoter activity.

Taken together, these results indicate that (i) the bulk of the basal promoter activity is contained within –102 and –7 upstream of the ATG; (ii) the C/EBP binding site is not involved in the establishment of the basal promoter activity; and (iii) cleavage of the basal promoter at –73 bp leads to total loss of activity from all initiation regions on both sides of the bisection point.

An upstream *cis*-acting element is required for the function of the downstream initiators

As shown above, deletion of the sequence from –102 to –73 abolishes all transcriptional activity for Inr 1, 2 and 3 indicating that these Inrs require a *cis*-acting element from the –102 to –73 region. Conversely, deletion of the sequence from –46 to –73 bp abolishes all transcriptional activity for Inr 4, indicating that this

Inr requires a *cis*-acting element from the –46 to –73 region. In essence then, cleavage of the –102 to –7 fragment at –73 destroys the activity of both components. We have asked if we can restore transcriptional activity by rejoining these fragments and reintroducing these putative *cis*-elements into linear integrity. This experiment was performed so that the fragments were rejoined with an additional 6, 11, 14 and 17 bp of DNA inserted at the joining point. As indicated in Figure 5B, rejoining does indeed restore activity. The nature of the constructs and the number of base pairs used in the rejoining were such that we could assess the effects of both directional and rotational orientation, as well as distance, on the recovery of transcriptional activity. The extent of the recovery of activity seems to be more dependent upon the distance separating the two fragments rather than the rotational orientation of the binding domain, though a detailed assessment of the latter effect would require more constructs to be made. Interestingly, the restoration of transcription is not sensitive to directional orientation of the upstream fragment. This observation is consistent with the need to bring an orientation-independent *cis*-acting element which resides within –102 to –73 into proximity with the –73 to –7 fragment in order to activate Inr 1, 2 and 3.

Comparison of transcription start sites *in vivo* and *in vitro*

The –102 to –7 region contains the four transcriptional start sites detected *in vivo*. These initiators map to positions –10 to –22 (Inr 1), –30 to –35 (Inr 2), –48 to –60 (Inr 3) and –73 to –85 (Inr 4) with activities of the initiators following the order of Inr 1 > Inr 2 > Inr 3 > Inr 4. We asked whether the XDH/XO-luciferase reporter constructs recapitulate the initiator activity seen in the intact cell. In Figure 6, we show the results of primer extension analyses of start site usage from transient transfection studies in HeLa cells. It is clear that the same initiators are used following transfection as is observed for the endogenous XDH/XO mRNA molecules. However, although the initiator usage is qualitatively unchanged, the relative preference for specific initiators in the transfection study as well as in the *in vitro* transcription analysis differs from that seen for the endogenous RNA in liver cells and bone marrow-derived macrophages. The quantitative differences may reflect a tissue-specific difference in relative initiator preference, but the important observation is that the –102 to –7 region clearly contains all the information for correct position of initiation. Possibly, additional sequences outside the basal promoter may be required for a correct quantitative usage of the various initiation sites.

Transcription from initiator regions is stimulated by Sp1

Since the usage of individual start sites differed quantitatively from that seen *in vivo*, we have assayed the extent to which the initiation regions in the XDH gene can be categorized as canonical Inr sequences (30,39,40). The criteria for assignment of a sequence as a bona fide Inr are (i) the sequence should direct transcription initiation from a TATA-less promoter *in vivo*; (ii) the sequence should match canonical sequences identified from well documented Inrs; (iii) transcription from the presumptive Inr should be orientation dependent; and (iv) the Inr activity should be stimulated by adding Sp1 binding sites to the construct. The XDH basal promoter Inrs satisfy the first three criteria (Figs 5A, 6 and 7 from ref. 11) and we have assayed whether they are responsive to Sp1. As shown in Figure 7, the presence of an Sp1

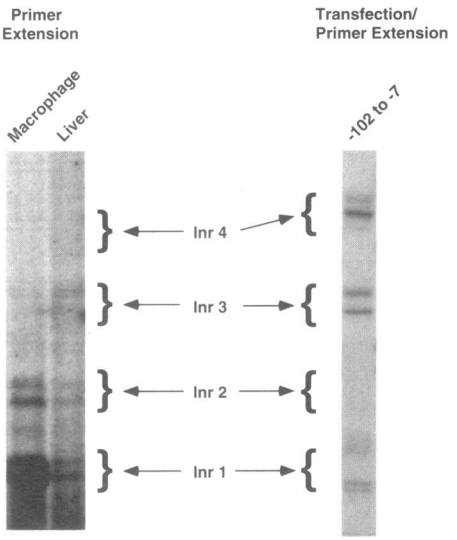

Figure 6. Comparision of the transcriptional start sites of the transfected XDH/XO reporter construct with the endogenous XDH/XO mRNA molecules. The –102 to –7 construct was transfected into HeLa cells and total RNA was isolated for primer extension analysis. Total RNA from rat liver and rat bone-marrow derived macrophages was isolated for primer extension as described previously. Corresponding position of the four initiators (Inr 1, 2, 3 and 4) in the XDH/XO construct and the endogenous RNA messages are indicated.

binding site upstream of the fragment containing either Inr 1, 2 and 3 (–73 to –7) or Inr 4 (–102 to –73) stimulated transcription substantially. Thus all the initiator sites appear to behave in the fashion expected for 'classical' non-TATA containing Inrs.

DISCUSSION

In this report we have extended our studies of the rat XDH/XO gene promoter. We have identified a hypersensitives site located upstream of the XDH/XO promoter. This hypersensitive site is co-localized with the basal promoter previously reported. No other hypersensitive site could be detected within 7.5 kb upstream of the XDH/XO promoter.

Within the identified hypersensitive site, at least six factor-binding footprints have been detected. Two C/EBP binding sites have been identified among these footprints. Since high XDH/XO enzyme activity has been found in liver, the presence of C/EBP binding site [a binding site that has been found in several liver genes (12,34,36,41)] on the XDH/XO promoter was not surprising. Using gel mobility shift assays, C/EBP α and β, but not C/EBP δ, have been shown to be able to bind to both of the C/EBP binding sites. Results from deletion and mutation analyses indicate that the P1-C/EBP binding site is not important for basal promoter activity. However, the expression of different C/EBP isoforms have been shown to be regulated by cytokines such as IL-6 (1,3,24). During inflammation, C/EBP β has been shown to be up-regulated while the C/EBP α isoform is down-regulated (24). Thus, the presence of C/EBP binding sites in the rat XDH/XO gene promoter may imply a possible mechanism for transcriptional regulation of the gene during inflammation. We surmise that increased levels of the C/EBP β isoform could activate XDH/XO gene expression during inflammation; and that

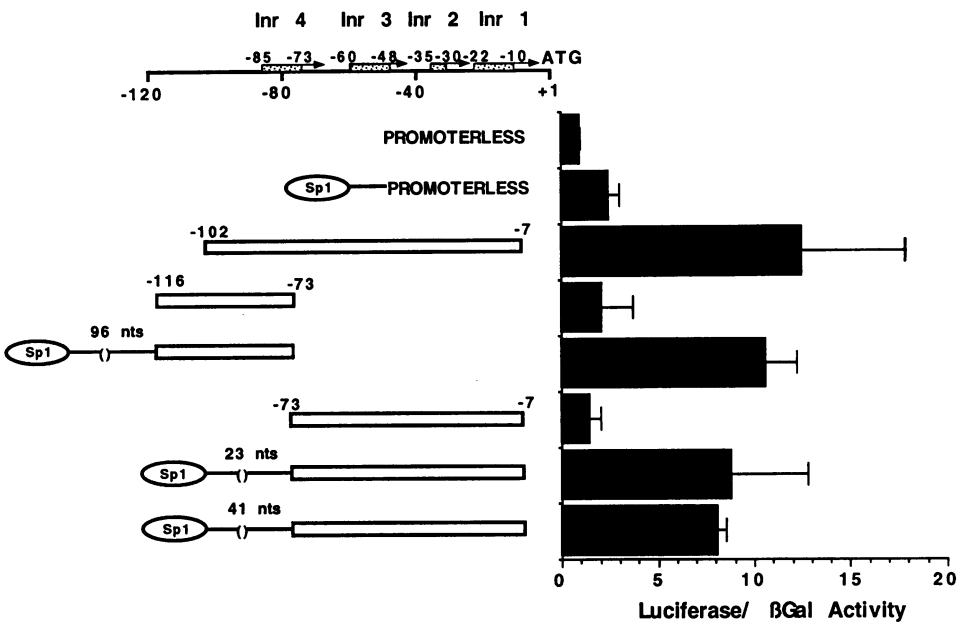

Figure 7. Transcription factor Sp1 stimulates individual Inr fragments. An exogenous Sp1 binding site was subcloned 5′ of the –116 to –73 (Inr 4) and –73 to –7 (Inr 1, 2 and 3) constructs. The constructs were transfected into HeLa cells and assayed as described in Materials and Methods.

the elevated amount of XDH may lead to increased levels of XO, and to the increased amount of reactive oxygen species.

Previous work from our laboratory has shown that there are multiple transcriptional start sites in the rat XDH/XO gene promoter (11). In this report, we have extended this analysis and showed that all information for basal transcription is found in the –102 to –7 bp fragment of the promoter. This fragment contains multiple initiator elements (Inr 1, 2, 3 and 4) that are important for transcription initiation. The linear integrity of this fragment is critical. Disruption of the fragment at –73 bp leads to complete loss of basal transcription; however, this can be restored by the introduction of a Sp1 binding site upstream of the different initiators or by rejoining the fragment, though the orientation of attachment was not important. This latter observation implies (i) that the DNA containing Inr 4 also contains additional information for a *cis*-acting factor which stimulates the activity of Inr 3 in an orientation-independent manner and (ii) that in addition to Inr 3, there is also a *cis*-acting element in the –46 to –73 sequence which activates Inr 4. These two *cis*-acting elements appear to be different and do not compete for binding factors when assayed in competition gel shifts (data not shown). This offers an interesting possibility for an additional level of regulation for this promoter. Thus if there were to be a decrease in the amount of factor binding to the Inr 4 sequence, this would necessarily result in a decrease in initiation from Inr 3, most possibly accompanied by a concomittant increase in Inr 4 transcription due to release of steric hindrance as less of this factor was bound. Presumably the converse situation could apply for the factor binding to sequences within the Inr 3 domain.

The presence of multiple initiators also raises the possibility of regulation by synthesis of different mRNA molecules which might be post-transcriptionally differentiated due to variation in 5′ structures and sequences. In Figure 8, we show the possible RNA secondary structures which might be generated if transcription was initiated from Inr 3. Several such structures, with an energy difference of <5%, were generated by using an energy minimization analysis (25,45). In one of these structures, an extra stem–loop structure is introduced. Interestingly, a canonical iron-responsive element (CAGUGA) is present in this extra stem–loop. The presence of an iron responsive element in such a position has been shown to be an important mechanism for post-transcriptional regulation of the ferritin and the transferrin genes (29,43). Thus, transcripts initiated from Inr 3 or 4 might be expected to be especially responsive to variations in iron concentration, whereas transcripts from Inr 1 or 2 would be insensitive. Rinaldo and Gorry (46) has shown that XDH/XO enzymatic activity is dramatically down-regulated by exposure of cells to deferoxamine, which depletes cellular iron levels. However, to our knowledge, the effect of iron concentration on XDH/XO transcription has not been examined. This clearly offers a possible route to a complex regulatory program in which changes in Inr selection might lead to changes in sensitivity to other agents. Factors which could affect Inr choice might include cytokines, glucocorticoids or even oxygen tension, all of which have been described as modifiying agents for the XDH/XO transcription (13,20). We note that in liver cells or bone marrow-derived macrophages, the major Inr employed is Inr 1 and 2 whereas in transient transfection of the promoter constructs into HeLa cells, Inr 3 and 4 appear to be the major initiation sites. This raises the possibility of tissue-specific Inr selectivity which again might lead to the production of different mRNA molecules. Thus, particular physiological needs and/or changes in a given tissue might activate different modes of regulation.

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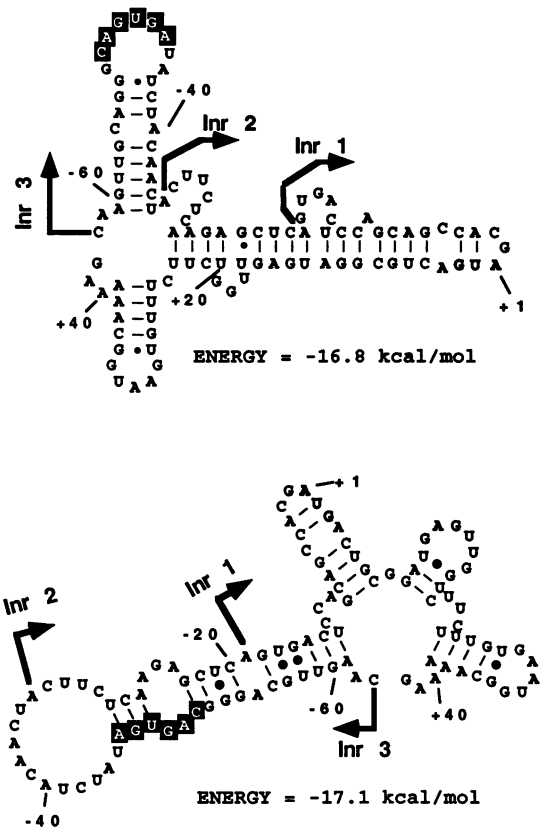

Figure 8. Potential secondary structures of the 5′ end of the XDH/XO mRNA. Using a free energy minimization program (25,45), the secondary structure of the XDH/XO mRNA was predicted. Sequences highlighted in filled boxes represent the putative iron-responsive element. Adenine of the translational start site is numbered as +1. Up-and-arrow heads represent the initiation sites.

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