Molecular Basis for the Dual Mitochondrial and Cytosolic Localization of Alanine:Glyoxylate Aminotransferase in Amphibian Liver Cells*

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To gain further insights into the molecular basis of the evolution of alanine:glyoxylate aminotransferase (AGT) intracellular targeting in vertebrates, we have studied the molecular basis of its dual mitochondrial and cytosolic distribution in amphibian liver cells. The AGT gene in Xenopus laevis encodes a polypeptide of 415 amino acids, which includes a 24-residue N-terminal mitochondrial targeting sequence (MTS), at either end of which are located two in-frame potential translation start sites. This MTS is necessary to target Xenopus AGT and sufficient to target a green fluorescent fusion protein to mitochondria in transfected COS cells. The C-terminal tripeptide (KKM), despite being similar to the nonconsensus type 1 peroxisomal targeting sequence in human AGT (KKL), was unable to target Xenopus AGT or human AGT to peroxisomes. The Xenopus AGT gene produces two types of transcript. The longer form encodes a polypeptide that contains the MTS and is targeted to mitochondria. The shorter form encodes a polypeptide that does not contain the MTS and remains in the cytosol. These results are discussed not only in terms of the molecular evolution of AGT targeting but also in terms of the ancillary requirements for the peroxisomal targeting of human AGT.

The intermediary metabolic enzyme alanine:glyoxylate aminotransferase 1 (AGT, EC 2.6.1.44) is unusual in that it is targeted to different organelles in different mammalian species (1, 2). Although there are a number of exceptions, there appears to be a general rule that AGT in the hepatocytes of carnivores or insectivores (e.g. cat, dog, shrew, mole, and hedgehog) tends to be mainly mitochondrial, whereas that in herbivores (e.g. gorilla, orangutan, saki monkey, rabbit, guinea pig, fruit bat, koala, and wallaby) tends to be peroxisomal. In omnivores (e.g. marmosets, tamarins, rodents, and opossum) AGT is usually more evenly distributed between mitochondria and peroxisomes. The guinea pig has significant levels of cytosolic AGT in addition to peroxisomal AGT (3). No mammals have been identified so far with both mitochondrial and cytosolic AGT. However, such a distribution has been found in the common frog (1). We have suggested previously (1, 2) that the variable dual distribution of organellar AGT in mammalian hepatocytes reflects a dual metabolic role of gluconeogenesis (in the mitochondria) and glyoxylate detoxification (in the peroxisomes). Whether cytosolic AGT, when present, has any particular metabolic function is unknown. Gluconeogenesis might be expected to be more important in evolutionary terms for carnivores and insectivores because of their high protein and low carbohydrate diets, whereas glyoxylate detoxification might be expected to be more important for herbivores because their diets are more likely to contain relatively high levels of oxalate and oxalate precursors, most of which are metabolized to oxalate via glyoxylate. Oxalate cannot be metabolized further in mammals, and when present above certain levels oxalate causes severe problems due to the very low solubility of its calcium salt, which can crystallize out in the kidney and urinary tract as stones (4–6). Humans seem to be an exception to the general diet-AGT distribution rule by having peroxisomal AGT (7), even though most people could probably be categorized as omnivores.

In all the species studied so far (i.e. human, marmoset, cat, rat, guinea pig, and rabbit), AGT is encoded by a single gene that has the potential to encode N-terminal mitochondrial and C-terminal peroxisomal targeting sequences (3, 8–11). The variable targeting of AGT in mammals appears to be dependent on the variable use of two in-frame translation start sites, which straddle the region encoding the 22-amino acid-cleavable N-terminal mitochondrial targeting sequence (MTS), and two groups of transcription start sites, one upstream of both translation start sites and one between them (10, 12) (see Fig. 9A). Transcription and translation from the more 5′-sites yield a polypeptide possessing an MTS, whereas in the polypeptide produced following transcription or translation from the more 3′-sites, the MTS is missing.

Whereas the mitochondrial targeting of AGT appears to be fairly straightforward, its peroxisomal targeting is rather unusual. Most peroxisomal proteins are targeted by a C-terminal tripeptide based on the consensus motif S/A/C-K/R/H-L/M (13, 14). This type 1 peroxisomal targeting sequence (PTS1) interacts directly with the tetrapeptide repeat domain of the recycling PTS1 import receptor Pex5p (15, 16). Although the peroxisomal import of AGT is also mediated by Pex5p (17), and is therefore presumably imported via the PTS1 pathway, its C-terminal tripeptides in mammals rarely achieve better than a two out of three match with the consensus PTS1. At least in the case of human AGT, its nonconsensus PTS1 (i.e. KKL) is necessary for peroxisomal import but insufficient to direct the peroxisomal import of a variety of reporter proteins (17, 18).

The importance of hepatic AGT in minimizing the endog-
nous oxalate production in at least some mammals is clearly shown by the autosomal recessive disorder of glyoxylate metabolism primary hyperoxaluria type 1 (PH1) (4), a potentially lethal condition in which AGT deficiency leads to excessive oxalate synthesis and excretion and the deposition of insoluble calcium oxalate in the kidney. PH1 also shows the importance of the correct intracellular compartmentalization of AGT. Although most patients have a complete absence of AGT, a significant subset do have catalytically active AGT, but it is mistargeted from the peroxisomes to the mitochondria (19) where it is unable to detoxify glyoxylate efficiently, thus allowing more to be oxidized to oxalate.

It is quite common to find peroxisomal enzymes in the cytosol, especially the classical peroxisomal marker catalase (20, 21). In the case of guinea pig AGT, its presence in the cytosol appears to be due to an intrinsic inefficiency with which the guinea pig peroxisomal import machinery can deal with the nonconsensus PTS1 HRL (3). However, it is rare to find mitochondrial enzymes in the cytosol to any significant extent, presumably because a failure of mitochondrial import leads to rapid degradation. The few examples that are known result from alternative transcription or translation initiation, similar to that found for mammalian AGT, or from import being partially thwarted due to the presence of tightly folded import-incompetent conformations in the protein cargo (22).

In the present study, we have attempted to gain a better understanding of the molecular evolution of AGT targeting by studying its distribution in a phylum not studied before (i.e. the Amphibia). We have determined the molecular basis for its dual mitochondrial and cytosolic localization in the liver of an amphibian exemplar, Xenopus laevis. In addition, we have embarked on preliminary studies that might help explain some of the unusual characteristics of human AGT peroxisomal targeting.

EXPERIMENTAL PROCEDURES

Animals—Fresh liver samples from common frog (Rana temporis), bullfrog (Rana catesbeiana), palmate newt (Triturus helvetica), pobblebonk (Limnodynastes dumerilii), and Xenopus (X. laevis) were kindly provided by Dr. Andrew Cunningham, Institute of Zoology, London, UK, or the Biological Services Department, University College London, UK.

Subcellular Distribution of AGT—Fresh samples of liver were fixed in 1% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, and prepared for post-embedding protein A-gold immunoelectron microscopy as described previously (7, 19, 23). Immunoperoxidase AGT was detected using monospecific rabbit anti-human AGT antiserum and protein A-gold (10 nm) to label the peroxisomes.

Isolation of Xenopus AGT cDNA Clones—Standard recombinant DNA methodologies (24) were used unless indicated otherwise. The primers used to make the various Xenopus AGT cDNA clones are described in Table I, and the overall cloning strategy is outlined in Fig. 1. A total of 300,000 plaques from a Xenopus liver cDNA expression library in the Uni-ZAP XR vector (Stratagene) were screened using rabbit anti-human AGT antiserum. Following secondary and tertiary screens, seven positive plaques were identified, one of which (pXS) had a strong similarity to AGT cDNAs isolated from other species. For expression studies, pXS was cloned into pcDNA3 (Invitrogen) to give pXAGTshort.

On comparison with AGTs from other species, it was clear that, although the library clone pXS extended to the poly(A) tail at the 3'-end, at the 5'-end it extended to only 23 bp upstream of the more 5'-end of the ancestral translation start sites. Therefore, it did not include the region homologous to the more 5'-translation start site (see Fig. 1). To acquire more 5'-sequence, especially to determine the presence or absence of this 5'-site, rapid amplification of cDNA ends (RACE) was carried out (25). This was performed using the 5'3'-RACE kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using Xenopus liver total RNA and primer P9 (Table I and Fig. 1). The cDNA was purified using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using Xenopus liver total RNA and primer P9 (Table I and Fig. 1). The cDNA was purified using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using Xenopus liver total RNA and primer P9 (Table I and Fig. 1). The cDNA was purified using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using Xenopus liver total RNA and primer P9 (Table I and Fig. 1). The cDNA was purified using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using Xenopus liver total RNA and primer P9 (Table I and Fig. 1). The cDNA was purified using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

To check that RACE-L and pXS were derived from the same transcript, reverse transcriptase-PCR was carried out on total Xenopus liver RNA, using oligo(dT) as the primer for first strand synthesis and then primer pairs P1/P2 and P3/P2. The longer P1/P2 product (Xall) appeared to continu...
The position of the targeting sequence.

**Results**

**AGT Is Both Mitochondrial and Cytosolic, but Not Peroxisomal, in Amphibian Liver Cells**—The subcellular distribution of immunoreactive AGT was determined in *Xenopus*, *pobblebonk*, bullfrog, and common newt livers obtained from both adults and tadpoles. As we have found previously in the case of the common frog (1), AGT in amphibian livers appears to be both mitochondrial and cytosolic (Fig. 2). There was no evidence of any peroxisomal labeling. Insofar as these five species are representative of their phylum, amphibians appear to have a distribution of AGT that is distinct from that found in any mammal so far studied. Interestingly, the distribution of AGT in the only reptile to have been studied to date (i.e. the pond terrapin) shows it also to be mitochondrial and cytosolic. The presence of mitochondrial, but not peroxisomal, AGT is compatible with the carnivorous diets (e.g. insects, slugs, worms, etc.) of adult, if not larval, amphibians and suggests a gluconeogenic, rather than glyoxylate detoxification, role for the enzyme.

The Xenopus AGT Gene Encodes a Long Transcript That Includes the MTS within the Open Reading Frame and a Short Transcript from Which It Is Excluded—The composite *Xenopus AGT* cDNA sequence (GenBank™ AJ278065) shown in Fig. 3 contains an open reading frame that encodes a protein of 415 amino acids. Two in-frame potential translation initiation sites can be identified 24 codons apart. The polypeptide initiating at the more 5'-site is predicted to contain 391 amino acids (one less than found in the equivalent region in AGTs from mammalian sources) and is 66% identical and 75% similar to human AGT. The first 24 amino acids (from the more 5'-initiation site) contains basic, hydroxyl, and hydrophobic amino acids and is deficient in acidic amino acids, as is typical of MTSs. Almost no primary sequence identity between this putative MTS and the 22-residue MTS in AGT of mammals, such as marmoset, cat, and rat, could be discerned (Fig. 4). On the other hand, the C-terminal region of *Xenopus AGT* is well conserved when compared with mammalian AGTs, except that the C-terminal tripeptide in *Xenopus AGT* is KKM, compared with KKL, SQL, NKL, and HRL found in various mammalian AGTs (Fig. 4).

The Xenopus AGT Gene Encodes a Long Transcript That Includes the MTS within the Open Reading Frame and a Short Transcript from Which It Is Excluded—RNase protection analysis generated two fragments (Fig. 5), the 5'-ends of which mapped to −39 and −89 bp downstream of the 5'-end of pXAGT(long). This indicates that the *Xenopus AGT* gene encodes at least two different kinds of transcript, the longer including both of the putative translation initiation sites and the putative MTS in its open reading frame and the shorter excluding the more 5'-site and the MTS. Although the 5'-ends of the long and short transcripts determined by RNase protection and RACE do not coincide, the functional consequences as far as the nature of the polypeptides synthesized are the same.

The subcellular localizations of the long and short forms of *Xenopus AGT* can be seen following their expression in COS cells.
AGT (long) is its MTS, although other regions of the protein Xl strongly suggests that the N-terminal 24 amino acids of diffuse labeling was also apparent (Fig. 7, pressed it was localized to the mitochondria, although some do or do not possess an MTS. 

Alternative transcription initiation and the production of both, and probably other amphibians, can be explained by Xenopus mitochondrial and cytosolic distribution of AGT in the liver cells of tadpole, and palmate newt adult and tadpole gave similar results. Adult bullfrog, common frog tadpole, adult pobblebonk, Xenopus and marmoset (8, 9), NKL in cat and rat (11, 26), HRL in guinea pig (3), and SQL in rabbit (9)). In no cases are these better than a two out of three match to the consensus PTS1 of S/A/C/K/R/H-L/M (13, 14). The C-terminal tripeptide of Xenopus AGT is KKM which is also a two out of three match and rather similar to the C terminus of human AGT. The nonperoxisomal distribution of both XIAGT (long) and XIAGT (short) clearly shows that KKM cannot direct the peroxisomal targeting of Xenopus AGT. To test whether it was able to target a mammalian AGT to peroxisomes, a construct was made in which the C-terminal KKL of human AGT was replaced by the C-terminal tripeptide of human AGT, KKL. When this construct (pHsAGT-KKM) was expressed in COS cells the distribution was cytosolic, no peroxisomal labeling was evident (Fig. 7, E and F), showing that KKM is not capable of directing either human or Xenopus AGT to peroxisomes.

The Consensus PTS1 SKL Is Able to Direct the Peroxisomal Targeting of Xenopus AGT—Unlike AGT in mammalian livers, there is no evidence that AGT is at all peroxisomal in amphibian livers. However, it is very difficult to provide definitive proof against the presence of a protein in an intracellular compartment. Peroxisomal targeting of mammalian AGTs is, at least in part, dependent on their C-terminal tripeptides that seem to function as rather atypical type 1 peroxisomal targeting sequences (PTS1s). In an attempt to gain further insights into whether Xenopus AGT has any potential for peroxisomal targeting, we have functionally characterized its C terminus further.

Unlike the N-terminal 24 amino acid MTS of XIAGT (long), the C terminus of XIAGT is very well conserved with 16 of the last 20 residues being found at the same position in the AGTs from at least one mammal (see Fig. 4). In all mammals studied so far, AGT is at least partly peroxisomal even though the tripeptides at the C termini are highly variable (i.e. KKL in human and marmoset (8, 9), NKl in cat and rat (11, 26), HRL in guinea pig (3), and SQL in rabbit (9)). In no cases are these better than a two out of three match to the consensus PTS1 of S/A/C/K/R/H-L/M (13, 14). The C-terminal tripeptide of Xenopus AGT is KKM which is also a two out of three match and rather similar to the C terminus of human AGT. The nonperoxisomal distribution of both XIAGT (long) and XIAGT (short) clearly shows that KKM cannot direct the peroxisomal targeting of Xenopus AGT. To test whether it was able to target a mammalian AGT to peroxisomes, a construct was made in which the C-terminal KKL of human AGT was replaced by the C-terminal tripeptide of Xenopus AGT (i.e. KKM). When this construct (pHsAGT-KKM) was expressed in COS cells the distribution was cytosolic, no peroxisomal labeling was evident (Fig. 7, E and F), showing that KKM is not capable of directing either human or Xenopus AGT to peroxisomes.

The C-terminal Tripeptide of Xenopus AGT Is Not Sufficient to Direct the Peroxisomal Targeting of Human AGT—Unlike AGT in mammalian livers, there is no evidence that AGT is at all peroxisomal in amphibian livers. However, it is very difficult to provide definitive proof against the presence of a protein in an intracellular compartment. Peroxisomal targeting of mammalian AGTs is, at least in part, dependent on their C-terminal tripeptides that seem to function as rather atypical type 1 peroxisomal targeting sequences (PTS1s). In an attempt to gain further insights into whether Xenopus AGT has any potential for peroxisomal targeting, we have functionally characterized its C terminus further.

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The Consensus PTS1 SKL Is Able to Direct the Peroxisomal Targeting of Xenopus AGT but the Nonconsensus PTS1 of Human AGT Is Not—To check that XIAGT did not contain any sequence or structural elements incompatible with peroxisomal targeting and import, the C-terminal tripeptide of XIAGT (short) was replaced by the canonical PTS1 SKL. When this construct (pXIAGTshort-SKL) was expressed in COS cells its distribution was peroxisomal (Fig. 7, G and H), indicating that such inhibitory elements were not present.

The C-terminal tripeptide of human AGT, KKL, is necessary for its peroxisomal targeting but insufficient to direct the peroxisomal import of reporter proteins. One possible explanation for this is that human AGT contains additional targeting information that makes KKL acceptable as a PTS1. XIAGT is 66% identical and 75% similar to HsAGT and therefore might

### Table II

| Construct | Description | Distribution |
|-----------|-------------|--------------|
| pXIAGT\(^{\text{long}}\) | Xenopus AGT encoded by the long transcript containing a putative N-terminal mitochondrial leader sequence and a C-terminal KKM | Mitochondrial |
| pXIAGT\(^{\text{short}}\) | Xenopus AGT encoded by the short transcript containing a C-terminal KKM | Cytosolic |
| pXIAGT\(^{\text{long}}\)-KKL | Xenopus AGT encoded by the long transcript in which the C-terminal KKM is replaced by KKL | Mitochondrial |
| pXIAGT\(^{\text{short}}\)-KKL | Xenopus AGT encoded by the short transcript in which the C-terminal KKM is replaced by KKL | Cytosolic |
| pXIAGT\(^{\text{short}}\)-SKL | Xenopus AGT encoded by the short transcript in which the C-terminal KKM is replaced by SKL | Peroxisomal |
| pHsAGT | Human AGT containing the non-consensus PTS1 KKL at the C terminus | Peroxisomal |
| pHsAGT-KKM | Human AGT in which the C-terminal KKL is replaced by KKM | Cytosolic |
| pGFP | Green fluorescent protein | Cytosolic |
| pXIAGT\(^{\text{MTS}}\)-GFP | A construct in which the first 24 residues of XIAGT\(^{\text{long}}\) are fused to the N terminus of GFP | Mitochondrial |

The N-terminal 24 Amino Acids of Xenopus AGT Are Necessary for Targeting to Mitochondria and Sufficient for the Mitochondrial Targeting of a GFP Fusion Protein—The observation that XIAGT\(^{\text{long}}\) is mitochondrial and XIAGT\(^{\text{short}}\) is cytosolic clearly shows the necessity of the N-terminal 24 amino acids for the mitochondrial targeting of Xenopus AGT. To test its sufficiency, a construct (pXIAGT\(^{\text{MTS}}\)-GFP) was made in which the N-terminal 24 amino acids of XIAGT\(^{\text{long}}\) is its MTS, although other regions of the protein might also be involved in improving its efficiency.

![Image of AMPHIBIAN LIVER CELLS](http://www.jbc.org/)
also contain this putative extra targeting information even though it is not targeted to peroxisomes. To test this hypothesis, constructs were made in which the C-terminal KKM of *Xenopus* AGT was replaced by KKL. When either pXlAGT(short)-KKL or pXlAGT(long)-KKL was expressed in COS cells, no peroxisomal labeling was detectable. XlAGT(short)-KKL was cytosolic and XlAGT(long)-KKL was mitochondrial (Fig. 8). Therefore, whatever is present in *Hs*AGT that enables KKL to target it to peroxisomes, it appears to be absent from XIAGT.

**DISCUSSION**

**Evolution of AGT Targeting in Amphibians**—The dual mitochondrial and cytosolic localization of AGT in *Xenopus* liver can be explained by the use of alternative transcription start sites such that the region encoding an N-terminal MTS is either included or excluded from the open reading frame (see Fig. 9).
other enzymes, such as the products of the HTS1, VAS1, MOD5, CCA1 and LEU4 genes in *Saccharomyces cerevisiae* (22). Alternative transcription initiation also explains the rather different mitochondrial and peroxisomal distribution of *AGT* in some mammals, such as the marmoset and rat (9, 10, 12). The absence of peroxisomal *AGT* in *Xenopus* liver can be attributed to the absence of a PTS1. However, it is not clear why the C terminus of *Xenopus* AGT should be so well conserved between species, the N-terminal leader sequence is very poorly conserved.

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**Fig. 4.** N-terminal and C-terminal amino acid alignments. The predicted N- and C-terminal amino acid sequences of *Xenopus* AGT are aligned with the homologous regions of various mammalian AGTs. The positions of the ancestral translation start sites 1 and 2 are shaded, and the initiating methionines are in *bold* and *underlined*. The C-terminal tripeptides are in *bold*. *, residues conserved in mammals if contained within the open reading frame.+, residues in *Xl* AGT that are found in at least one mammalian AGT. Unlike the C terminus, which is very well conserved between species, the N-terminal leader sequence is very poorly conserved.

**Fig. 5.** Transcript analysis of *Xenopus* AGT by RNase protection. *A* shows the autoradiograph resulting from the RNase protection assay. *B* shows the relative sizes of the antisense riboprobe and the protected fragments compared with various *Xl* AGT clones. The numbers indicate the estimated sizes of the protected fragments in nucleotides (i.e. ~259 and ~209) compared with the size of the probe (i.e. 298).

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**Fig. 6.** Intracellular distribution of the long and short forms of *Xenopus* AGT in transfected COS cells. COS cells were transfected with either pXlAGTlong (A–D) or pXlAGTshort (E–H). Cells were double-labeled for AGT (A, C, E, and G) and either the peroxisomal marker catalase (B and F) or the mitochondrial marker MitoTracker (D and H). *Xl* AGTlong co-localized with MitoTracker, but not catalase. *Xl* AGTshort was diffusely distributed throughout the cell with no evidence of co-localization with either catalase or MitoTracker. Bar, 10 μm.
served and especially why the C-terminal tripeptide should be so similar to that of human and marmoset AGT (i.e. KKM compared with KKL). Although amphibian AGT genes other than that of *Xenopus* have not been sequenced, it is highly probable that the same molecular basis for dual mitochondrial and cytosolic distribution of AGT also occurs in common frog, pobblebonk, bullfrog, and common newt.

The 5′-structure of the *Xenopus* AGT gene and its ability to produce two transcripts would suggest that dual AGT compartmentalization was present early in the evolution of vertebrates. Although the lack of any conservation between the *Xenopus* and mammalian AGT MTSs might suggest that mitochondrial targeting of AGT in amphibians and mammals was acquired separately, it is perhaps more likely that mitochondrial targeting was present in a common ancestor and that lack of conservation simply reflects the well recognized primary sequence degeneracy of MTSs (27). What is much less clear is whether the nonmitochondrial AGT in early vertebrates was cytosolic or peroxisomal.

Unlike other peroxisomal enzymes, the C termini of AGT in different mammalian species are highly variable. For example, they are KKL in human and marmoset, NKL in cat and rat, SQL in rabbit, and HRL in guinea pig. In all cases, these tripeptides can direct the peroxisomal import of the protein, but none fit the conservative consensus PTS1 (see Introduction). The only mammalian species identified so far in which such a PTS1 is present is in bovine AGT (GenBank™ accession number BE750720), the C terminus of which is SKL. AGT cDNAs have been isolated in a number of invertebrates and higher plants. In *Drosophila melanogaster* (GenBank™ accession number CAA58024) and *Caenorhabditis elegans* (GenBank™ accession number Q94055) the C terminus of AGT is SKI, and in *Arabidopsis thaliana* (GenBank™ accession number AAD28669) and *Fritillaria agrestis* (GenBank™ accession number AAB95218) it is SRL. AGT is peroxisomal in *A. thaliana* (28) and is predicted to be peroxisomal in the other species as well. Amphibian AGT is the only naturally occurring form of AGT shown not to be able to targeted to peroxisomes, and thus KKM is the only naturally occurring AGT C-terminal

![Figure 7](http://www.jbc.org/)

**Fig. 7.** Intracellular distribution of various *Xenopus* and human fusion proteins. COS cells were transfected with either pXlAGT<sup>MTS</sup>-GFP (A and B), pHsAGT (C and D), pHsAGT-KKM (E and F), or pXlAGT<sup>short</sup>-SKL (G and H). Cells were double-labeled for GFP autofluorescence (A) or AGT (C, E and G) and MitoTracker (B) or catalase (D, F and H). XlAGT<sup>MTS</sup>-GFP co-localized mainly with MitoTracker (A and B) but with some diffuse staining also. HsAGT co-localized with the peroxisomal marker catalase (C and D), but when its C-terminal KKL is replaced by KKM it was diffusely distributed with no catalase co-localization (E and F). Unlike pXlAGT<sup>short</sup>, which was diffusely distributed (see Fig. 6, E–H), pXlAGT<sup>short</sup>-SKL co-localized with the peroxisomal marker catalase (G and H). Bar, 10 μm.

![Figure 8](http://www.jbc.org/)

**Fig. 8.** Intracellular distribution of short and long *Xenopus* AGT in which the C-terminal KKM has been replaced by KKL. COS cells were transfected with either pXlAGT<sup>short</sup>-KKL (A and B) or pXlAGT<sup>short</sup>-KKL (C–F). Cells were double-labeled for AGT (A, C, and E) and either catalase (B and D) or MitoTracker (F). XlAGT<sup>short</sup>-KKL was diffusely distributed with no evidence for co-localization with catalase. XlAGT<sup>short</sup>-KKL was co-localized with MitoTracker, again with no evidence of any co-localization with catalase. Bar, 10 μm.
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![Diagram of transcription start sites](image)

**Fig. 9.** Alternative transcription and translation initiation and the dual compartmentalization of AGT. A, diagrammatic structure of the archetypal AGT gene. A and B, the two ancestral transcription start sites. 1 and 2, the two ancestral translation start sites. MTS, region with potential to encode an N-terminal mitochondrial targeting sequence. PTS1, region with potential to encode a non-consensus PTS1 in some mammalian species. B, presence (+) or absence (−) of translation start sites A and B and translation start sites 1 and 2 in AGT from various species, together with the presence or absence of a C-terminal PTS1. Subcellular distribution: MITO, mitochondrial; PEROX, peroxisomal; CYTO, cytosolic (case represents the approximate proportional distribution). This site is presumed to be present due to an appropriate cDNA being isolated (8) but not detected by primer extension (M. Sato, S. Tone, T. Ishikawa, P. E. Purdue, C. J. Danpure, and Y. Minatogawa, submitted for publication). This site is shown to be predominant by primer extension (M. Sato, S. Tone, T. Ishikawa, P. E. Purdue, C. J. Danpure, and Y. Minatogawa, submitted for publication). This site is presumed to be present due to an appropriate cDNA having been isolated (9). 4 Transcription from this site is induced by gluconeogenic stimuli (35). 5 Distribution of AGT in rat liver is highly variable depending on diet and presence of gluconeogenic stimuli (36).

The similarity between the C-terminal tripeptides of Xenopus AGT (i.e., KKM) and human/marmoset AGT (i.e., KKL) is difficult to understand unless either peroxisomal targeting has only recently been lost in the evolutionary history of amphibians or the C terminus of AGT contributes to some other essential characteristic of the protein. As peroxisomal AGT targeting was probably lost before the divergence of Xenopus, common frog, bullfrog, pobblebonk, and common newt, the former possibility seems unlikely. As far as the latter possibility is concerned, it is interesting to note that the C-terminal 21 amino acids of Xenopus AGT are 81% identical to human AGT, which is much higher than the average for the whole molecule (i.e., 66%). Pressure for such sequence conservation could come from the requirement to maintain correct folding and catalytic activity. Whether this is actually the case is not currently known, but it is relevant to note that human AGT still dimerizes when the C-terminal KKL is deleted (29) and is still catalytically active when a C-terminal His tag is attached (30).

**Xenopus AGT Lacks the Ancillary Peroxisomal Targeting Information Predicted to Be Present in Human AGT**—The peroxisomal targeting of mammalian AGTs is far from being fully understood. Although it is dependent on the presence of the PTS1 import receptor Pex5p, but not the PTS2 import receptor Pex7p (17), the C-terminal tripeptides found in different species are highly variable and in most cases do not fit the PTS1 consensus sequence of S/A/C-K/R/H-V/L/M.

The peroxisomal targeting of human AGT has been studied in more detail than that in the other species. Although the C-terminal KKL is necessary for the peroxisomal targeting of human AGT, it is insufficient to target reporter proteins such as chloramphenicol acetyltransferase, firefly luciferase, and GFP to peroxisomes (17, 18). The unusual nature of the PTS1 in human AGT has been confirmed recently when it was shown that AGT does not interact with human Pex5p in the yeast two-hybrid system, cannot compete with the peroxisomal import of other PTS1 proteins, and its peroxisomal import can be inhibited by overexpression of Pex5p (31). In an attempt to explain this atypical behavior, we have suggested that human AGT might possess ancillary peroxisomal targeting information other than at the C terminus and that interaction with Pex5p might require the presence of a an additional adaptor molecule (31).

The observation that Xenopus AGT cannot be targeted to the peroxisomes, despite its high similarity to human AGT, appears to be due to the fact that KKM cannot act even as a nonconsensus PTS1. In addition, the combined failure of KKM to direct the peroxisomal targeting of human AGT and KKL to direct the peroxisomal targeting of Xenopus AGT strongly suggests that Xenopus AGT does not contain the ancillary peroxisomal targeting information predicted to be present in human AGT. The significance of this for our understanding of the evolution of AGT targeting remains to be seen. Nevertheless, the sequence similarity between Xenopus and human AGT opens up the possibility that identification of this ancillary targeting information might be possible by studying the ability of various human-Xenopus chimeric AGTs to target to peroxisomes.

Whatever the results of such studies, they are likely to add yet another chapter to the remarkable story of the evolution of AGT targeting, which so far includes the following: (a) the repeated loss of one or other of the alternative transcription and translation initiation sites in mammals with the inclusion or exclusion of the MTSs from the open reading frame (12); (b) the positive selection for loss or decreased efficiency of the MTS in primates (32); (c) the generation and high population frequency of a cryptic MTS in humans (33, 34); (d) the manifestation of this cryptic MTS in patients with a lethal hereditary disease resulting in peroxisome-to-mitochondria mistargeting (30, 34).

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