

# Antizyme2 Is a Negative Regulator of Ornithine Decarboxylase and Polyamine Transport\*

(Received for publication, April 13, 1999, and in revised form, June 22, 1999)

Chang Zhu‡, D. Wayne Lang‡, and Philip Coffino‡§¶

From the ‡Department of Microbiology and Immunology, §Department of Medicine, University of California, San Francisco, California 94143-0414

**The antizyme family consists of closely homologous proteins believed to regulate cellular polyamine pools. Antizyme1, the first described, negatively regulates ornithine decarboxylase, the initial enzyme in the biosynthetic pathway for polyamines. Antizyme1 targets ornithine decarboxylase for degradation and inhibits polyamine transport into cells, thereby diminishing polyamine pools. A polyamine-stimulated ribosomal frameshift is required for decoding antizyme1 mRNA. Recently, additional novel conserved members of the antizyme family have been described. We report here the properties of one of these, antizyme2. Antizyme2, like antizyme1, binds to ornithine decarboxylase and inhibits polyamine transport. Using a baculovirus expression system in cultured Sf21 insect cells, both antizymes were found to accelerate ornithine decarboxylase degradation. Expression of either antizyme1 or 2 in Sf21 cells also diminished their uptake of the polyamine spermidine. Both forms of antizyme can therefore function as negative regulators of polyamine production and transport. However, in contrast to antizyme1, antizyme2 has negligible ability to stimulate degradation of ornithine decarboxylase in a rabbit reticulocyte lysate.**

The mammalian antizyme (AZ)<sup>1</sup> was first described as an inhibitor of ornithine decarboxylase (ODC) (reviewed in Ref. 1). ODC is a key enzyme in polyamine metabolism. It is induced by growth signals, and overexpression is observed in many tumor cells. Further, forced expression of ODC can transform mouse fibroblast cells (2).

AZ is induced when cellular polyamine levels rise. AZ mRNA has two overlapping open reading frames, a short ORF1 and a second ORF2, which encodes most of the AZ protein, but lacks an initiation methionine (3). A +1 translational frameshift, favored by elevated polyamines, aligns the two ORFs, thus producing the full-length functional AZ protein. AZ1 regulates ODC activity by dissociating the enzymatically active ODC dimer, forming the inactive ODC:AZ1 heterodimer (4, 5). ODC is a substrate for degradation by the 26 S proteasome, and is much more efficiently degraded when associated with AZ1 (6, 7). This accelerated form of ODC proteolysis is ATP-dependent

but, distinct from most proteasome substrates, does not require ubiquitination (8–10). AZ1 thus takes part in a form of feedback regulation that restricts polyamine pools. Two activities of AZ1 are relevant to its limitation of ODC activity. The first is stoichiometric with respect to ODC, depends on dissociation of the ODC homodimer, and is, in principle, reversible. The second is catalytic with respect to ODC, because the enzyme is destroyed while AZ1 is recycled. In addition to its effects on ODC, AZ1 also negatively regulates polyamine transport into cells (11, 12).

Both the structure of AZ proteins and the polyamine-induced frameshifting mechanism are highly conserved; they have been found in a spectrum of organisms from man to *Drosophila*. Recently, a second AZ (AZ2) gene has been reported in human and mouse (13, 14); their transcripts also retain the characteristic pseudoknot structure that mediates AZ1 frameshifting (3, 15). GenBank™ search shows the additional existence of a third form of AZ (AZ3) in humans. AZ1 and AZ2 are each more conserved across species than they are within a single species, implying that AZ1 and AZ2 have maintained independent lineages since their divergence from a common ancestral gene. This suggests that they mediate distinct functions. AZ1 is known to limit polyamine accumulation in three ways: 1) it binds to and inactivates ODC; 2) it causes the degradation of ODC; and 3) it inhibits cellular uptake of polyamines. Here we assess the capacity of AZ2 to carry out these activities, using AZ1 as a reference for comparison. We find that AZ2, like AZ1, binds to ODC and inhibits polyamine transport. AZ2 expression accelerates ODC degradation in cultured cells but has negligible degradative activity compared with AZ1 in an *in vitro* system.

## EXPERIMENTAL PROCEDURES

**Plasmids and Baculovirus**—Throughout this paper, we enumerate the first amino acid or the first nucleotide of AZ ORF1 as position 1. Z1 is a rat AZ1 partial cDNA clone, which lacks the first 45 nucleotides of ORF1 (3). GST-AZ1<sup>69–227</sup>, a fusion of Z1 to GST, has been described previously (16). AZ2 sequences were amplified from a human cDNA library (17). Primers used were upstream primer AZ2b (5'-ccgaggatgataaacacc), and downstream primer AZ2c (5'-gctatactcaggagccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ2<sup>33–189</sup> was made by PCR (primers: 5'-cgggaattc(EcoRI)ggcctgatgccctgac and 5'-cgggaattc(EcoRI)cccggctccccttaggc) and then cloning of the product into the EcoRI site of pGex2TK (Amersham Pharmacia Biotech). For baculovirus-based expression we used the BacPAK system (CLONTECH). To construct a baculovirus vector for expression of AZ1, we excised the AZ1Δ<sup>205</sup> sequence, containing a single T nucleotide deletion that aligns ORF1 with ORF2, from pGem4AZ1Δ<sup>205</sup> (3) by *NcoI*/*EcoRI* digestion and cloned this fragment into the *NcoI*/*EcoRI* sites of pBakPAKHis1 (CLONTECH) to make pBakPAK.AZ1Δ<sup>205</sup>. To create a comparable baculovirus vector for expression of His<sub>6</sub>-tagged AZ2, AZ2Δ<sup>197</sup>, which contains a single T nucleotide deletion analogous to that present in AZ1Δ<sup>205</sup>, was engineered by overlapping PCR (18) and cloned into the *EcoRV* site of Bluescript KS (Stratagene) by TA cloning (19). This construct was cleaved by double digestion with *KpnI*/*SmaI* and the

\* This work was supported by Public Health Service Grant GM45335 from the NIGMS, National Institutes of Health and by Grant RG0384 from the Human Frontiers Science Project. 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.

¶ To whom correspondence should be addressed: Tel.: 415-476-1783; Fax: 415-476-8201; E-mail: pcoffin@itsa.ucsf.edu.

<sup>1</sup> The abbreviations used are: AZ, antizyme; EGFP, enhanced green fluorescent protein; His<sub>6</sub>, repeat of 6 histidine residues; ODC, ornithine decarboxylase; ORF, open translational reading frame; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

resultant fragment was subsequently inserted into pBacPAKH1 at *KpnI* and blunted *HindIII* sites to make pBacPAK.His<sub>6</sub>AZ2ΔT<sup>97</sup>. To create a baculovirus vector that requires frameshifting for expression of AZ2, AZ2 sequences were excised from pCR2.1 by *EcoRV/KpnI* digestion and inserted into pBakPAKH1 digested with *HindIII/KpnI* to make pBakPAK.His<sub>6</sub>AZ2. The amino acid tag sequences that precede native AZ2 are for His<sub>6</sub>AZ2: MG(H)<sub>6</sub>VVICRIRLPRM and for His<sub>6</sub>AZ2ΔT<sup>205</sup>: MG(H)<sub>6</sub>VVDKLGCRNSIPRM. The underlined M indicates the native initiation methionine of AZ2. A baculovirus expression vector for His<sub>6</sub>-tagged mouse ODC, pBakPAK.His<sub>6</sub>ODC, was made by PCR cloning. An ODC fragment containing the whole ORF<sup>1</sup> (461 amino acids) of ODC was PCR amplified from pOD48 (20) using the primers 5'-agcagctttactaaggagc and 5'-gggtacc(*KpnI*)tggtcccccaaatgccc. The product was blunt-ended by treating with T4 DNA polymerase, digested with *KpnI* and inserted into the *PmlI/KpnI* sites of pBakPAKH1 to make pBakPAK.His<sub>6</sub>ODC. These constructs were packaged for delivery to Sf21 insect cells as recombinant baculovirus and cells infected following the protocols recommended by CLONTECH. Virus purification, amplification, and expression of heterogeneous proteins were according to standard protocols (21). pBacPAK6 virus encoding *Escherichia coli* β-galactosidase was purchased from CLONTECH.

**ODC Activity and Inhibition Assay**—GST-AZ1<sup>69–227</sup> and GST-AZ2<sup>33–197</sup> were expressed in *E. coli* and purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Proteins were eluted from the beads with 30 mM glutathione, 75 mM Hepes (pH 8.0), 150 mM NaCl, 10 mM β-mercaptoethanol, and 2.5% Sarkosyl. Sf21 cell extracts were prepared by freezing and thawing of cells in ODC assay buffer (66 mM Tris (pH 7.5), 4 mM dithiothreitol, 80 μM EDTA, 50 μM pyridoxal 5-phosphate, 400 μM L-ornithine). To measure inhibition of ODC enzymatic activity, crude extract from ODC baculovirus-infected Sf21 cells was mixed with purified GST-AZ or crude extracts from AZ-infected Sf21 cells. The mixture was pre-incubated on ice for 30 min, and ODC activity was then assayed as described (5).

**Metabolic Labeling, Immunoprecipitation, and Western Blotting**—Baculovirus-infected Sf21 cells were labeled with <sup>35</sup>S-Express (NEN Life Science Products) as described (21). Cells were starved for 1 h, labeled for 1 h, and chased for 30 min in medium containing excess cold methionine. Cells were extracted in 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM sodium orthovanadate, and 10 μg/ml each of phenylmethylsulfonyl fluoride, aprotinin, pepstatin A, and leupeptin. Radiolabeled proteins were immunoprecipitated with rabbit polyclonal antibody prepared against recombinant mouse ODC. For Western blotting, cell extracts were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with peroxidase-coupled anti-His<sub>6</sub> antibody (Roche Molecular Biochemicals). To assess association of ODC with AZs, *in vitro* translated and metabolically labeled ODC was incubated with purified GST-fusion proteins bound to glutathione-Sepharose beads in phosphate-buffered saline plus 0.05% Triton X-100. Bound ODC was analyzed by SDS-PAGE and autoradiography or by using a PhosphorImager (Molecular Dynamics).

**ODC *in Vitro* Degradation**—[<sup>35</sup>S]methionine-labeled ODC and AZ were generated by coupled *in vitro* transcription and translation in rabbit reticulocyte lysate (Promega) as described (22). A DNA template for transcription by T7 RNA polymerase was generated by PCR. Primers used were the following: ODC<sup>1–461</sup> (5'-gtaatacgcactcactatagggacatgagcagctttactaag and 5'-ccggaattctacacattgatctagc), AZ1<sup>70–227</sup> (5'-gt-aatacgcactcactatagggacatggatgctcctcaccacc and 5'-gggtcgactagctctctcagccgg), AZ2<sup>33–189</sup> (5'-gtaatacgcactcactatagggacatggatgcccctcaccact and 5'-gggtcgacttagctctcctcagacaag). T7 promoter sequences are underlined. [<sup>35</sup>S]methionine labeling of AZs and ODC, and ODC degradation were as described (22) with modification. An AZ dilution series was prepared using a 1:1 v/v mixture of reticulocyte lysate and ATP regenerating system as diluent, and 8 μl of the resulting diluted AZ1<sup>70–227</sup> or AZ2<sup>33–189</sup> translation product and 5 μl of ODC translation product were mixed and placed on ice for 5 min. The samples were then removed from ice and 13 μl of an ATP regenerating system (60 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 4 mM dithiothreitol, 2 mM ATP, 20 mM creatine phosphate, 3.2 mg/ml phosphocreatine kinase) were added. Degradation was allowed to take place at 37 °C for 1 h. The reaction was stopped by adding SDS-PAGE loading buffer. ODC and AZ were analyzed by SDS-PAGE and autoradiography, or by PhosphorImager analysis.

**Polyamine Transport**—Sf21 cells were infected with recombinant baculovirus carrying the indicated constructs. Approximately 48 h post-infection, polyamine transport was measured as described (23) with modification. Cells were resuspended in 100 μl of serum-free Grace's medium (~5 × 10<sup>6</sup> cells/ml). [<sup>3</sup>H]spermidine (NEN Life Science Products) was added (2.2 μM), and cells were incubated at room temperature for the indicated time with gentle rotation. Cells were then washed two

times with 1 ml of Grace's medium and lysed in 200 μl of 1% SDS at 65 °C for 30 min. [<sup>3</sup>H]spermidine uptake was measured by scintillation counting, and transport activity was normalized to cell extract, determined by OD measurement at 260 nm; normalization factors differed among experimental samples by less than 25%.

## RESULTS

**Antizymes Are Encoded by a Gene Family**—Recently a second mammalian AZ gene has been cloned (13, 14). Using sequence data presented by Kajiwara *et al.* (13) and in EST data bases (GenBank<sup>TM</sup> accession number W76088), we independently cloned the putative coding region of AZ2 from a human cDNA library (17) using PCR amplification. Nucleotide sequencing confirmed that AZ2 is related to AZ1. First the predicted AZ2 protein is structurally similar to AZ1 (Fig. 1). Also, as is true for AZ1, AZ2 has two overlapping ORFs, and the second ORF lacks an initiation codon. Translation of the second ORF therefore requires a programmed +1 or -2 ribosomal frameshift. Notational alignment and translation of the two ORFs predicts a protein of 21 kDa. Second, the putative translation product of human AZ2 shares 54% sequence identity with the human AZ1. Third, the frameshift region is more conserved between AZ1 and AZ2 than the rest of the coding regions, 62 of 77 nucleotides (81%) compared with 60% for the whole of both coding regions. Furthermore, the proposed pseudoknot structure that promotes the frameshift of rat AZ1 mRNA (3, 15) is also conserved in human AZ2. The sequence of the gene we cloned agrees exactly with that reported (14). There is also evidence of a third AZ gene in humans. A single EST sequence (GenBank<sup>TM</sup> accession number AI186032) distinct from AZ1 and AZ2 has been deposited in the GenBank<sup>TM</sup> (Fig. 1).

**AZ Is Ubiquitous in Vertebrates**—Two forms have been reported in the zebra fish *Danio rerio* (GenBank<sup>TM</sup> accession numbers AB017117 and AB017118). In invertebrates, AZ has been found in *Drosophila* (24, 25). The *Caenorhabditis elegans* genome contains one AZ-like ORF encoding an 80-amino acid peptide (26) (GenBank<sup>TM</sup> accession number 2746910). Interestingly, that sequence is also preceded by an upstream overlapping ORF. A +1 frameshift that aligns the two ORFs would extend the putative translation product to 160 amino acids and would improve slightly its similarity to other AZ proteins. Consistent with this hypothesis, a search of *C. elegans* ESTs showed that the transcription start site is at least 45-nucleotide 5' of the putative ATG of ORF1.

Sequence comparisons of AZ1 and AZ2 of rodents and humans show that each is more highly conserved across species lines than are AZ1 and AZ2 when compared within species (Fig. 1) (14). Such conservation of both AZ1 and AZ2 suggests they may have distinct cellular functions. We therefore compared AZ1 with AZ2 to determine which activities of the former are also found in the latter.

**AZ2 Binds to ODC and Inhibits Its Activity *in Vitro***—Sequence similarities between AZ1 and AZ2 suggest that AZ2 may also be a negative regulator of ODC. To test this idea, the second ORF of AZ1 and AZ2 were each expressed as GST fusion proteins in *E. coli*. Similar to GST-AZ1<sup>69–227</sup>, GST-AZ2<sup>33–189</sup> bound to ODC (Fig. 2A). As expected, both AZ1 and AZ2 inactivated ODC enzymatic activity. A parallel dilution series of each fusion protein showed that they were approximately equipotent in inhibiting ODC activity (Fig. 2B). Similar to AZ1, the functional domain of AZ2 for ODC binding and inactivation is within ORF2.

**AZ Activity in Sf21 Cells**—Native AZ proteins are the product of expression of both ORF1 and 2. To test *in vivo* function of the full proteins in cells, we used a point deletion of a single nucleotide to align the two ORFs. We thereby made expression independent of cellular polyamine status and avoided the re-

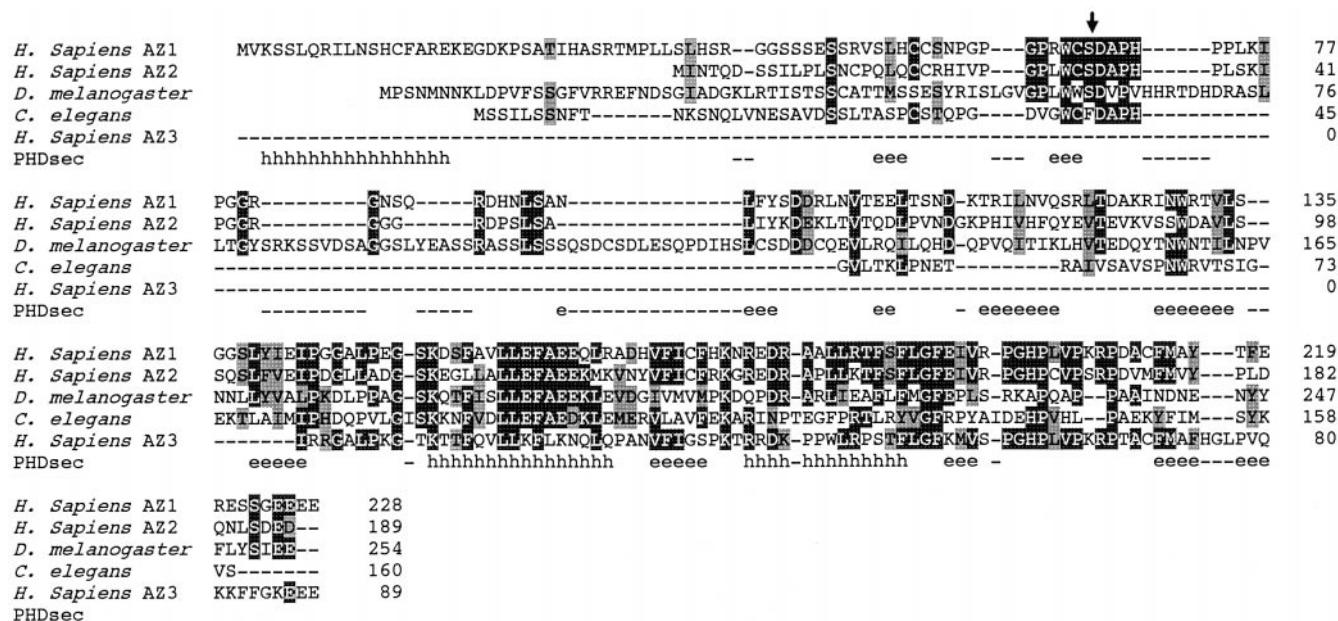


FIG. 1. Multiple sequence alignment of AZ proteins. The alignment was done using CLUSTAL W multiple sequence alignment program (version 1.7) (36). Gaps were manually introduced to allow the optimal alignment of the N terminus. The frameshift site is marked by an arrow. Human AZ1 (GenBank<sup>TM</sup> accession number D89870), AZ2 (GenBank<sup>TM</sup> accession number AF057297), and fruit fly AZ (GenBank<sup>TM</sup> accession number AF038597) sequences are from the GenBank<sup>TM</sup>. The *C. elegans* AZ amino acid sequence shown is that predicted from its genomic sequence (GenBank<sup>TM</sup> accession number 2746910), and assumes a +1 translational frameshift at the marked site. The partial sequence of human AZ3 is predicted from an EST sequence (GenBank<sup>TM</sup> accession number AI186032). Dark shading with white characters indicates amino acid identity among at least three proteins. Light shading indicates amino acid similarity among at least three proteins. Predicted secondary structure for AZ1 is listed below the amino acid sequences. The secondary structure is predicted by the PHDsec computer program (31, 32). *h*, helix; *e*, extended (sheet); *blank*, other (loop).

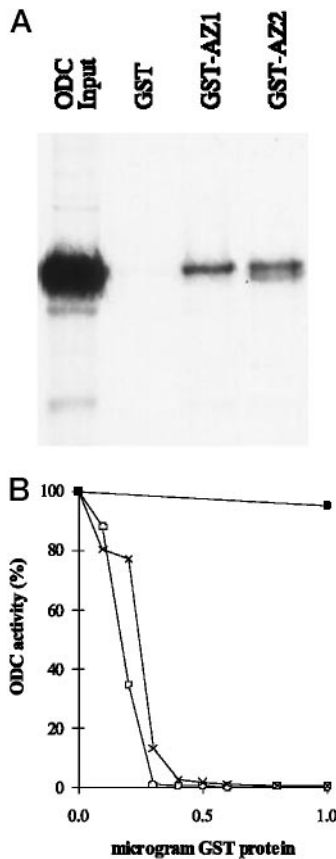
requirement for frameshifting, a process that reduces the efficiency of expression. We expressed full-length AZ1 and AZ2 using a baculovirus expression system (21). This system was developed to facilitate high level expression of cloned gene products in insect cells, and uses virus-derived vectors for transient expression. The two ORFs were aligned by a single base deletion,  $\Delta T^{205}$  for AZ1 and  $\Delta T^{97}$  for AZ2. Infection of Sf21 cells with viruses encoding AZ1 or AZ2 resulted in the cellular production of similar amounts of ODC inhibitory proteins. Titration of extracts prepared from cells infected with AZ1 (AZ1 $\Delta T^{205}$ ) or AZ2 (His<sub>6</sub>AZ2 $\Delta T^{97}$ ) against active ODC showed that they differed by only 2-fold (AZ1 > AZ2) in inhibitory activity (Fig. 3). Full-length AZ1 (AZ1 $\Delta T^{205}$ ) or AZ2 (His<sub>6</sub>AZ2 $\Delta T^{97}$ ), when co-expressed with His<sub>6</sub>-tagged ODC in Sf21 cells, form a complex (Fig. 4). ODC-AZ co-infected Sf21 cells were metabolically labeled and extracts immunoprecipitated with an anti-ODC antibody. Both AZ1 $\Delta T^{205}$  and His<sub>6</sub>AZ2 $\Delta T^{97}$  were pulled down together with His<sub>6</sub>ODC by anti-ODC antibody (Fig. 4).

**AZ2 Frameshift *In Vivo***—To test whether AZ2 is capable of frameshifting *in vivo*, we also expressed AZ2 cDNA in Sf21 cells with an N-terminal His<sub>6</sub> tag. As no mutagenesis was performed on this construct to align ORF1 and ORF2, expression of the protein should require an *in vivo* frameshift. Although expressed at a lower level compared with His<sub>6</sub>AZ2 $\Delta T^{97}$ , which does have the reading frames artificially aligned, His<sub>6</sub>AZ2 was nevertheless expressed in Sf21 cells (Fig. 4). Note that His<sub>6</sub>AZ2 migrated a little faster than His<sub>6</sub>AZ2 $\Delta T^{97}$ , because they differ slightly in sequence between the His<sub>6</sub> tag and the first native amino acid of AZ2. That the frameshift has produced functional protein was supported by the following evidence. First, it bound to ODC and was immunoprecipitated with ODC by an anti-ODC antibody (Fig. 4). Second, Sf21 cells expressing His<sub>6</sub>AZ2 but not cells infected with a control virus contained ODC inhibitory activity (data not shown). We also constructed a fusion of the AZ2 frameshift region to EGFP such

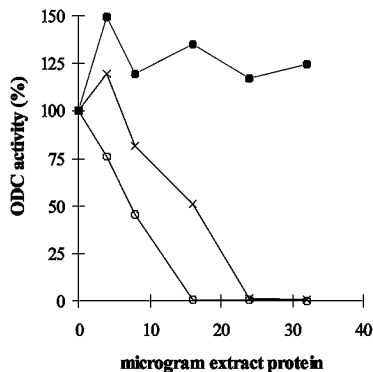
that ORF2 of AZ2 was in frame with the EGFP reading frame. When this construct was transiently expressed in COS-7 or ODC-deficient Chinese hamster ovary C55.7 (27) mammalian cells and protein products detected with an anti-EGFP antibody, protein of the size expected for the frameshift product was observed.<sup>2</sup> These data are consistent with the *in vitro* data showing AZ2 frameshift reported earlier (14).

**AZ2 Targets ODC for Degradation in Sf21 Cells**—In addition to inactivating ODC by dissociating the functional ODC homodimer, AZ1 also targets ODC for degradation. To test whether AZ2 can also target ODC for degradation, we co-infected Sf21 cells with baculoviruses expressing ODC and AZ. We first measured ODC activity. As expected, co-infection with baculovirus expressing ODC and AZ1 or AZ2 from AZ1 $\Delta T^{205}$  and His<sub>6</sub>AZ2 $\Delta T^{97}$ , respectively, resulted in much less ODC activity than was expressed upon co-infection with ODC and PAK6 vectors, a control construct encoding  $\beta$ -galactosidase, or with virus encoding only ODC (Fig. 5A). There are two possibilities to explain the lesser ODC activity caused by co-expression of either AZ. The first is that ODC is inactivated by AZ binding, but ODC protein remains present. The second is that ODC is actively degraded in Sf21 cells expressing AZ. In the latter case AZ expression should cause steady-state levels of ODC to decline. Western blotting with an antibody against the His<sub>6</sub> tag at the N terminus of the ODC protein (Fig. 5B) revealed that ODC protein was indeed reduced to an undetectable level by expression of AZ, compared with a minor reduction seen with the control  $\beta$ -galactosidase construct. Similar results were obtained when the anti-ODC antibody was used for Western blotting (data not shown), which ruled out the possibility that the N terminus of His<sub>6</sub>ODC fusion had been cleaved in AZ-infected cells. It is not likely that the reduced ODC level results from reduced replication of the virus encod-

<sup>2</sup> C. Zhu and P. Coffino, unpublished observations.

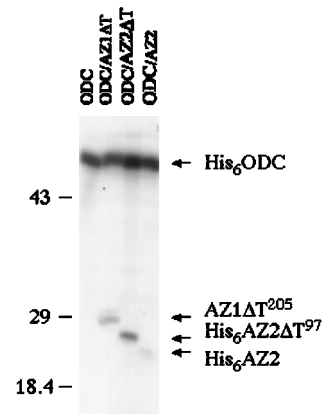


**FIG. 2. Binding and inhibition of ODC.** AZ binds to and inhibits ODC *in vitro*. *Panel A*,  $^{35}\text{S}$ -labeled *in vitro* translated ODC was allowed to interact with immobilized GST, GST-AZ1, or GST-AZ2. The ODC input protein (*left lane*) and bound protein was visualized by SDS-PAGE and autoradiography. *Panel B*, titration of ODC activity by GST (●), GST-AZ1 (○), or GST-AZ2 (×). 4  $\mu\text{g}$  of extract protein from ODC vector-infected Sf21 cells was used per assay point as the source of ODC activity. Data is plotted as a percent of activity present without addition of GST proteins and was approximately 400 nmol/min/mg of protein.

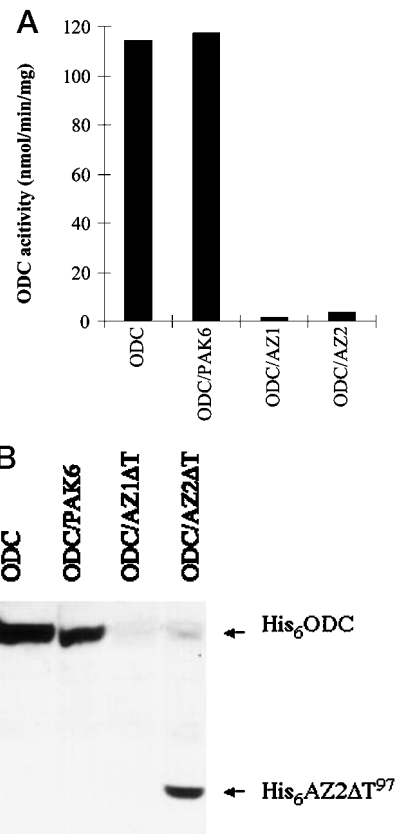


**FIG. 3. ODC inhibitory activity of AZ expressed in Sf21 cells.** ODC activity was titrated by extracts of Sf21 cells expressing AZ1 $\Delta\text{T}^{205}$  (○), AZ2 $\Delta\text{T}^{97}$  (×), or PAK6 encoding  $\beta$ -galactosidase (●). 4  $\mu\text{g}$  of extract protein from ODC-infected Sf21 cells was used per assay point as the source of ODC activity. Data is plotted as a percent of activity present without addition of inhibitory proteins and was approximately 400 nmol/min/mg of protein.

ing ODC in cells infected by the virus encoding AZs. AZ expression had little or no effect on ODC expression as measured by metabolic labeling (Fig. 4), which detects newly synthesized proteins instead of steady state protein levels. Taken together, the data strongly suggest that ODC degradation in Sf21 cells is accelerated by both AZ1 and AZ2. (Pulse-chase experiments were not carried out to confirm this conclusion, as we could not



**FIG. 4. ODC and AZ form intracellular complexes.** Sf21 cells were singly or doubly infected with the viruses indicated and metabolically labeled with  $^{35}\text{S}$ methionine. Radiolabeled proteins were immunoprecipitated with an anti-ODC antibody and visualized by SDS-PAGE and autoradiography. *Arrows* mark the positions of His<sub>6</sub>ODC and of co-immunoprecipitated proteins encoded by AZ1 $\Delta\text{T}^{205}$ , His<sub>6</sub>AZ2 $\Delta\text{T}^{97}$ , or His<sub>6</sub>AZ2 vectors. The position of migration of marker proteins of the indicated molecular masses (kDa) are shown on the *left*.



**FIG. 5. Reduction of ODC activity and protein level by AZ co-expression in Sf21 cells.** ODC levels were assessed in Sf21 cells infected with ODC alone or in combination with AZ1 $\Delta\text{T}^{205}$ , His<sub>6</sub>AZ2 $\Delta\text{T}^{97}$  or PAK6, a  $\beta$ -galactosidase control. *A*, ODC enzymatic activity; *B*, steady state level of His<sub>6</sub>ODC detected by Western blotting with an anti-His<sub>6</sub> antibody. Note that AZ1 $\Delta\text{T}^{205}$  was not detected, because it was not His<sub>6</sub>-tagged.

establish effective chase conditions for these cells.) The expression level of AZ2 from His<sub>6</sub>AZ2 $\Delta\text{T}^{97}$  in Sf21 cells was much lower than the ODC expression level when cells were infected with ODC baculovirus alone (Fig. 5*B*). This suggests that in this experimental system, 1 molecule of AZ2 can catalyze the degradation of more than 1 ODC molecule.

*AZ2 Does not Cause ODC Degradation in Vitro*—Extensive

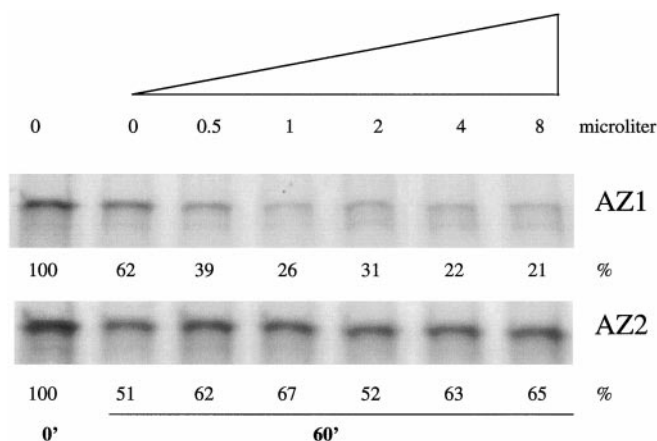


FIG. 6. **In vitro degradation of ODC directed by AZ1 or by AZ2.** ODC was incubated with 0–8  $\mu$ l of extracts in which AZ1 or AZ2 had been translated, as indicated. After mixing and preincubation of ODC and the AZ on ice and addition of an ATP generating system, samples were immediately analyzed (left lanes) or incubated at 37  $^{\circ}$ C for 60 min before analysis. The amount of ODC that remained undegraded is shown below each lane as a percentage of that present in the sample not subject to 60 min of incubation at 37  $^{\circ}$ C.

previous studies have used both crude and purified cellular extracts as constituents of an *in vitro* system for study of ODC degradation (10, 16, 28, 29). Such investigations have shown proteolysis to be independent of ubiquitination and dependent on ATP, AZ1, and the 26 S proteasome. Using a rabbit reticulocyte extract supplemented with an ATP regenerating system, we examined the capacity of proteins corresponding to AZ1 or AZ2 ORF2 to direct degradation of ODC. The AZs and ODC were produced by *in vitro* translation. They were radiolabeled using incorporation of [ $^{35}$ S]methionine to provide a means for following their amount and stability. A semi-quantitative assessment of the relative potencies of AZ1 and AZ2 was obtained by comparing the degradative activity of a dilution series of the two proteins (Fig. 6). Relative intensity of labeling, normalized to the respective methionine content of each protein, was used to estimate relative protein stoichiometry. At the highest concentration of each AZ used in the experiment shown, the AZs and ODC were initially present at approximately equimolar concentrations.

In the case of AZ1, an 8-fold dilution resulted in approximately the same extent of ODC degradation as the highest concentration examined, and a 16-fold dilution produced more ODC degradation than a control with no AZ1 added. In the control with no exogenous AZ1 added, the intensity of the ODC signal was reduced about 2-fold compared with an identical sample, but one not subjected to the 1-h incubation period used to elicit degradation. This “AZ-independent background degradation” is prevented by the proteasome inhibitor *N*-acetyl-leu-leu-norleucinal peptide (results not shown) and is probably due to the presence of endogenous AZ in the reticulocyte lysate (28). The effect of adding AZ2 is very different from that seen with AZ1. AZ2 produced no degradation, even when added at a 1:1 molar ratio with respect to ODC. In fact, even the lowest concentration used provided a modest ODC-protective effect compared with a control sample incubated without either AZ1 or AZ2. These results imply that under the experimental conditions used, AZ2 is at least 16-fold less potent than AZ1 in directing the degradation of ODC.

**AZ Inhibits Spermidine Uptake**—In addition to regulating ODC activity, AZ1 also inhibits polyamine transport into cells. In mammalian cells transfected with AZ1 under the control of an inducible promoter, polyamine uptake was reduced several-fold when AZ1 was expressed (11, 12). We measured spermi-

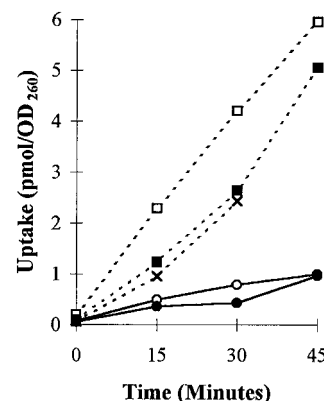


FIG. 7. **Inhibition of spermidine uptake by AZ.** Sf21 cells were infected with vectors expressing AZ1 $\Delta$ T<sup>205</sup> (○), AZ2 $\Delta$ T<sup>97</sup> (●), His<sub>6</sub>ODC (■),  $\beta$ -galactosidase (X), or were uninfected (□), and [ $^3$ H]spermidine uptake then measured.

dine uptake in Sf21 cells infected with AZ-expressing baculoviruses (Fig. 7). Spermidine uptake was measured approximately 48 h post-infection, by which time almost all the cells would have been infected and expressing high levels of AZ protein. Compared with cells infected with a control virus, spermidine uptake was reduced 3–5-fold in AZ1- or AZ2-expressing cells. These data support the conclusion that AZ2, like AZ1, functions as a negative regulator of polyamine pools by diminishing uptake.

#### DISCUSSION

AZ1 is known to have two activities that diminish cellular polyamine levels: it reduces the level of ODC, a key enzyme in polyamine synthesis and inhibits polyamine uptake. AZ1 directly inhibits the enzyme activity of ODC and accelerates its degradation. The degradation depends on ATP and the 26 S proteasome but is ubiquitin-independent. AZ1 activity is induced by elevated polyamines, which induces the +1 translational frameshift. It has now become apparent that multiple copies of AZ genes are present in vertebrates and AZ genes are also found in invertebrates such as *Drosophila* and *C. elegans*. It is not clear, however, whether all AZ family members share the same biochemical functions of mammalian AZ1.

In this paper we compared the biochemical activities of rat AZ1 and human AZ2 and found that they have similar biochemical activities. First, both AZ1 and AZ2 bound to and inactivated ODC. ORF2 was sufficient for binding and inactivation. Second, using the baculovirus system, we demonstrated that ODC protein level in ODC-AZ2-co-infected Sf21 cells was reduced. The result suggested that AZ2 also regulated ODC activity by targeting it for degradation. Third, both AZ1 and AZ2 inhibited spermidine uptake in Sf21 cells. Similarities between AZ1 and AZ2 goes even further, we also demonstrated an AZ2 translational frameshift in Sf21 cells as well as in mammalian cells,<sup>2</sup> which is consistent with the *in vitro* data reported earlier (14). In summary, AZ2, like AZ1, is a negative regulator of polyamine metabolism.

The evidence suggests that all known AZ genes (except perhaps AZ3, whose 5' sequences have not yet been reported) require a frameshift to produce functional full-length proteins. AZ1 frameshifting has been demonstrated both *in vitro* and *in vivo* (3). For AZ2, polyamines enhance frameshifting efficiency *in vitro* (14), and our present results show that frameshifting also takes place in Sf21 cells (Fig. 4) and in mammalian cells (data not shown). The mRNA secondary structure that promotes polyamine-induced frameshifting of rat AZ1 gene is conserved in all known vertebrate AZ genes. The characteristic secondary structure, however, is not apparent in the *Drosophila*

*ila* and *C. elegans* AZ genes (24, 26), which could be interpreted to imply that invertebrate AZ does not use a programmed frameshift. Sequence data strongly suggests otherwise. Although the pseudoknot secondary structure is absent in invertebrate AZ, approximately 20 nucleotides of the mRNA sequences surrounding the frameshift site, including the ORF1 stop codon UGA, are highly conserved for all known AZs (where sequences are available). Indeed, translational frameshifting of *Drosophila* AZ, which lacks the pseudoknot, and its enhancement by spermidine has been demonstrated *in vitro* in wheat germ and rabbit reticulocyte lysate (24).

AZ proteins are most highly conserved in the C terminus where the functional domains required for binding to ODC have been mapped. The N terminus, including all of ORF1 and the initial part of ORF2, is more diverged, and the N terminus, at least for AZ1, is dispensable for all known biochemical functions. We have shown here that ORF1 of AZ2 is not required for ODC binding and inactivation. It is possible that the only evolutionary constraint on the N terminus is that a polyamine-responsive frameshift signal has to be maintained. On the other hand, the N terminus may also contain additional signals for regulating AZ activities. For instance AZ itself could be subject to regulation of proteolysis.

Using a baculovirus expression system, we found that ODC is efficiently degraded when AZ1 or AZ2 is present. We also tested AZ-dependent ODC degradation in rabbit reticulocyte lysate, an *in vitro* system that has been widely used for studying ODC degradation (10, 16, 28, 29). In this system, we found that *in vitro* translated ODC was degraded in the presence of AZ1 ORF2 protein but no degradative activity was detected using AZ2 ORF2 protein. What explains this apparent contradiction between our data from cultured insect cells and the *in vitro* system? There are several possibilities. 1) AZ2 may be capable of directing ODC degradation but does so inefficiently compared with AZ1. Expression at high level in insect cells may produce amounts sufficient to elicit degradation. The data shown in Fig. 6 makes it possible to estimate the minimum difference in relative potency of AZ1 versus AZ2. AZ1 is at least 16-fold more active than AZ2 using this assay. 2) The *in vitro* system lacks components that promote degradative function *in vivo*. AZ1 compared with AZ2 is less dependent on such components or depends on different components that are present in amounts sufficient to evoke activity. 3) AZ2 is more susceptible than AZ1 to loss of activity *in vitro*. 4) AZ2 may fail to become active *in vitro*. For example, AZ2 may not fold properly, or post-translational modification of AZ2 may be required for activating its degradative function.

Ichiba *et al.* (30) have identified a 6-amino acid sequence (113–118, TRVLSI for rat and TRILNV for human) in AZ1, deletion of which maintains the ODC binding/inhibition activity of AZ1 but disrupts its *in vitro* ODC degradation activity. The equivalent sequences present in AZ2 (77–82, PHIVHF) bear no similarity to those in AZ1. If these sequences are indeed critical determinates of AZ1 degradative activity observed *in vitro*, this might explain the observation that AZ2 does not target ODC for degradation. Closer examination of the sequence shows that residues 113–118 are part of a predicted  $\beta$ -sheet secondary structure that follows a long region of predicted loop structure (31, 32). A  $\beta$ -sheet structure following a loop structure is also predicted for AZ2 (Fig. 1). Deletion of 113–118 in AZ1 might have disturbed the overall three-dimensional structure of the AZ protein, rather than removed residues specifically critical for degradative function. The capacity of AZ1 residues 16–112 to confer lability on ODC when grafted to the N terminus of ODC (22) is consistent with this interpretation. It is therefore unclear whether dissimilarity of amino

acids 113–118 will prove to explain the distinctive properties of AZ1 versus AZ2.

The opposite degradative properties of AZ2 we observed in two different systems suggest that this function may be subject to physiologic regulation. AZ2 may have the capacity to reversibly inhibit ODC, and thus provide transient inhibitory regulation or a means to store inactive ODC in a form available for rapid use. AZ2 cDNA was originally described in a screen for seizure-inducible brain mRNAs (13). ODC has been found to be present in adult mouse brain in an inactive complex with antizyme that can be activated on further purification (33, 34). The AZ activity present in mouse brain has been found to be unreactive with a series of monoclonal antibodies reactive with rat liver AZ (34), a result consistent with distinctive tissue distributions of the AZs. If the brain ODC complex contains AZ2, it may represent a stored form of ODC available for activation, perhaps by displacing AZ2 with antizyme inhibitor, a catalytically inactive AZ-binding homolog of ODC (35).

*Acknowledgments*—We thank Sudarsi Desta for technical assistance.

#### REFERENCES

- Hayashi, S., and Cannellakis, E. S. (1989) in *Ornithine Decarboxylase: Biology, Enzymology, and Molecular Genetics* (Hayashi, S., ed) pp. 47–58, Pergamon Press, New York
- Auvinen, M., Paasinen, A., Andersson, L. C., and Hölltä, E. (1992) *Nature* **360**, 355–358
- Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F., and Hayashi, S. (1995) *Cell* **80**, 51–60
- Murakami, Y., Fujita, K., Kameji, T., and Hayashi, S. (1985) *Biochem. J.* **225**, 689–697
- Li, X., and Coffino, P. (1992) *Mol. Cell. Biol.* **12**, 3556–3562
- Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) *Nature* **360**, 597–599
- Murakami, Y., Matsufuji, S., Miyazaki, Y., and Hayashi, S. (1994) *Biochem. J.* **304**, 183–187
- Glass, J. R., and Gerner, E. W. (1987) *J. Cell. Physiol.* **130**, 133–141
- Rosenberg-Hasson, Y., Bercovich, Z., Ciechanover, A., and Kahana, C. (1989) *Eur. J. Biochem.* **185**, 469–474
- Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A., and Kahana, C. (1989) *J. Biol. Chem.* **264**, 15949–15952
- Mitchell, J. L. A., Judd, G. G., Bareyal-Leyser, A., and Ling, S. Y. (1994) *Biochem. J.* **299**, 19–22
- Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S., and Igarashi, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8930–8934
- Kajiwara, K., Nagawawa, H., Shimizu-Nishikawa, K., Ookura, T., Kimura, M., and Sugaya, E. (1996) *Biochem. Biophys. Res. Commun.* **219**, 795–799
- Ivanov, I. P., Gesteland, R. F., and Atkins, J. F. (1998) *Genomics* **52**(2), 119–129
- Matsufuji, S., Matsufuji, T., Wils, N., Gesteland, R., and Atkins, J. (1996) *EMBO J.* **15**, 1360–1370
- Li, X., and Coffino, P. (1994) *Mol. Cell. Biol.* **14**, 87–92
- Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1993) *Genes Dev.* **7**, 555–569
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77**, 51–59
- Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. (1991) *Nucleic Acids Res.* **19**, 1154
- Gupta, M., and Coffino, P. (1985) *J. Biol. Chem.* **260**, 2941–2944
- O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1994) *Baculovirus Expression Vectors*, Oxford University Press, New York
- Li, X., Stebbins, B., Hoffman, L., Pratt, G., Rechsteiner, M., and Coffino, P. (1996) *J. Biol. Chem.* **271**, 4441–4446
- Sakata, K., Fukuchi-Shimogori, T., Kashiwagi, K., and Igarashi, K. (1997) *Biochem. Biophys. Res. Commun.* **238**, 415–419
- Ivanov, I. P., Simin, K., Letsou, A., Atkins, J. F., and Gesteland, R. F. (1998) *Mol. Cell. Biol.* **18**, 1553–1561
- Salzberg, A., Golden, K., Bodmer, R., and Bellen, H. J. (1996) *Genetics* **144**, 183–196
- Wilson, R., Ainscough, R., and Anderson, K. (1994) *Nature* **368**, 32–38
- Steglich, C., and Scheffler, I. E. (1982) *J. Biol. Chem.* **257**, 4603–4609
- Murakami, Y., Matsufuji, S., Tanaka, K., Ichihara, A., and Hayashi, S. (1993) *Biochem. J.* **295**, 305–308
- Li, X., and Coffino, P. (1993) *Mol. Cell. Biol.* **13**, 2377–2383
- Ichiba, T., Matsufuji, S., Miyazaki, Y., Murakami, Y., Tanaka, K., Ichihara, A., and Hayashi, S. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1721–1727
- Rost, B., Sander, C., and Schneider, R. (1994) *Comput. Appl. Biosci.* **10**, 53–60
- Rost, B., and Sander, C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 7558–7562
- Laitinen, P. H., Hietala, O. A., Pulkka, A. E., and Pajunen, A. E. (1986) *Biochem. J.* **236**, 613–616
- Onoue, H., Matsufuji, S., Nishiyama, M., Murakami, Y., and Hayashi, S. I. (1988) *Biochem. J.* **250**, 797–803
- Murakami, Y., Ichiba, T., Matsufuji, S., and Hayashi, S. (1996) *J. Biol. Chem.* **271**, 3340–3342
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680