Molecular Cloning of Mouse Glycolate Oxidase

HIGH EVOLUTIONARY CONSERVATION AND PRESENCE OF AN IRON-RESPONSIVE ELEMENT-LIKE SEQUENCE IN THE mRNA*

(Received for publication, October 5, 1998, and in revised form, November 5, 1998)

Stefan A. Kohler‡, Eric Menotti, and Lukas C. Kühn§

From the Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges s/Lausanne, Switzerland

Iron regulatory proteins (IRPs) control the synthesis of several proteins in iron metabolism by binding to iron-responsive elements (IREs), a hairpin structure in the untranslated region (UTR) of corresponding mRNAs. Binding of IRPs to IREs in the 5′ UTR inhibits translation of ferritin heavy and light chain, erythroid aminolevulinic acid synthase, mitochondrial aconitase, and Drosophila succinate dehydrogenase b, whereas IRP binding to IREs in the 3′ UTR of transferrin receptor mRNA prolongs mRNA half-life. To identify new targets of IRPs, we devised a method to enrich IRE-containing mRNAs by using recombinant IRP-1 as an affinity matrix. A cDNA library established from enriched mRNA was screened by an RNA-protein band shift assay. This revealed a novel IRE-like sequence in the 3′ UTR of a liver-specific mouse mRNA. The newly identified cDNA codes for a protein with high homology to plant glycolate oxidase (GOX). Recombinant protein expressed in bacteria displayed enzymatic GOX activity. Therefore, this cDNA represents the first vertebrate GOX homologue. The IRE-like sequence in mouse GOX exhibited strong binding to IRPs at room temperature. However, it differs from functional IREs by a mismatch in the middle of its upper stem and did not confer iron-dependent regulation in cells.

RNA-binding proteins play a central role in RNA processing, nucleo-cytoplasmic transport, localization, translation, or stability. This makes it desirable to identify all the targets of a given RNA-binding protein. Because many RNA recognition elements cannot be identified by hybridization or predicted with certainty by search programs, we developed an experimental approach based on the interaction between an RNA-binding protein and its target RNAs. As a model system we are investigating the iron regulatory proteins 1 and 2 (IRP-1 and IRP-2),† which become active in iron-deprived cells and bind with high affinity to structural RNA motifs, the iron-responsive elements (IREs) (1–5). IRPs repress translation of mRNAs with a cap-proximal IRE, as found in the mRNA of ferritin heavy (H) and light (L) chain (1, 6), mitochondrial aconitase (7, 8), erythroid 5-aminolevulinic acid synthase (9, 10), and Drosophila succinate dehydrogenase b (SDHB) (8, 11, 12). As a consequence, IRP binding lowers iron storage and utilization. Moreover, binding of IRPs to five IREs in the 3′ untranslated region (UTR) of transferrin receptor mRNA protects this otherwise unstable mRNA from degradation (3, 13). This leads to more receptor synthesis and enhanced iron uptake.

Functional IREs form stem loop structures with a conserved CAGUGN loop sequence (where N is any nucleotide except G). An upper stem of five perfectly paired bases is separated from a lower stem by a single cytosine on the 5′ side or by a cytosine preceded by two nucleotides with one unpaired nucleotide on the 3′ side. Adopting an in vitro selection procedure, we and others have identified IREs with alternative loop sequences that bind to IRPs and of which some show a preferential interaction with IRP-1 or IRP-2 (14–16). Such IRE mutants confer translational control only when inserted into the 3′ UTR of a reporter construct (17). However, none of these alternative IREs was detected in naturally occurring mRNAs to date.

Here we devised a method to enrich IRE-containing mRNAs by using recombinant human IRP-1 as an affinity matrix (Fig. 1). We then constructed an enriched cDNA library from mouse liver mRNA and screened it for IREs by RNA-protein band shift assays. This revealed a clone with strong homology to plant glycolate oxidase (GOX, or short-chain α-hydroxy acid oxidase, EC 1.1.3.15). In plants, this enzyme participates in the glyoxylate cycle and catalyzes the oxidation of glycolate to glyoxylate. An IRE-like sequence was found in the 3′ UTR of the mouse mRNA and analyzed with respect to its possible involvement in iron-dependent, post-transcriptional regulation.

EXPERIMENTAL PROCEDURES

Cell Culture—HL-60 and Ltk− cells were cultured in a minimal essential medium; FTO2B rat hepatoma cells were cultured in 45% Dulbecco’s modified Eagle’s medium and 45% F-12 (Life Technologies, Inc.), and B16.F1 mouse melanoma cells were cultured in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10% fetal calf serum. Cells were deprived of iron in medium with 100 μM desferrioxamine (Desferal, a gift from Novartis, Basel, Switzerland) for 16–20 h or iron-loaded in medium with ferric ammonium citrate (60 μg/ml) for 4–5 h.

*This work was supported by the Swiss National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Present address: Institute of Pharmacology and Toxicology, University of Lausanne, CH-1005 Lausanne, Switzerland.

‡To whom correspondence should be addressed: Swiss Institute for Experimental Cancer Research, Genetics Unit, 155 Ch. des Boveresses, CH-1066 Epalinges s/Lausanne, Switzerland.

§To whom correspondence should be addressed: Swiss Institute for Experimental Cancer Research, Genetics Unit, 155 Ch. des Boveresses, CH-1066 Epalinges s/Lausanne, Switzerland.

* The abbreviations used are: IRP, iron regulatory protein; GOX, glycolate oxidase; GST, glutathione S-transferase; HGH, human growth hormone; IRE, iron-responsive element; UTR, untranslated region; SDHB, succinate dehydrogenase B; H, heavy; L, light.

This paper is available on line at http://www.jbc.org
IRP-1 recovered between 140 and 200 mM KCl.

Isolation of RNA—Total RNA was extracted as described by Chromczyk and Sacchi (19). Poly(A)\(^+\) RNA was prepared using the Poly(A)Tract mRNA isolation kit (Promega).

Affinity Purification of RNA—GST-tagged human IRP-1 (100 \(\mu\)g) was immobilized on 50 \(\mu\)l of glutathione-Sepharose (Amersham Pharmacia Biotech). Beads were washed extensively with binding buffer (10 mM HEPES, pH 7.6, 3 mM MgCl\(_2\), 40 mM KCl, 5% glycerol) containing 5 mg/ml heparin (Serva) and binding buffer alone. Total RNA (5 mg), renatured for 5 min at 70 °C and 5 min at 42 °C, was adsorbed to the IRP-coated beads in 4 ml of binding buffer with 0.2% 2-mercaptoethanol, 50 mM NaCl, and 1 unit/ml RNasin. After gentle agitation for 30 min at 25 °C, unbound RNA was removed, and the beads were washed three times with binding buffer containing 5 mg/ml heparin and twice with binding buffer alone. Bound RNA was recovered by phenol-chloroform extraction and precipitated with 2.5 volumes of ethanol.

cDNA Synthesis and Library Construction—cDNA was synthesized from enriched RNA using Superscript II RNaseH\(^-\) reverse transcriptase (Life Technologies) and an oligo(dT) primer with a NotI site. EcoRI adaptors were then ligated. The cDNAs were digested with NotI for 16 h at 37 °C, ligated into an EcoRI-NotI-cleaved pBluescript II SK\(^+\) (Stratagene) vector (Stratagene), and transformed into Escherichia coli XL1-Blue. Bacteria were plated on Luria-Bertani plates containing 100 \(\mu\)g/ml ampicillin (Sigma), 1.5 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (Boehringer Mannheim), and 60 \(\mu\)g/ml 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (Boehringer Mannheim). White colonies were manually transferred onto 18 16 × 16 array master plates.

Library Screening—Ferritin H and L chain cDNAs were identified by hybridization (on GeneScreen Plus membranes; DuPont NEN) with \(\alpha\)-\[^{32}\text{P}\]ATP-labeled (Amersham Pharmacia Biotech), random-prime dDNA probes. Hybridization was carried out at 65 °C overnight in hybridization buffer (10% [w/v] dextran sulfate and 1% SDS) with 150 \(\mu\)g/ml salmon sperm DNA.

To screen for new IR-containing mRNAs, groups of 16 colonies (excluding the ferritin clones) were grown in 5 ml of L broth, and plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen). The eluted DNA was transcribed (with a GST tag in bacteria, purified to remove bacterial RNA, and adsorbed on glutathione-Sepharose) and used as a source for isolation of target mRNAs. For library construction of cDNA synthesis and library construction, see Fig. 1A. The plasmid mixture was linearized with RI and NotI and purified on a 0.8% agarose gel. For protein expression, the coding region of the GOX cDNA was amplified by polymerase chain reaction and cloned into the RI site of pBluescript II SK\(^+\) (Stratagene). For protein expression, the coding region of the GOX cDNA was amplified by polymerase chain reaction and cloned into the RI site of pBluescript II SK\(^+\) (Stratagene).

Enrichment of IRE-containing mRNAs on an IRP-1 column—Library screening by RNA-protein band shift assays. As shown in Fig. 1A, an affinity column with recombinant IRP-1 can selectively bind IRE-containing mRNAs. For this, the human IRP-1 mutant C437S that binds IREs constitutively (23) was expressed with a GST tag in bacteria, purified to remove bacterial RNA, and adsorbed on glutathione-Sepharose. Total input RNA, unbound RNA, washes, and bound RNA were analyzed by Northern hybridization (on GeneScreen Plus membranes; DuPont NEN) with \[^{32}\text{P}\]CTP-labeled (Amersham Pharmacia Biotech) ferritin tracer RNA. Nonspecifically bound RNA was removed by three washes with binding buffer containing 5 mg/ml heparin and two washes with binding buffer alone. Bound RNA was recovered by phenol-chloroform extraction. Equal fractions of the total input RNA (lane T), unbound RNA (lane U), washes (lanes 1–5), and bound RNA (lane B) were analyzed on a Northern blot and sequentially hybridized with probes for transferrin receptor (TIR), \(\beta\)-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and SDHb. The ferritin tracer mRNA was directly detected by autoradiography.

with 165 \(\mu\)M 2,6-dichloroindophenol (sodium salt hydrate; Fluka), 660 \(\mu\)M sodium glycolate (ICN) in 66 mM potassium phosphate, pH 8.3, and 1 mM EDTA, and the decrease in absorption was measured at 605 nm (20).

Construction of Plasmids—pBSII-GOXIRE for transcription of band shift probes was constructed by cloning a Dral-NotI fragment of the GOX cDNA (nucleotides 1853–2026 plus the poly(A) tail) between sites EcoRI and NotI of pBluescript II SK\(^+\). For protein expression, the coding region of the GOX cDNA was amplified by polymerase chain reaction and cloned into the SalI and NotI sites of pGEX-5X3 (Amersham Pharmacia Biotech) in frame with the GST sequence. The GOX IRE was inserted into the vector pLS-GH (6) as described (17). Plasmid pER-GH was kindly provided by Dr. Matthias Hentze (EMBL, Heidelberg, Germany).

Translation Assay in Cells—Ltk\(^-\) cells were stably transfected by the calcium phosphate method (21). Incorporation of \[^{35}\text{S}\]methionine and immunoprecipitation of secreted human growth hormone (hGH) were carried out as described previously (17, 22).

RESULTS

GST-tagged Human IRP-1 Can Be Used as an Affinity Matrix for IRE-containing mRNAs—The approach for the identification of targets of IRP-1 is depicted in Fig. 1A. It comprises the selection IRE-containing mRNA on an IRP-1 affinity column, the construction of an enriched cDNA library, and its screening by RNA-protein band shift assays. As shown in Fig. 1B, an affinity column with recombinant IRP-1 can selectively bind IRE-containing mRNAs. For this, the human IRP-1 mutant C437S that binds IREs constitutively (23) was expressed with a GST tag in bacteria, purified to remove bacterial RNA, and adsorbed on glutathione-Sepharose. Total input RNA, unbound RNA, washes, and bound RNA were analyzed by Northern blotting (Fig. 1B). Quantification by the PhosphorImager.
revealed specific retention of 50% of the IRE-containing ferritin mRNA as well as human transferrin receptor mRNA. In contrast, <0.5% of the control β-actin and glyceraldehyde-3-phosphate dehydrogenase mRNA without an IRE was retained on the column. Thus, immobilized IRP-1 can be used to enrich IRE-containing mRNAs at least 100-fold.

The column was also tested for the binding of human SDHb mRNA when we noticed an IRE in Drosophila SDHb mRNA (11). The Northern blot was rehybridized with a rapid amplification of cDNA ends polymerase chain reaction product of the human SDHb cDNA 5’ end including part of the coding sequence (24). Human SDHb mRNA was not retained on the IRP-coated beads (Fig. 1B). This agrees with conclusions drawn from the human SDHb gene sequence that revealed no IRE-like motif upstream of the coding region (25). It suggests that human SDHb expression is not regulated by IRP-1.

Analysis of Enriched mRNAs: Putative Glycolate Oxidase mRNA Is Bound by IRP-1 in Vitro—To isolate new mRNAs with an IRE, we incubated the IRP-1 column at a preparative scale with total mouse liver RNA. Bound mRNAs were eluted, reverse-transcribed, and cloned directionally to obtain an oligo(dT)-primed cDNA library enriched in IRE-containing inserts. 4608 recombinant clones were identified by blue-white selection and transferred to 18 master plates with 256 clones each. Among 22 randomly picked recombinant cDNAs, we found clones coding for mouse ferritin H and L chains. Additional ferritin cDNAs were expected at high frequency in the library and identified by hybridization of colony lifts. 56 ferritin cDNAs were found clones coding for mouse ferritin H and L chains. Additional ferritin cDNAs were expected at high frequency in the library and identified by hybridization of colony lifts. 56 ferritin H chain and 214 ferritin L chain clones were found, representing a frequency of 1.2 and 4.6%, respectively.

Clones that did not code for ferritins were screened for the presence of an IRE by a RNA-protein band shift assay. Plasmids were linearized behind the cDNA insert, transcribed in vitro with T7-RNA polymerase, and incubated with recombinant purified IRP-1, first in groups of 16, then in subgroups of 4, and finally as single clones. Because some transcripts were RNase T1-resistant and resembled IRE-IRP band shift complexes in their migration, we had to exclude false positives by omitting IRP-1 in the assay. 14 cDNA clones were finally identified as IRE-positive and sequenced. Of these, 11 had escaped detection by hybridization and coded for ferritin L or H chain. The remaining three clones originated of a single mRNA species with homology to plant glycolate oxidase and contained in the 3’ UTR an IRE-like sequence close to the polyadenylation site (Fig. 2A, bold). However, a mismatch in the upper stem distinguished this putative IRE from other known IREs (Fig. 2B). We also noticed different 3’ end sequences among the isolated clones, one with the poly(A) tail beginning at 20 bases from the polyadenylation site and another one extending 40 bases further (Fig. 2A, underlined) to a second poly(A) tail start site. Both classes of cDNAs were also found in an independent cDNA library (data not shown). This difference was not further investigated and probably represents alternative polyadenylation sites.

Rehybridization of our cDNA library with the longest cDNA insert identified three more clones, one of them with a full-length cDNA of 2.0 kb. This clone contained an open reading frame of 1110 nucleotides coding for a protein of 370 amino acids with a predicted molecular mass of 41.0 kDa and a pI of 7.6 (GenBank accession number AI104312). In vitro transcription and translation of the plasmid yielded a protein with the predicted size (Fig. 2C). A search in the data bases revealed that this clone had ~55% amino acid identity (74% similarity) to GOX from plants (Fig. 3), suggesting strongly that it might encode a mouse GOX. A kidney-specific isozyme from rat long-chain hydroxyacid oxidase (26) was likewise related but less homologous than the plant enzyme (48% identical and 59% similar). We also identified several expressed sequence tag clones of human origin, which could be aligned to the mouse sequence and served to reconstruct part of the human GOX sequence (Fig. 3). It exhibited 87.9% amino acid identity (92.2% similarity) with the mouse protein. Moreover, a genomic sequence from Caenorhabditis elegans (GenBank accession number AF016448) previously predicted to encode GOX (27) was 48.9% identical (60.2% similar) to that of mouse GOX (Fig. 3).

**cDNA Codes for a Liver-specific Mouse Glycolate Oxidase—**Spinach GOX has been crystallized, and amino acid residues involved in the active site, the substrate binding, and tight interaction with FMN are known (28). Here we found that all of these residues are conserved in our cDNA (Fig. 3), supporting the idea that it encodes a mouse GOX. To test this hypothesis, we investigated the enzymatic activity of recombinant protein. The cDNA was subcloned into the plasmid pGEX-5X3 for expression with an amino-terminal GST tag in bacteria. A spectrophotometric assay for GOX activity (20) was set up for crude bacterial extracts. Control extracts carrying plasmid without insert showed no reduction of 2,6-dichloroindophenol in the presence of excess sodium glycolate (660 μM) as a substrate (Fig. 4A). In contrast, extracts from GOX-transformed bacteria oxidized glycolate at a rate of 69 pmol/s per μg of crude protein. Moreover, GOX activity co-purified with the fusion protein on glutathione-Sepharose beads (Fig. 4B). This confirmed that the newly isolated cDNA encodes mouse GOX.

In animals, two isozymes of α-hydroxyacid oxidase were re-
ported (29, 30), one expressed in the kidney with preference for long chain α-hydroxyacids and the liver-specific short chain α-hydroxyacid oxidase, also known as GOX. This prompted us to investigate the tissue specificity of the mouse GOX mRNA. Northern blot analysis of RNA from various mouse tissues and fibroblast cells revealed GOX mRNA expression exclusively in liver but not in spleen, skeletal muscle, kidney, embryos, or fibroblasts (Fig. 5A).

Mouse GOX IRE Binds to IRPs with High Affinity in Vitro but Not in Cells—The presence of an IRE-like sequence in the 3′ UTR suggested that GOX mRNA might be regulated by IRPs. Therefore, rat hepatoma cells (FTO2B) were either treated with iron or the iron chelator desferrioxamine or with iron after desferrioxamine. GOX mRNA levels were measured on Northern blots and normalized to β-actin mRNA (Fig. 5B). No effect was observed when iron-deprived cells were compared with controls. But when iron salt was added, GOX mRNA was up-regulated 2.3- and 2.8-fold, respectively. To test the RNA binding activity of IRPs in FTO2B cells, band shift experiments were performed and responded as expected (31) to different iron-loading conditions (not shown).

These results suggested that IRP might be involved in the control of GOX expression. Therefore, the IRP-1 binding region of the mRNA between nucleotides 1884 and 1974 in the 3′ UTR encompassing the IRE-like sequence (Fig. 2A, bold) suggested, however, that it might affect protein binding. Therefore, the affinity of the GOX IRE for IRPs was compared with that of ferritin H chain IRE in competition band shift assays (Fig. 6). Surprisingly, at 25 °C, both probes bound equally well to IRP-1 and IRP-2 (Fig. 6). Encouraged by this result, we inserted the putative IRE into the 5′ UTR of vector pL5-GH containing a constitutive ferritin promoter and the human growth hormone (hGH) (6). A wild-type ferritin IRE in this vector confers IRP-mediated repression of hGH translation in iron-deprived cells (Ref. 10 and Fig. 7A). However, we were unable to detect translational regulation in response to iron deprivation in stably transfected mouse L cells with the GOX IRE construct (Fig. 7A). Yet, control band shift experiments with extracts from the same cells revealed strong induction of IRP-1 in response to control of GOX expression.

Fig. 3. Evolutionary conservation of glycolate oxidase. Using the program Fileup of the Genetics Computer Group package (52), the amino acid sequence of rat kidney long chain 2-hydroxyacid oxidase (L-HAO; GenBank accession number X671786) was aligned to the deduced amino acid sequence of the mouse GOX cDNA as well as to other GOX sequences from human (partial sequence reconstructed from several expressed sequence tags), C. elegans (GenBank accession number AF016448), ice plant (GenBank accession number U80071), pumpkin (GenBank accession number D14044), spinach (GenBank accession number J03492), Arabidopsis thaliana (GenBank accession number AL021710), and rice (GenBank accession number AF022740). Active site residues (A) and amino acids involved in FMN (F) or substrate (S) binding of the spinach enzyme (28) are indicated.
iron deprivation (Fig. 7B). Thus, it seemed plausible that the
GOX IRE was not binding to IRPs in cells. Therefore, we
measured carefully the IRE-IRP interaction at higher temper-
atures in vitro (Fig. 8). We found a strong temperature depend-
ence, the interaction being entirely lost between 34 and 37 °C.
The stem loop of the GOX IRE is probably thermodynamically
unstable because of the A:A mismatch in the upper stem.

**DISCUSSION**

Screening for Targets of mRNA-binding Proteins—We ex-
plor whether affinity chromatography with immobilized
RNA-binding IRP-1 can contribute to the identification of new
targets for post-transcriptional gene regulation by this protein.
Such a method would be generally useful in view of the rapidly
growing list of RNA-binding proteins. Only few methods to
search for targets of RNA-binding proteins have been de-
scribed. They comprise either systematic evolution of ligands
by exponential enrichment with naturally occurring mRNAs (32)
or immunoprecipitation of mRNA-protein complexes fol-
lowed by reverse transcription-polymerase chain reaction (33).
The present study indicates that mRNA affinity purification on
recombinant protein and construction of an enriched cDNA
library combined with screening by RNA-protein band shift
assays is a viable approach. Our cDNA library was clearly
enriched for IRE-containing clones. Although a normal human
liver cDNA library comprises 0.5% ferritin H and L chain
clones (34), the frequency was increased to 5.8% in the enriched
library (1.2% ferritin H chain clones and 4.6% L chain clones).
This was less than expected from the mRNA enrichment step
but can be explained by a high frequency of void plasmids.

**FIG. 4.** Enzymatic activity of recombinant mouse glycolate ox-
idase. The activity of GOX was measured at 25 °C by the reduction of
2,6-dichloroindophenol (DCIP) coupled to the oxidation of glycolate.
Enzyme activity was detected in crude extract of bacteria expressing
GST-tagged GOX protein (A; ●) but not in extract from mock-trans-
fected bacteria with the parental plasmid (A; ○). The enzyme was
adsorbed on glutathione-Sepharose (B; ■), and only low residual activity
was found in the soluble supernatant (B; □). No activity could be
adsorbed from extracts of mock-transfected bacteria (B; ○).

**FIG. 5.** Liver-specific expression of glycolate oxidase mRNA
and effect of iron levels. A, total RNA (20 µg) extracted from mouse
Ltk- cells or mouse tissues was analyzed on Northern blots with an
antisense riboprobe specific for mouse GOX mRNA. Cross-hybridization
to 28 S rRNA provides a loading control. B, FTO2B rat hepatoma cells
were cultured for 20 h in medium with either 60 µg/ml ferric ammo-
nium citrate (FAC), 100 µM desferrioxamine (Des), or 100 µM desferri-
oxamine for 20 h followed by 60 µg/ml ferric ammonium citrate (Des/

**FIG. 6.** RNA-protein band shift assay analyzing mutual compe-
tition of mouse GOX IRE and human ferritin H IRE. Equimolar
amounts of the human ferritin H IRE (Fer H; 31 pg, 2 × 10^4 cpm) or
mouse GOX IRE (150 pg, 7.6 × 10^4 cpm) were mixed with the indicated
molar excess of unlabeled competitor RNA (ferritin H IRE, mouse GOX
IRE, or yeast tRNA) and analyzed by band shift assay as described
under "Experimental Procedures" with 2 µg of protein extract from
iron-deprived FTO2B cells.

The data with human SDHb mRNA (Fig. 1B) illustrate that
by affinity purification it is also possible to determine whether
a candidate mRNA binds to a regulatory protein in vitro.
from a constitutive promoter. Mouse Ltk− cells were stably transfectected and labeled for 4 h with [35S]methionine, and secreted hGH was immunoprecipitated (A). To detect IRP-mediated inhibition of translation, cells were preincubated overnight with 100 μM iron chelator desferri-oxalate (Des) and compared with cells treated with 60 μg/ml ferric ammonium citrate (Fe). A 10-fold inhibition of hGH translation was observed with the positive control construct harboring the ferritin IRE (Fe-GH) (10), but no difference was visible with the GOX IRE construct (Gox-GH). B, cytoplasmic extracts of the same cells were analyzed for RNA binding activity of IRPs with a 32P-labeled ferritin H chain IRE. The IRP-IRE band shift assay shows strong activity of IRP-1 and IRP-2 in iron-deprived cells (Des; −2-me) but very weak activity in iron-loaded cells (Fe; −2-me). The in vitro addition of 2% 2-mercaptoethanol (+2-me) to extracts activates fully all cytoplasmic IRP-1. This serves as a control for equal loading.

Knowledge of the entire mRNA or precise target sequence is not required, because a Northern blot with column fractions can be hybridized with a probe corresponding to any fragment of the candidate RNA. From the results with this particular mRNA, the IRPs are probably not involved in regulation of human SDHB expression.

Our data demonstrate that screening by in vitro RNA-protein band shift requires certain controls, notably for the specificity and temperature of the interaction. In the mouse GOX mRNA, the IRE-related sequence binds IRPs at 25 °C but not at 37 °C. It fits with the lack of translational repression of the GH construct with the 5′ GOX IRE despite IRP activation (Fig. 7). These results indicate that GOX mRNA is not regulated by IRPs and that the stimulation of expression by high iron in a liver cell line must be attributable to a different mechanism.

At 25 °C, quite unexpectedly, the GOX IRE bound as strongly to rat IRP-1 and IRP-2 as the ferritin H chain IRE (Fig. 6), despite an A:A mismatch in the center of the upper stem (Fig. 2B). We have previously made a similar observation with a mutant ferritin H chain IRE containing a G:G mismatch in the same position and that displayed ~25% of wild-type binding affinity (14). These results seem to reflect a certain tolerance for noncanonical base pairing in the upper stem. Its thermodynamic instability explains probably why this IRE does not bind at higher temperature and, when placed in the 5′ UTR, does not render translation of a human growth hormone reporter mRNA iron-dependent in cells.

Considering the relatively limited number of clones analyzed in the present screen, we cannot exclude that further IRE-containing candidate mRNAs may exist in the liver. However, if further clones are to be found, they can be expected at a frequency at least as low as that of the GOX mRNA, which was 50–100-fold less frequent than that of ferritin mRNA.

Possible Role of Mammalian Glycolate Oxidase—The second main conclusion from our study concerns the first isolation of a mammalian GOX cDNA. Its amino acid sequence was 55% identical (74% similar) to GOX (also termed short-chain 2-hydroxyacid oxidase) from plants. The plant enzyme contains an FMN cofactor and catalyzes oxidation of glycolate to glyoxylate. The three-dimensional structure of spinach GOX has been resolved by x-ray crystallography (28), which allowed determination of active site residues and side chains involved in FMN binding. All of these amino acids are perfectly conserved in the isolated clone (Fig. 3), suggesting that it codes for mouse GOX. This conclusion is supported by the enzymatic activity of the purified recombinant protein (Fig. 4).

From these results and evidence detailed below we conclude that our clone is full length. The sequence encompassing the start codon (GCCACAAUGU) matches well the Kozak consensus (GCCAAGU) for translation start sites (35), and the size of the predicted protein was in good agreement with the in vitro transcription-translation product analyzed on SDS-PAGE (Fig. 2C). Second, several homologous clones were isolated from a commercial mouse liver cDNA phage library. Only one of those clones contained a slightly longer 5′ sequence (not shown) but had no additional upstream start codon. Third, a probe derived from the isolated clone detected a single band of 2.2 kb on a Northern blot, the size expected for the predicted, polyadenylated mRNA (Fig. 5A). Fourth, alignment to homologous sequences in the database revealed high conservation close to the amino terminus (Fig. 3).

Mammalian GOX activity had been discovered as early as 1940 (36), but to our knowledge no vertebrate GOX cDNA or gene has been cloned to date. In animals, two 2-hydroxy acid oxidases have been reported with expression in either liver or kidney, one with specificity for short chain 2-hydroxyacids and the other for long chain 2-hydroxyacids, respectively (30, 37). Our cDNA corresponds to the liver enzyme (Fig. 5B). The sequence of a rat kidney long chain 2-hydroxyacid oxidase (26) is similar and belongs to the same family of proteins (Fig. 3). Mammalian liver GOX has been located in peroxisomes (38–40). This localization might be mediated by the carboxyl-terminal sequence SKI (Fig. 3), which resembles the consensus SKL sequence for peroxisomal import (41, 42). However, studies with mutants of the localization tripeptide behind luciferase suggested that SKI is a poor import signal (43). Further work is required to analyze this issue for mammalian GOX.

Glycolate oxidase contains an FNM cofactor (20, 44) that catalyzes the oxidation of glycolate to glyoxylate and possibly to oxalate (45–47). GOX could be implicated in the detoxification of glycolate via the synthesis of oxalate. Alternatively, it might provide building blocks for the synthesis of glycine and serine, because the carbon atoms of 14C-labeled glycolate are detected in these amino acids (48).
In oil seed plants, GOX acts as part of the glyoxylate cycle. This pathway is required in early stages of seedling growth for the conversion of fatty acid stores into sugars (49). Because of its importance in plant growth, GOX cDNAs have been cloned in several plant species. The question arises of whether lipid mobilization and gluconeogenesis involving glyoxylate cycle enzymes are a specialization of plants only. Both isocitrate lyase and malate synthase activities have been detected in liver peroxisomal fractions and in adipose tissue of mammals (50, 51), where they are induced by fasting. This suggests that under certain conditions, mammalian GOX might also feed glyoxylate into this pathway. The present cDNA clone should help clarify the physiological role of GOX.

Acknowledgments—We thank Jovan Mirkovich for the FTO2B cell line, Martin Irmler for carrying out the in vitro transcription-translation experiment, and Markus Nabholz for critically reading the manuscript.

REFERENCES

1. Leibold, E. A., and Munro, H. N. (1988) Proc. Natl Acad. Sci. U. S. A. 85, 2171–2175
2. Casey, J. L., Hentze, M. W., Koehler, D. M., Caughman, S. W., Rouault, T. A., Klauser, R. D., and Harford, J. B. (1988) Science 240, 924–928
3. Muller, E. W., Neupert, B., and Kuhn, L. C. (1989) Cell 58, 373–382
4. Haile, D. J., Hentze, M. W., Rouault, T. A., Harford, J. B., and Klauser, R. D. (1989) Mol. Cell. Biol. 9, 5055–5061
5. Barton, H. A., Eisenstein, R. S., Bomford, A., and Munro, H. N. (1990) J. Biol. Chem. 265, 7000–7008
6. Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., Harford, J. B., and Klauser, R. D. (1987) Science 238, 1570–1573
7. Zheng, L., Kennedy, M. C., Blondin, G. A., Reisert, H., and Zalkin, H. (1992) Arch. Biochem. Biophys. 299, 356–360
8. Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996) Proc. Natl Acad. Sci. U. S. A. 93, 4925–4930
9. Cox, T. C., Bawden, M. J., Martin, A., and May, B. K. (1991) EMBO J. 10, 1891–1902
10. Dandekar, T., Stripecke, R., Gray, N. K., Caughman, B., Constantine, A., Johansen, H. E., and Hentze, M. W. (1991) EMBO J. 10, 1903–1909
11. Kohler, S. A., Harford, B. R., and Kuhn, L. C. (1996) J. Biol. Chem. 270, 30781–30786
12. Melefas, O. (1996) Biochem. Biophys. Res. Commun. 221, 437–441
13. Koehler, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L. N., Klauser, R. D., and Harford, J. B. (1989) Proc. Natl Acad. Sci. U. S. A. 86, 3574–3578
14. Henderson, B. R., Menotti, E., Bonnard, C., and Kuhn, L. C. (1994) J. Biol. Chem. 269, 17481–17489
15. Butt, J., Kim, H. Y., Basilion, J. P., Cohen, S., Iwai, K., Philpott, C. C., Ailschul, S., Klauser, R. D., and Rouault, T. A. (1996) Proc. Natl Acad. Sci. U. S. A. 93, 4345–4349
16. Henderson, B. R., Menotti, E., and Kuhn, L. C. (1996) J. Biol. Chem. 271, 1821–1824
17. Menotti, E., Henderson, B. R., and Kuhn, L. C. (1998) J. Biol. Chem. 273, 387–389
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J. Biol. Chem. 1999, 274:2401-2407.
doi: 10.1074/jbc.274.4.2401

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Vol. 274 (1999) 2401–2407

Molecular cloning of mouse glycolate oxidase. High evolutionary conservation and presence of an iron-responsive element-like sequence in the mRNA.

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Page 2403, left-hand column, seventh line from the bottom: The GenBank™ accession number should read AF104312.

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