The Human Hydroxyacylglutathione Hydrolase (HAGH) Gene Encodes Both Cytosolic and Mitochondrial Forms of Glyoxalase II* [S]

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In yeast and higher plants, separate genes encode the cytosolic and mitochondrial forms of glyoxalase II. In contrast, although glyoxalase II activity has been detected both in the cytosol and mitochondria of mammals, only a single gene encoding glyoxalase II has been identified. Previously it was thought that this gene (the hydroxyacylglutathione hydrolase gene), comprised 8 exons that are transcribed into mRNA and that the resulting mRNA species encoded a single cytosolic form of glyoxalase II. Here we show that this gene gives rise to two distinct mRNA species transcribed from 9 and 10 exons, respectively. The 9-exon-derived transcript encodes two protein species: mitochondrially targeted glyoxalase II, which is initiated from an AUG codon in a previously uncharacterized part of the mRNA sequence, and cytosolic glyoxalase II, which is initiated by internal ribosome entry at a downstream AUG codon. The transcript deriving from 10 exons has an in-frame termination codon between the two initiating AUG codons and hence only encodes the cytosolic form of the protein. Confocal fluorescence microscopy indicates that the mitochondrially targeted form of glyoxalase II is directed to the mitochondrial matrix. Analysis of glyoxalase II mRNA sequences from a number of species indicates that dual initiation from alternative AUG codons is conserved throughout vertebrates.

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[†] The on-line version of this article (available at http://www.jbc.org) contains Supplementary Fig. 1.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY576804 for the extended glyoxalase II 5' UTR sequence.

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1 The abbreviations used are: MG, methylglyoxal; CMV, cytomegalovirus; C, amplification threshold cycle; DsRed2, modified Discosoma sp. red fluorescent protein; EGFP, enhanced green fluorescent protein; exAAATG, exon A initiating ATG codon; ex1TATG, exon 1 initiating ATG codon; EYFP, enhanced yellow fluorescent protein; 6-FAM, 6-carboxyfluorescein; HAGH, hydroxyacylglutathione hydrolase; RACE, rapid amplification of cDNA ends; TAMRA, carboxytetramethylrhodamine; UTR, untranslated region; RT, reverse transcriptase; IRS, internal ribosome entry site.
been reported to prevent MG accumulation and advanced glycation end product formation under hyperglycemic conditions (16). In vertebrates there is a single gene encoding glyoxalase II, HAGH (hydroxyacylglutathione hydrolase) (5, 17, 18).

The mRNA sequence of glyoxalase II identified by Mannervik and co-workers (5) contains eight coding exons that encode the 260-amino acid cytosolic form of the enzyme. In this paper, we describe the identification of extended transcripts from the human HAGH gene encode both cytosolic and mitochondrial isoforms of glyoxalase II, and that the previous mRNA sequence is incomplete. The cytosolic form originates by internal priming of protein synthesis from the initiating AUG codon previously identified in exon 1 (5). The mitochondrial form arises from initiation of translation at an upstream AUG codon encoded on an upstream exon that can be fused in-frame to exon 1. Examination of GenBank nucleic acid sequences indicates that alternative translation initiation of HAGH transcripts is conserved throughout vertebrates giving a possible explanation for the production of vertebrate intramitochondrial glyoxalase II.

EXPERIMENTAL PROCEDURES

Determination of the 5' end of the glyoxalase II mRNA and the production of constructs to test its function are described below. As the 5' end of the mRNA is very (G + C) rich, 5' MeSO (BDH) and 1 M betaine (Sigma) were included in all PCR to allow amplification. PCR amplification was performed using the Expand high fidelity system (Roche Diagnostics) except where a blunt end was produced with Pfu polymerase (Stratagene). All constructs were sequenced on an ABI310 automated sequencer (Applied Biosystems) using BigDye® Terminator version 3.1 sequencing reagents. All primers were synthesized by Invitrogen. All restriction endonuclease enzymes were purchased from New England Biolabs.

5'-RACE—RNA ligase-mediated RACE was performed using the GeneRacer kit (Invitrogen) as directed by manufacturer's instructions using total RNA extracted from HepG2 cells by the TRIzol method (Invitrogen). Thermoscript reverse transcriptase (Invitrogen) was used in the presence of 1.5 M betaine and 10% MeSO. For priming of cDNA synthesis the primer 5'-GCAGCAGACCTGCTACATCATCATAATG-3' (primer 3, Fig. 1) was used. For secondary amplification the primer 5'-GCAGCAGACGATGTTCTTTCC-3' was used. Nested PCRs were performed using the primary primer 5'-GGGCAGCTCGGCAGTCCAG-3' (primer 3, Fig. 1) in all cases. For secondary amplification the primer 5'-CCGTCAGCTTCCGCGACTTCTTTATCT-3' was used to amplify from exon 1 and the primer 5'-GCGCGGAGCTCCGCGCTTTTTT-3' was used to amplify from the 5' end of exon A.

Reverse Transcriptase (RT)-PCR—Total RNA from HepG2 cells, HT1080 cells, and from human liver and kidney (Clontech total RNA Banan Collection of Cell Cultures) were cultured in Eagle's modified essential medium (Sigma) supplemented with 10% fetal calf serum (Promega). This construct was digested with NcoI and blunted as above. The PCR product was then ligated into pCR2.1 TOPO vector to give pPrTOPO. The promoter sequence was subcloned into pGL3 basic digested with NcoI and blunted as above to give pPrGL3. The latter vector was digested with StuI to give pPrGL3-STOP. The products were amplified from in vitro transcription of SacI and XhoI to give the positive control construct shown in Fig. 2.

A negative control construct fused the same promoter sequence as in the positive control to firefly luciferase from which the initiating ATG had been deleted. pGL3 basic vector with NcoI and blunted as above was then digested with SacI and the glyoxalase II promoter insert was introduced as a SacI-EcoRV fragment.

Ex/AiATG → TTG, ex/iATG → TTG mutants and ex/A+ iATG → TTG) double mutants were generated from pPrA1-GL3 using the QuikChange® site-directed mutagenesis kit (Stratagene). The phosphorylated oligonucleotides (with mismatched base emboldened) used were 5'-TGGTCGCTGGGCGG-3' (forward) and 5'-P-CCGTTAGGCGACCCTTGAAGTTAGAGG-3' (reverse). To ensure a high level of luciferase expression for Western blotting, pCMV-A1-GL3 and pCMV-AB1-GL3 were derived from pPrA1-GL3 and pPrAB1-GL3. The glyoxalase II core promoter region was removed from these constructs by digestion with EcoRI and PvuII, an enzyme that cuts 2 bp downstream from the experimentally determined 5' end (Fig. 1A). This was replaced with the CMV immediate/early promoter sequence and a T7 promoter. First the CMV and Renilla luciferase region of pRL-CMV (Promega) were excised using BglII and XbaI and subcloned into pGEMZf+ (Promega). This construct was digested with NheI, the 5' overhangs were filled with the Klenow fragment of DNA polymerase I (New England Biolabs). The appropriate fragment was excised with EcoRI digestion and the resulting fragment was ligated to digested pPrA1-GL3 and pPrAB1-GL3 constructs (above). Constructs pCMV-ex/AiATG → TTG) and pCMV-ex/iATG → TTG were made using the same strategy.

Real Time PCR—Constructs pCMV-A1-GL3 and pCMV-AB1-GL3 were in vitro transcribed with T7 polymerase (Ambion) to yield 2-kb transcripts from each that spanned exons A and 1 or A,B and 1, respectively. Equal concentrations of each transcript derived by A500 absorption and agarose gel electrophoresis were mixed and used as a control for quantification by real time RT-PCR. HepG2 cell RNA and in vitro transcribed were used to synthesize cDNA with random hexamers.

Reverse transcription was performed on an ABI PRISM 7700 sequence detection system with TaqMan® Universal PCR mixture (Applied Biosystems). Primers and probes were designed to cross exon boundaries using the Primer Express program version 1.5 (Applied Biosystems, in each case) to specifically detect products deriving from mRNA and concentrations of each were optimized. For detecting exon A-exon 1 mRNA, primers 5'-CCGAGGCTCCTGCCGGA-3' (forward) and 5'-GTCAAGTCTTCCGCAAAATCTTG-3' (reverse, primer 4, Fig. 1). Plasmid Construction and Site-directed Mutagenesis—Constructs pPrA1-TOPO and pPrAB1-TOPO containing the promoter (Pr) and alternative 5' UTR sequences (A-1 or A,B-1) up to and including the 5' end of exon A were amplified using 5'-ACCGAGAGATTTGTTCTTTCC-3' (forward, primer 1, Fig. 1) and 5'-GGGACGACGACTTCTATTCTCTT-3' (reverse, primer 3, Fig. 1). Amplification was performed using the Expand high fidelity system (Roche Diagnostics) except where a blunt end was produced with Pfu polymerase (Stratagene). All constructs were sequenced on an ABI310 automated sequencer (Applied Biosystems) using BigDye® Terminator version 3.1 sequencing reagents. All primers were synthesized by Invitrogen. All restriction endonuclease enzymes were purchased from New England Biolabs.

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The mRNA sequence of glyoxalase II identified by Manner-
transfection reagent (Roche) according to manufacturer's instructions. Cells were lysed 48 h post-transfection in passive lysis buffer (Promega) and assayed using the dual luciferase assay system (Promega) according to manufacturer's instructions with a Mediators Phl. luminometer.

For Western blotting cells were seeded at 3 × 10^4/well in 6-well plates cultured for 24 h and transfected with 1 µg of each firefly luciferase construct per well using FuGENE 6. Whole extracts of transfected cells were harvested using SDS-PAGE loading buffer (19) heated to 100 °C added directly to cells (150 µl/well). The lysate was cleared by centrifugation (10,000 × g, 10 min). Total protein was measured using the BCA assay system (Pierce) and samples were electrophoresed on an 8% polyacrylamide gel (19) and blotted to the polyvinylidene difluoride membrane (Pierce). Blots were blocked with 5% (w/v) nonfat milk powder in Tris-buffered saline at room temperature for 1 h. Goat anti-firefly luciferase primary antibody (Promega) was used at a 1:1000 dilution in blocking buffer for 1 h at room temperature. After washing with Tris-buffered saline containing 0.05% Tween 20, horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Promega) was used for incubation at a dilution of 1:5000 at room temperature for 1 h. Antibody complexes were visualized by incubation with 3,3',5,5'-tetramethylbenzidine (Calbiochem) for 30 min.

Confocal Microscopy—HepG2 cells were seeded at 4 × 10^4/well on 13-mm/0.17-mm thick coverslips in 24-well plates. Cells were transfected with pGII-DsRed2 alone or co-transfected with a carnitine palmitoyltransferase I-EGFP fusion construct (20) or with plasmid pEFPP-Mito (Clontech) (400 ng total DNA/well in each case). After 72 h cells were fixed for 30 min with 4% paraformaldehyde in Dulbecco's modified phosphate-buffered saline (Sigma), washed twice with saline (10 min each), and mounted using Vectashield Hard Set (Vector Laboratories). Cells were prepared for immunocytochemistry as described by Satoh et al. (21) using an anti-cytchrome c monoclonal antibody (6H2.B4, BD Pharmingen) and rabbit anti-mouse IgG fluorescein isothiocyanate conjugate (Dako). Images were obtained using a ×63 oil immersion objective of a Leica DMIRE2 confocal laser scanning microscope at 1024 × 1024 pixel resolution using a pinhole diameter of 76 μm and averaged 30 times. For cells transfected with multiple fluorescent proteins, signals were collected over a narrow wavelength band after excitation with one laser line at a time to avoid bleed through of signal from other fluorophores.

RESULTS

Examination of Available HAGH mRNA Sequences—The first reported human cDNA sequence of HAGH (X90999) had 8 coding exons (5). However, the NCBI Reference Sequence for HAGH mRNA (NM_005326) contains an additional 500-bp exon at the start of the sequence (termed exon A in this report). In the genomic sequence MapViewer (NCBI) shows that exon A is located some 3.7 kb upstream of the previously assigned first coding exon (exon 1, Ref. 5) and around 100 bp downstream of gene DKFZP566F2046. Examination of exon A shows the presence of an in-frame ATG (exA1ATG) 144 bp upstream of the previously recognized initiation codon (ex1iATG) with no intervening stop codons. Initiation at exA1ATG would add 48 extra amino acids to the amino terminus of glyoxalase II (Fig. 1) giving replicate values in parentheses) were 25.66 (25.44, 25.55, 26.00) and 30.07 (29.81, 30.19, 30.21), respectively, giving ΔCt = 4.41 and for HepG2 total RNA was 26.76 (26.82, 27.01, 26.45) and 30.25 (30.28, 30.37, 30.11), respectively, giving ΔCt = 3.49. This equates to a relative abundance of the A,1–8 isoform may be intrinsic difference compared to the other two isoforms, where A,1–8 isoform is more abundant in HepG2 cells (results not shown). Fragment (ΔCt) was included in constructs designed to examine the initiation of translation (described below).

Real Time PCR—To estimate the relative abundance of the A,1–8 and A,B,1–8 isoforms of the glyoxalase II mRNA in HepG2 cells, real time RT-PCR was carried out. Both cDNA synthesis and PCR amplification of the A,B,1–8 isoform may be intrinsically less efficient than of the A,1–8 isoform in view of the high G/C content of exon B (80%). To correct for this possibility, data from total cellular RNA were normalized to data obtained with a defined mixture of in vitro transcripts spanning exons A,B,1 and A,1 (i.e. covering the amplon). The average Ct values for A,1 and A,B,1 in vitro transcripts (at a constant threshold value, and giving replicate values in parentheses) were 25.66 (25.44, 25.55, 26.00) and 30.07 (29.81, 30.19, 30.21), respectively, giving ΔCt = 4.41 and for HepG2 total RNA was 26.76 (26.82, 27.01, 26.45) and 30.25 (30.28, 30.37, 30.11), respectively, giving ΔCt = 3.49. This equates to a relative abundance of the A,1–8 and the A,B,1–8 transcripts in HepG2 cells of ~1:2, i.e. both forms are similarly represented in cells. In contrast, uncorrected PCR data would suggest a marked preponderance of the A,1 splice form as implied in Fig. 1C.

Nucleotide and Amino Acid Sequence Analysis of Glyoxalase II Orthologues—The mRNA sequences of a number of HAGH orthologues were examined to determine the extent of their conservation. All vertebrate sequences contained a highly conserved cytosolic coding region of 260 amino acids and all possessed an in-frame upstream ATG corresponding to human exA1ATG, adding between 43 and 48 amino acids depending on the species. Using MitoProt analysis (22) the amino-terminal extension of all sequences except Gallus gallus was predicted to contain a mitochondrial targeting sequence with a score greater than 50% and all sequences were predicted to contain a cleavage site. Similar analysis of the glyoxalase II proteins

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FIG. 1. Annotated mRNA sequences of two glyoxalase II mRNA splice forms. A, the 5′ most and 3′ most sequences of the A,1–8 splice form are shown. A continuous open reading frame encoding 308 amino acids is predicted from the ATG codon at nucleotide 408 (open box). A second ATG codon at nucleotide 552 (filled box) corresponds to the amino-terminal methionine of the previously described 260-amino acid protein. The upstream ATG codon is located in a novel exon (exon A) and the splice junction with the previously described exon 1 is indicated (gg at nucleotides 483 and 484). Nucleotide 1 corresponds to the first nucleotide in the mRNA determined by 5′ RACE in the current study and is 33 bases upstream of the first cytosine nucleotide (c) of the curated mRNA sequence, NM_005326. The first nucleotide (g) of the previously reported cDNA sequence (5) is shown at position 516. This sequence lacks the initiating ATG for the longer open reading frame. Primers used for expressing predicted protein sequences (1–4) are shown along with restriction sites XmaI and StuI. A PvuII site in the genomic sequence that overlaps the 5′ end of the cDNA was also used for expressing predicted protein sequences (see text). The protein sequence between Met-49 and Asp-308 is as shown in Ref. 5. B, partial sequence of the A,B,1–8 splice form is shown, covering the region where in some transcripts a second additional exon (exon B)
without the amino-terminal extensions gave scores of less than 10% in all cases except for Bos taurus (37%), and none of the proteins contained a predicted cleavage site. These data are summarized in Table I.

**Investigation of Translational Efficiency and ATG Usage in Both HAGH Splice Forms**—To determine whether each splice form of human glyoxalase II mRNA could be translated efficiently, the core promoter region and 5’ mRNA sequence for both splice forms up to and including the ex1iATG were fused in-frame to firefly luciferase that had had its own initiating ATG removed. In these constructs expression of luciferase should only arise by translation initiated from within the HAGH sequence. This was confirmed by a negative control made by fusing the same promoter sequence (i.e. lacking any initiating ATG from the HAGH sequence) to the firefly luciferase from which the initiating ATG had been deleted. A signal of <0.1% of positive control signal was obtained excluding the possibility that the functional protein could be produced by initiation from downstream ATG codons in the firefly luciferase gene. In contrast, constructs with either exons A and 1 or exons A, B, and 1 directed luciferase production at ~50 and 25% of the positive control value, respectively (Fig. 2). These results suggest that internal initiation at ex1iATG can occur.

To determine the contribution of each ATG to translation initiation in the pPrA1-GL3 construct they were mutated singly and in combination to produce Exa(iATG → TTG), ex1(iATG → TTG), and exA+ex1 (iATG → TTG). Mutation of exA/iATG reduced the luciferase signal by <10%, whereas mutation of ex1/iATG reduced the signal by ~90% and mutation of both ATGs reduced the signal by 99% (Fig. 5). Thus it appears that exA/iATG is responsible for ~10% of initiation and ex1/iATG is responsible for ~90%.

Immunoblotting of luciferase fusion proteins verified that initiation occurs at both exA/iATG and ex1/iATG and can occur from either ATGs in the A,1 transcript (Fig. 3). Thus in the top row, two separate proteins are detected and the ratio of these proteins fits well with the relative expression apparent from the luciferase activity data (above). Addition of 48 amino acids to the amino-terminal of glyoxalase II would increase its size by 3 kDa, whereas an increase of 2–3 kDa is estimated. This may suggest cleavage within the mitochondrial targeting sequence, as predicted above.

**Subcellular Targeting Conferred by the Amino-terminal Extension of Glyoxalase II**—To determine whether the 48-residue extension of glyoxalase II initiating at ex1iATG affected subcellular targeting, this sequence was added in-frame to the amino terminus of the fluorescent protein DsRed2 and the fusion was expressed in HepG2 cells. pGII-DeRed2-transfected cells showed a punctate distribution of red fluorescence (Fig. 4, A–C), whereas a diffuse cytosolic distribution was seen with unmodified DeRed2 protein (not shown).

To determine whether the punctate distribution was because of mitochondrial targeting, HepG2 cells were co-transfected with pGII-DeRed2 and pEYFP-mito (Clontech), a plasmid that expresses a fusion of the mitochondrial matrix targeting sequence of cytochrome c oxidase subunit VIII linked to enhanced yellow fluorescent protein. The two signals appeared to be extensively co-localized (Fig. 4A).

Previous studies suggested that a pool of glyoxalase II that is immunologically related to the cytosolic form is present in the intermembrane space (9, 11), in contrast to the matrix distribution observed above. To determine whether the subcompartments of the mitochondria can be adequately resolved by confocal fluorescence microscopy, pGII-DeRed2 was co-expressed with GFP-tagged carnitine palmitoyltransferase I (CPT-EGFP), a fusion protein previously used as an outer membrane marker (20). In other experiments, cells expressing pGII-DeRed2 were immunostained for cytochrome c, a marker previously used to visualize the intermembrane space (21).

CPT-EGFP protein showed a reticular distribution (Fig. 4B). Although the red and green signals did not co-localize, a significant proportion of the red signal was enclosed within the reticular structures bounded by green fluorescence (Fig. 4B), which is consistent with matrix targeting by the glyoxalase II leader sequence.

Immunolabeling of cytochrome c also showed a reticular distribution. Permeabilization may have lead to poorer preservation of structure, but again the signal was distinct from that of the glyoxalase II leader-DeRed2 protein. Because both the inner and outer mitochondrial membranes give a reticular

### Table I

| Species          | ORF length (codons) | Sequence alignment of putative amino-terminal extensions | MitoProt data |
|------------------|----------------------|----------------------------------------------------------|---------------|
| Mus musculus     | 309                  | MVLRGRS—L-CLRSLSALGATCARRGLQ—ALLG—LSLCHSDF—RRNLTVQODIM | **0.9901 33** |
| Rattus norvegicus| 309                  | MVLRGRS—L-CLRSLSALGATCARRGLQ—ALLG—LSLCHSDF—RRNLTVQODIM | **0.9753 33** |
| Homo sapiens     | 308                  | MVVRGRDL—L-GRSSLALAGACPRRLGP—ALLG—V-FCHTDL—RRNLTVQODIM | **0.9372 33** |
| Bos taurus       | 308                  | MVLRGRDL—L-GRSSLALAGACPRRLGP—ALLG—V-FCHTDL—RRNLTVQODIM | **0.9438 12** |
| Takifugu rubripes| 307                  | MLL-KSL—VGSACTL—LTATVWK—FAPVFKQAAALLHSV—RRKSVVEGAMN | **0.5748 42** |
| Danio rerio      | 303                  | MWF-RSLS—V-SACTVQVIG—ALSKR—FAP—TALFHSAA—RRKSVVBQDSM | **0.8712 36** |
| Gallus gallus    | 310                  | M-LGGQWRLS—GT-ALVALQAGALLR—APQ—AQLR—AVFHLTEHEQRKSKTVQNNM | **0.3654 25** |

* Table I: Analysis of amino-terminal extensions of the glyoxalase II protein in vertebrates.

mRNA sequences were obtained from GenBank as curated sequences, or were assembled from EST sequences and genomic clones (35), and were then translated using ORFFinder (NCBI). Alignments were performed using the ClustalW program (36). Full protein sequence alignments and the NCBI accession numbers of the clones considered are submitted as a supplementary figure (Fig. S1). Predicted sizes of ORF are shown and the amino-terminal extensions are aligned with the human sequences between the exon A and exon 1 Met residues. Asterisks indicate identical amino acids, colons indicate conserved substitutions, and dots indicate semiconserved substitutions. For the full-length proteins (Fig. S1), MitoProt (22) strongly predicted mitochondrial targeting in mammals, and there was support for this in fish (*Danio* and *Takifugu*) and to a lesser extent in chicken. The predicted cleavage site position in each case is the residue number in the unspaced peptide sequence. Visual inspection of the nucleotide sequences surrounding the first ATG showed they conformed well to Kozak’s consensus for initiation (34) in all mammalian species and in the chicken. The first ATGs were predicted to be in a poor context in fish, where the accepted consensus may not apply.
distribution clearly resolved from the glyoxalase II leader-
DsRed2 protein, then its co-localization with the EYFP-mito
protein must reflect matrix targeting.

DISCUSSION

In this report we have demonstrated the production of a
novel mitochondrial isoform of glyoxalase II from HAGH tran-
scripts, in addition to that of the previously characterized cyto-
solic isoform. The novel isoform results from initiation of
translation at an in-frame ATG codon (exAiATG) in exon A.
This is a previously uncharacterized exon upstream of exon 1,
the exon that contains the previously described translation
initiation site (ex1iATG) of glyoxalase II (5). The ability of the
amino-terminal extension initiated at exAiATG to direct fluo-

FIG. 2. The 5′ mRNA sequences of glyoxalase II enables translation of a luciferase reporter from initiation codons both in exon A and exon 1. The left-hand panel shows the structures of reporter plasmids and the right-hand panel shows the activity of these constructs assessed by their ability to direct firefly luciferase expression in transfected HepG2 cells. Human glyoxalase II core promoter (Prom) sequence (−103 to +1) and 5′ mRNA sequences including exon A (exA) and exon 1 (ex1) up to and including ex1iATG, with or without exon B (exB) were fused in-frame to firefly luciferase (Fluc). In all constructs except the positive control, Fluc had its own initiatory ATG removed so that translational initiation was dependent on glyoxalase II sequence. The predicted initiating ATGs in the glyoxalase II sequence were removed singly and in combination by site-directed mutagenesis to examine their contribution to initiation of translation. In the negative control, there is no initiating ATG in either sequence. Firefly luciferase levels were measured in cells harvested after 48 h and were normalized for expression against co-transfected Renilla luciferase (pRL-TK vector). Data are expressed as the mean ± S.D. of three independent experiments with three replicate wells in each case. The results show that initiation at either ATG can support translation. The ATG in exon 1 also supports translation in the presence of exon B.

FIG. 3. Immunoblotting of glyoxalase II-luciferase fusion proteins. The left-hand panel shows the structures of reporter plasmids derived from the constructs in Fig. 2 but with a CMV promoter replacing the endogenous core promoter. The fusion conserved all but the first two bases of the glyoxalase II 5' UTR as determined in the present study (sequence AY578804). The right-hand panel shows luciferase protein expression in whole cell extracts of HepG2 cells that were transfected and harvested after 48 h and blotted using an anti-firefly luciferase antibody. An arrow indicates 61 kDa (the size of native luciferase). Where both initiating ATG codons are present providing two continuous reading frames (top row) two polypeptides are observed. Where exon B introduces an in-frame stop codon, only the shorter product is observed (second row). Removal of either initiating ATG in the transcript lacking exon B gives a single product of the expected size (third and forth rows).
The amino-terminal extension of glyoxalase II directs DsRed2 to the mitochondrial matrix in HepG2 cells. HepG2 cells were transfected with a construct (pGII-DsRed2) encoding the 48-amino acid extension of glyoxalase II fused to the amino terminus of DsRed2. After 72 h cells were fixed with paraformaldehyde (panels A and B) or with paraformaldehyde supplemented with 0.05% Triton X-100 (panel C) to permeabilize the cells for immunofluorescence. Cells were examined using confocal microscopy. The scale bars represent 5 μm. A, co-transfection of the pGII-DsRed2 fusion construct with pEYFP-Mito (Clontech) indicated the co-localization of both proteins to the mitochondrial matrix. For clarity the EYFP has been false colored blue so that co-localization gives purple upon merging. Co-localization was apparent both at the default pinhole setting (not shown) and at a reduced pinhole setting chosen to maximize resolution as shown. B, HepG2 cells were co-transfected with the pGII-DsRed2 fusion construct and an outer mitochondrial membrane marker (CPT1-EGFP described in Ref. 20). At default pinhole settings, partial colocalization of the signals was apparent (data not shown). Using smaller pinhole settings to maximize resolution indicated that the DsRed2 signal was distinct from the outer membrane but in certain fields was clearly bounded by it (see inset). C, HepG2 cells were transfected with the pGII-DsRed2 fusion construct and processed for immunofluorescence with a previously characterized antibody against cytochrome c (21), which faces the intermembrane space. Immune complexes were detected with a fluorescein isothiocyanate conjugate. A reticular distribution of cytochrome c was observed similar to other reports (21). A punctate distribution of DsRed2 fluorescence was still observed as above but appeared weaker than in panels A or B presumably because of loss of protein upon permeabilization. Cytochrome c was apparent both in the transfected cell and in adjacent untransfected cells. The signals did not co-localize suggesting that the targeting sequence does not direct protein to the intermembrane space.

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Figure 5. Predicted secondary structure of glyoxalase II 5' mRNA regions. Bases 1–576 of A,B,1–8 mRNA (left) and 1–672 of A,B,1–8 mRNA (right) were folded using the mfold version 3.2 program (33). Default parameters were utilized and the most stable structure is shown for each splice form. The sequence predicted to constitute an IRES by UTRscan (25) is indicated and the free energy of each structure (dG) is given in kcal/mol. The regions displayed include a sequence deduced to confer IRES activity based on the luciferase fusion experiments (Figs. 2 and 3).

detected, consistent with IRES function (data not shown). In addition to the glyoxalase II mRNAs described here, other mRNAs have been described that utilize internal ribosome entry in combination with 5' cap-dependent initiation to generate alternative protein isoforms. For example, the Notch2 (27) and the PITSLRE protein kinase (28) mRNAs have been shown to contain an IRES within their coding region that directs the production of truncated isoforms with altered function. The requirement for separate A,1–8 and A,B,1–8 forms of the mRNA is currently not apparent. Speculatively the relative production of the two splice forms could be used to control the amount of cytosolic and mitochondrial enzymes, but further work will be required to establish if this is so.

Previous studies of rat liver mitochondria reported that multiple isoforms of glyoxalase II were present in the intermembrane space and matrix (9, 11). Although the isoforms were similar in size, only glyoxalase II present in the intermembrane space was reported to react with an antiserum raised to the polymerized cytosolic enzyme, whereas matrix glyoxalase II was found to be antigenically distinct (11), however, primary data was not shown. In contrast, we find that the mitochondrial targeting sequence present on glyoxalase II would give rise to a matrix form having 260 amino acids in common with the cytosolic form and so would be expected to be immunochemically similar. Within the resolution of our experiments we were unable to detect proteins targeted by this signal within the intermembrane space. The sequence and cleavage site of the amino-terminal pre-sequence predicted by MitoProt (22) are consistent with mitochondrial matrix targeting (29, 30), and this agrees with the matrix distribution of mitochondrial glyoxalase II in yeast (6).

The current study does not exclude the possibility of additional isoforms of mitochondrial glyoxalase II (as implied in the study of Talesa et al. (9, 11)), which are not accounted for by alternative targeting of HAGH encoded proteins, but there is no clear evidence on this point. Although yeast and higher plants do have distinct mitochondrial glyoxalase II genes (37, 45) it is not clear that this is the case in vertebrates. Besides glyoxalase II, three other GenBank gene entries (MGC2605, MF-1, and HSCO) encode human proteins that contain the GloB domain (characteristic of zinc-dependent hydrolases, including glyoxalases) as predicted by the conserved domain data base (NCBI). Of these proteins only HSCO has been characterized, but it can be discounted because it has been shown to lack glyoxalase II activity (31). MGC2605 encodes proteins that are highly homologous to glyoxalase II but lack a predicted amino-terminal extension that would target them to mitochondria. MF-1 is encoded by alternative mRNA splice forms both of which appear to encode a mitochondrial targeting sequence. However, these isoforms are larger than proteins known to possess glyoxalase II activity, and in particular are longer than the mitochondrial isoforms of glyoxalase II reported by Talesa et al. (9, 11).

In contrast, it may be that proteins encoded by the HAGH gene account for all mitochondrial glyoxalase II activity. We estimate that the ratio of transcripts A,1–8 to A,B,1–8 is –1:2 in HepG2 cells. Furthermore, luciferase experiments suggest that initiation at exAATG is –12% as efficient as that at ex1ATG in A,1–8. Assuming similar stability of luciferase and glyoxalase II in the cytosol and mitochondria this would account for a steady state of ~95% cytosolic and 5% mitochondrial protein. Within the limits of the analysis, this compares reasonably with measurements of rat liver glyoxalase II activity where 85–90% was estimated to be cytosolic and 10–15% mitochondrial (9, 11). It therefore seems plausible that all vertebrate mitochondrial glyoxalase II will derive from the HAGH gene.

The precise role of vertebrate mitochondrial glyoxalase II still remains unclear. In plants and yeast both cytosolic and mitochondrial glyoxalase II play complementary roles in protection against the toxic effects of MG (6, 14). It is possible that the mitochondrial enzyme hydrolyzes S-d-lactoylglutathione that has diffused or been transported into the mitochondrial matrix from the cytosol (12). It has also been suggested that uptake of S-d-lactoylglutathione is a possible pathway for the mitochondrial import of GSH (12), although direct import of GSH also occurs (32). Alternatively mitochondrial glyoxalase II activity may be required to hydrolyze thiol esters of glutathione formed by non-enzymatic or enzymatic acyl transfer from mitochondrial thiol esters of coenzyme A (9). For each of these putative functions, a matrix localization would seem appropriate. Defining the origin of a significant proportion of intramitochondrial glyoxalase II as we have done in this study will allow its functions to be defined, and also enable an evaluation of its possible role in disease processes.

REFERENCES
1. Uotila, L. (1989) in Coenzymes and Cofactors Glutathione: Chemical, Biochemical and Medical Aspects (Dolphin, D., Poulson, R., and Avromonic, O., eds) Vol. 3, pp. 767–804, John Wiley & Sons, New York
2. Thornalley, P. J. (1990) in Biochem. J. 269, 1–11
3. Vander Jagt, D. L. (1989) in Coenzymes and Cofactors Glutathione: Chemical, Biochemical and Medical Aspects (Dolphin, D., Poulson, R., and Avromonic, O., eds) Vol. 3, pp. 597–641, John Wiley & Sons, New York
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4. Daiyasu, H., Osaka, K., Ichino, Y., and Toh, H. (2001) FEBS Lett. 503, 1–6
5. Riddetstrom, M., Saccucci, F., Hellman, U., Bergman, T., Principato, G., and Mannervik, B. (1996) J. Biol. Chem. 271, 319–321
6. Bito, A., Haidr, M., Hadler, J., and Breitenbach, M. (1997) J. Biol. Chem. 272, 21509–21519
7. MacLean, M. J., Ness, L. S., Ferguson, G. P., and Booth, I. R. (1998) Mol. Microbiol. 27, 563–571
8. Bito, A., Haider, M., Hadler, I., and Breitenbach, M. (1997) J. Biol. Chem. 272, 21509–21519
9. MacLean, M. J., Ness, L. S., Ferguson, G. P., and Booth, I. R. (1998) Mol. Microbiol. 27, 563–571
10. Maiti, M. K., Krishnasamy, S., Owen, H. A., and Makaroff, C. A. (1997) Plant Mol. Biol. 35, 471–481
11. MacLean, M. J., Ness, L. S., Ferguson, G. P., and Booth, I. R. (1998) Mol. Microbiol. 27, 563–571
12. Bito, A., Haider, M., Hadler, I., and Breitenbach, M. (1997) J. Biol. Chem. 272, 21509–21519
13. MacLean, M. J., Ness, L. S., Ferguson, G. P., and Booth, I. R. (1998) Mol. Microbiol. 27, 563–571
14. Bito, A., Haider, M., Hadler, I., and Breitenbach, M. (1997) J. Biol. Chem. 272, 21509–21519
15. Bito, A., Haider, M., Hadler, I., and Breitenbach, M. (1997) J. Biol. Chem. 272, 21509–21519
16. MacLean, M. J., Ness, L. S., Ferguson, G. P., and Booth, I. R. (1998) Mol. Microbiol. 27, 563–571
17. MacLean, M. J., Ness, L. S., Ferguson, G. P., and Booth, I. R. (1998) Mol. Microbiol. 27, 563–571
18. Cho, M. Y., Baie, C. D., Park, J. B., and Lee, T. H. (1998) Exp. Mol. Med. 30, 53–57
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Broadway, N. M., Pease, R. J., Birdsey, G., Shayeugi, M., Turner, N. A., and David, S. E. (2003) Biochem. J. 370, 223–231
21. Satch, M., Hamamoto, T., Sato, N., Kagawa, Y., and Endo, H. (2003) Biochem. Biophys. Res. Commun. 300, 482–488
22. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2001) J. Biol. Chem. 276, 4788–4795
23. Singla-Pareek, S. L., Reddy, M. K., and Sopory, S. K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
24. Thornalley, P. J. (1998) Chem. Biol. Interact. 111–112, 137–151
25. Kozak, M. (1999) Mol. Cell. Biol. 21, 1899–1907
26. Lauring, A. S., and Overbaugh, J. (2000) Mol. Cell 6, 939–945
27. Kozak, M. (2000) FEBS Lett. 476, 27–31
28. Mihara, K. (2000) BioEssays 22, 364–371
29. Kozak, M. (2000) FEBS Lett. 476, 27–31
30. Muhar, K. (2000) BioEssays 22, 364–371
31. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
32. Singla-Pareek, S. L., Reddy, M. K., and Sopory, S. K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
33. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
34. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
35. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
36. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
37. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
38. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
39. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
40. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
41. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
42. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
43. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
44. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
45. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
46. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
47. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
48. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
49. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677
50. Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W., and Makaroff, C. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14672–14677