Structure, organization and expression of the mouse ornithine decarboxylase antizyme gene

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Ornithine decarboxylase antizyme is a protein that participates in the regulation of cellular polyamine levels. In this study we have isolated and sequenced the mouse gene encoding antizyme protein. Transfection of various cell lines with a 5.5 kb genomic fragment containing the antizyme locus resulted in the production of a 29 kDa antizyme protein, confirming that this locus contained a functional gene. Comparison of the mouse gene with the corresponding rat gene [Miyazaki, Matsufuji and Hayashi, (1992) Gene **113**, 191–197] revealed an identical exon/intron organization and high level of nucleotide sequence conservation that was 89% for the entire transcription unit. Protein-coding regions of the two genes exhibited 97% nucleotide sequence identity and there were only four amino acid differences between the 227-residue antizyme protein sequences of the mouse and rat. The promoter of the antizyme gene was functional in mouse (N2A and NIH/3T3) and hamster (CHO) cell lines. The presence of 0.1 mM spermidine in culture medium increased the amount of immunoreactive antizyme protein in cells transfected with the antizyme gene or antizyme cDNA, possibly owing to facilitated frameshifting in the translation of antizyme mRNA. Recombinant antizyme protein was also produced in *Escherichia coli* and used to raise specific polyclonal antibodies in rabbits and to devise immunological methods for the measurement of antizyme concentration.

INTRODUCTION

The polyamines spermidine and spermine, and their precursor putrescine, are ubiquitous components of mammalian cells. They play important roles in cell proliferation and differentiation as well as in neoplasia [1,2]. Cellular polyamine levels are tightly controlled, and ornithine decarboxylase (ODC; EC 4.1.1.17) is one of the key regulatory enzymes in the biosynthesis of polyamines [3]. Antizyme protein, in turn, has at least two known functions in the regulation of cellular polyamine concentrations. First, it binds to ODC and inhibits enzymic activity and recruits this protein for degradation in the 26 S proteasome [4,5], and secondly, it represses polyamine uptake [6,7]. In contrast, elevated polyamine levels stimulate the production of antizyme protein [8] that is, at least in part, due to translational frameshifting of antizyme mRNA, which is facilitated by polyamines [9–11].

Eukaryotic cells also contain a specific inhibitor for antizyme protein, the physiological function of which remains to be elucidated. This inhibitor binds very tightly to antizyme protein and bears a high degree of amino acid sequence similarity to ODC [12]. Moreover, antizyme seems to have important biological functions unrelated to the regulation of polyamine metabolism. For example, antizyme protein might be involved in the regulation of degradation of short-lived proteins other than ODC [13,14], and a *Drosophila* gene encoding an antizyme-like protein, the gutfeeling protein, is required for proper development of the embryonic peripheral nervous system [15]. In the present study we have elucidated the organization and the sequence of the mouse antizyme gene as the first step towards achieving genetic tools to understand better the regulation and functions of this intriguing gene and the encoded protein. During the course of these experiments we also developed a relatively convenient immunosorbent assay to quantify antizyme protein from biological samples.

EXPERIMENTAL

Isolation and sequencing of mouse antizyme cDNA and the chromosomal gene

Mouse antizyme cDNA was isolated from poly(A)$^+$ RNA of mouse hepatoma cells (BW-1 cells) by reverse transcriptase–PCR. The forward (5′-CGGAATTCATGGTGAAATCCTCCCTG-CA-3′) and reverse (5′-CGGAATTCCTAGTCCTCCTCACC-CGGGTCCTC-3′) primers contained *Eco*RI restriction sites at the 5′ ends and corresponded to the sequences at the beginning and the end of the open reading frame (ORF) of rat antizyme mRNA [16]. The PCR product was subcloned into the *Eco*RI site of pGEM-3Z (Promega, Madison, WI, U.S.A.) and sequenced.

Mouse genomic library (129SVJ; Stratagene, La Jolla, CA, U.S.A.) was screened with ^{32}P-labelled antizyme cDNA as the probe, under standard conditions [17]. Three positive clones were identified and plaque-purified; phage DNA was isolated and purified [18] and analysed by restriction enzyme digestion as well as by Southern blotting with ^{32}P-labelled antizyme cDNA as the probe. Restriction enzyme mapping and partial nucleotide sequencing revealed that two of the three genomic clones corresponded to intronless antizyme pseudogene sequence(s), whereas the third (designated gAZ-1) contained exonic and intronic sequences. A 5.5 kb *Eco*RI fragment of gAZ-1 was subcloned into the *Eco*RI site of pGEM-3Z; the resulting plasmid was termed pGEM-gAZ. The 5.5 kb insert was digested with

Abbreviations used: CHO, Chinese hamster ovary; ODC, ornithine decarboxylase; ORF, open reading frame.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U84291.

various restriction enzymes, and the cleaved fragments were subcloned into pGEM-3Z for nucleotide sequencing.

DNA sequencing was performed with Pharmacia ALFexpress sequencing system (Pharmacia Biotech, Uppsala, Sweden) with universal and specific primers. For both antizyme cDNA and gAZ-1, sequencing was performed on both strands.

cDNA expression constructs

The *Eco*RI site at the 3′ end of antizyme cDNA was replaced by a *Sal*I site by PCR, and the resulting 685 bp *Eco*RI–*Sal*I fragment of antizyme cDNA containing the entire protein-coding region was cloned into the corresponding site of pcDNA3.1 vector (Invitrogen, San Diego, CA, U.S.A.). This construct was termed pcDNA3.1-wtAZ. An expression vector containing the specific in-frame mutation (pcDNA3.1-ifmAZ) was assembled by using site-directed mutagenesis with PCR [19]. In this in-frame mutant construct, the T at position 205 was deleted, which resulted in the conversion of the in-frame stop codon (UGA) to a UGG codon. As a consequence, translation of the entire sequence coding for wild-type antizyme protein (residues 1–227) was possible without the need for frameshifting [9,10]. A fragment of antizyme cDNA coding for the N-terminal region until the in-frame stop codon (residues 1–68, termed here ORF1) was made by PCR with *Eco*RI and *Sal*I restriction sites at the 5′- and 3′-ends respectively and cloned into the corresponding site of pcDNA3.1. The resulting expression vector was termed pcDNA3.1-ORF1.

Cell culture and transfection

NIH/3T3 (mouse fibroblasts) and CHO (Chinese hamster ovary) cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.), and N2A cells (mouse neuroblastoma) were kindly provided by Dr. Eero Castrén (University of Kuopio, Kuopio, Finland). The cells were grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum (CHO and N2A cells) or calf serum (NIH/3T3 cells) with 1 mM aminoguanidine [20]. Tissue culture dishes (60 mm; 10^6 cells per dish) were transfected by the calcium phosphate precipitation method with 10 μg of plasmid DNA (pGEM-3Z, pGEM-gAZ, pcDNA3.1, pcDNA3.1-wtAZ, pcDNA3.1-ifmAZ or pcDNA3.1-ORF1). The precipitates were removed 24 h after transfection and the cells were provided with fresh medium with or without 0.1 mM spermidine. The cells were harvested 24 h later and soluble extracts were prepared for measurement of antizyme production.

Production of antizyme proteins in *Escherichia coli*

The in-frame mutant antizyme cDNA sequence was used to express the wild-type antizyme protein. A 684 bp fragment with *Sph*I–*Sal*I sites was cloned into the corresponding site of pQE-30 vector (Qiagen, Hilden, Germany), and the His_6-tagged full-length antizyme protein was expressed in *E. coli* strain M15 (Qiagen). The expressed protein was purified from bacterial cells by Ni^{2+}-nitrilotriacetic acid affinity chromatography. Briefly, cells from a 1 litre culture were collected by centrifugation and broken by sonication in 50 ml of 50 mM Tris/HCl (pH 8.0)/0.3 M NaCl/2 mM 2-mercaptoethanol. The sonicate was clarified by centrifugation at 40000 ***g*** and the supernatant was incubated with 2 ml of Ni^{2+}-nitrilotriacetic acid matrix for 60 min at 4 °C with continuous rotation. The matrix was washed successively with sonication buffer containing 0.1 % (v/v) Tween-80 alone, or together with 1 M NaCl and 40 mM imidazole. The His_6-tagged antizyme protein was released from the matrix with elution buffer composed of 250 mM imidazole, 50 mM Tris/HCl, pH 8.0, and 0.1 % Tween-80. Fractions (1 ml) were collected; those with the highest concentration of antizyme were pooled.

Purified antizyme protein was used for raising antibodies in rabbits (50 μg of protein per immunization at intervals of 4 weeks) and as a standard in the ELISA for antizyme. Sequences containing separately the two ORFs of antizyme, ORF1 (amino acid residues 1–68) and ORF2 (amino acid residues 69–227), were cloned as *Eco*RI–*Sal*I fragments into the corresponding site of pGEX-5X-1 vector (Pharmacia Biotech); the resulting GST fusion proteins were expressed in *E. coli* strain BL21 (DE3). GST–ORF1 and GST–ORF2 proteins were purified by glutathione–Sepharose (Pharmacia Biotech) affinity chromatography, and 2 mg of purified fusion proteins was immobilized on HiTrap *N*-hydroxysuccinimide-activated Sepharose (Pharmacia Biotech) columns (1 ml) in accordance with the manufacturer's instructions. These matrices were used for the purification of ORF1- and ORF2-specific antibodies respectively. Portions of ORF1- and ORF2-specific antibodies were biotinylated with sulpho-succinimidyl-6-(biotinamino)-hexanoate (Pierce, Rockford, IL, U.S.A.) in accordance with the manufacturer's instructions. ORF1-specific antibodies recognized GST–ifmAZ and GST–ORF1 proteins, but not GST–ORF2 protein on immunoblots, whereas ORF2-specific antibodies detected GST–ifmAZ and GST–ORF2 proteins, but not GST–ORF1 protein.

ELISA of antizyme protein

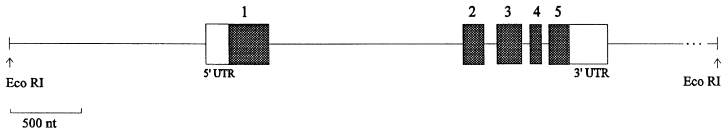
To establish a specific method for measurement of wild-type antizyme protein concentration, ORF1-specific antibodies (3 μg/ml, 100 μl/well) were used for coating of Cliniplate microtiter plates (Labsystems, Helsinki, Finland) and biotinylated ORF2-specific antibodies (1 μg/ml, 100 μl/well) as the secondary antibody. Biotinylated antibodies were detected with avidin–horseradish peroxidase conjugate (0.1 μg/ml, 100 μl per well; Pierce) with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) as the substrate.

Cultured cells were washed twice with PBS [150 mM NaCl/10 mM sodium phosphate (pH 7.4)] and collected in 1 ml of ice-cold PBS containing 2 mM EDTA/0.4 mM PMSF/0.02 unit/ml aprotinin. The cells were centrifuged for 5 min at 320 ***g*** and resuspended in 150 μl of the same buffer. The cells were lysed on ice by the addition of 150 μl of ice-cold lysis buffer [final concentrations: 0.1 M Tris/HCl, pH 7.4, 1 M NaCl, 1 % (v/v) Nonidet P40, 10 mM putrescine and protease inhibitors] and the incubation was continued on ice for 30 min. Then 750 μl of ice-cold 3 % (w/v) BSA in PBS with protease inhibitors was added and the samples were vortex-mixed and clarified by centrifugation for 5 min at 14000 ***g***. Aliquots from the clear supernatant were applied to a microtitre plate (100 μl per well). His_6-tagged antizyme protein standard was diluted in buffer with the same composition as that present in cell extracts and was used in the concentration range 0.02–2 ng/ml, 100 μl per well.

Values in ELISA that exceeded the mean blank signal by 3 S.D. were regarded as positive. The sensitivity of the antizyme-specific ELISA was 20 pg/ml and the linear measurement range was 20–200 pg/ml. When ORF1-specific antibodies were used for both coating and detection (biotinylated ORF1 antibodies) the sensitivity of the assay was about one-tenth of that obtained with the combined use of ORF1- and ORF2-specific antibodies as delineated above.

Immunoblotting

Soluble cell extracts were prepared and diluted in 3 % (w/v) BSA/PBS buffer as described for ELISA of antizyme protein.

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-1404  CCAAAGGTCC TGAGTTCAAT TTCCAGCAAC CACGTGGTGG CTCACAACCA TCTATAATGG GATCTCACAC CCTCTTCTGG TCTGTGTCTG AAGACCAGAA
-1304  ACAACAACAG TGTACTCATA TAAATAAAAT AAGTAAATCT TGAGAGAGAG AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG
-1204  AGAGAGAGAG AGAGAGAGAG CAGGGTGTGG TGCACAGGCC TTTAATCCCA ACACTCAGCT GGCAGAGTCA GGCAGATCTC TGAGTTTGAG GGTAGCTTAG
-1104  TTTATAGCGT AAGTTCCAGG ACATCCAGGG CTACACAGAA AAGCCCTGTC TCAAAAACAA AACATACAAA ACAAAACAAA ACAACAACAA CAAAAAACCC
-1004  ATAAAGAGAA AAAGAATACG AAATTCTTTA AGAATAAATT GTAAGCAGGG CAGTGATGGC GCACGCCTTT GATCCCAGCA CTTGGGAGGC AGAGGCAGGC
 -904  AAACAGAGAA ACCCTGTCTC AAAAAACAAA AATCAAAATA CAACAACAAA AAGCGGCGGC TGCGGGGGAA GGGGGGTTGG GATGAAGGAA ACCTTCAAGC
 -804  TGAGTCTATG AGAAAAGTTG TTTAAATTCC AATTTGACAT AAAATACTTC TCTAGATTGG GATACTTAAC TTTTGACAGA TTTTCTTTTA AATAGCAAGT
 -704  TGCTTTTAAA ACAAAGAGTC CCAATCTATT ACCCATTATT ACAAGGTCAT TAGAATAATT TTGCCCGCGT GGGGGATGGG CACCAAGCAA ACCGCCTAGG
 -604  TCAGCCTCAG GTACCCTTGA CAGTTATGTC CTGACAGTCT TCCCGGAGCG GGGCGTCCCA TGAGTGGGAC CATAATGGTT AAGATGGAAA GGATGACAGA
 -504  TGACAAAACT GAAGGGCCAC AGGTACTAGA GGAGGCGCAG AGTCCTAGCC TTGAGGCCTG GGCTAGATGG GGGTTTACGT CCTGGAGGCG ACTACAGAAG
 -404  CCCCGCCTCC GCCTCACGTC ATAAAGCCTC GAGGGCGGGC TTGTCAGAAC TCCAACCAAT CCTCAAAGGG GCCAATACGT CCGTTTTTCT ACATTGACCG
 -304  ATCAGAGCCT TGTAGGGGCG GGACGCGTCT GGCTTTTCTG TCTTCCCTCC CACAGAGTGA CGCAAACACG CGGGGCCGGG ATTCTCCTAT CGCCAACTAA
 -204  TCATCAACGG AAACCTTCTG TCTGTCTTTT TACTCTCGCC AATCAGAAGC CGGAGTAGGT GGGACGCATT TGGTTTTTCC TTGGGCTGCC ACTGTGGCCC
 -104  CACCCCGTTC CTCCGTCGCC TCACACTGCT GCGTTTACGC CTGCGTGTGG GGGCGTGGCC TGCCGTGACG ACGCATATAA AAGTGGGCGT CGGCGCCGGC
   -4  GCCATTTTGC GAACGGCGAG CAGCGGCGGC GGCGCGGGAA GTGCAGCGGA GGTTTTTGCT GGTTTCGGAC CCCAGCGGCC GGATGGTGAA ATCCTCCCTG
                                                                                                  M  V  K   S  S  L
    96  CAGCGGATCC TCAACAGCCA CTGCTTCGCC AGAGAGAAGG AAGGGGACAA ACGCAGCGCC ACGCTTCACG CCAGCCGCAC CATGCCGCTT CTTAGTCAGC
          Q  R  I   L  N  S  H   C  F  A   R  E  K   E  G  D  K   R  S  A   T  L  H   A  S  R  T   M  P  L   L  S  Q
   196  ACAGCCGCGG CGGCTGCAGC AGCGAGAGGT GAGAGCCCTG CCCCCGCCGC CGCGAACCTT CCAGAAACAG CGTCCGCGTA GGCCCCAGAG GGCAGCGCAG
          H  S  R  G   G  C  S   S  E
   296  GACGCGCAGG CCCGGAGCGG GGTCGGCCTC CGGCGCCAGA TAAGTCTCGG CAGCGCGAGA TCCGCCCCTT TGTGGCGGCC ACAATCGCGC AGGGGGCGGG
   396  GCGGGGCGGG GCGGGGCCTG TGGAGCGGGT CGCGGACTCG GATTTGGGAT TCGAGTGGAA TTAGGGAGTC TCAGGTTGAG GAATAGGGTT TGGTTTGATT
   496  GGAGGACCGG GAACATTTCG CGAAAATGTA GGATTCGATT TTGGGTTCGA ATCGGATTCG GGGGTTGTGA GTTGAGCCTA AGATTTGGGG AGGGATCTGA
   596  GAAGAGATTG GAGACTCGAA TTTGGGGTCT GCCTCTCGGA TATGTGAGCT AGTTCTAATG TCAGGTGGCG AAGTGTTTGT GGTTACCTGT AGGGCGTTTG
   696  GGTAATAAAT CGTATGCTAT GTAGAGGTGG GGCTCGTCCC GGTGTTTGGA GTTCTTTGCA GAATACTGGG ATTTTGGGTG CTGTTGGGGA GGAATCCTAT
   796  TATATTCTTG GGTACATGTT GAGGACTCAT TTCTTGGTGG GAGAGTATGG GATCAGATTT GGGGTGTTGA GAAAGAAGCT TGTGTCGGTT TAGGAAGTCA
   896  CAGGTGGGGA AGCTGGGATG AGGTTTAGGT TGGATTTAGT GATTGCGTGT TGAGGGCTCT AATACTTTGG AGGGATTTGG GTCAGAATTA GGACCTTGGA
   996  AGTGGTCAGG CTTGGGGGCG TCAACAGGAG GCTATGTGTG AGAGGACATG GAGGTGGTGT GGTGTGGTGA GTATGATGTG AGGTGACCTT AATCCCTGGT
  1096  CCACTTGGCA GTTAAAAGTC TGCAGTGGGT GGGATGGAGG GTATCACTTG AAGGGTTCAG AATGGGGACT GGTTTGGGAA GGGCGTCGAT ATAGGTGGTA
  1196  GGAACTTTGG ATATAGGCTG TGCAGACACC TATAGAGTGG CAGTAGGGAA GAGAGGAGAC TTGGGGTTGT CTGACTCTAG AGCATGAGTA GTTAGTGAGG
  1296  TAGAACTTTG GGGTCACTGG TAGCTGCTGA CCAGAGGGTA GGAAGGAGCG AGTGACCTGG TGTGAAGGGA GGATTGGCAG CATGGAGTGG GATGGGCCAC
  1396  AGTGCTGAGA TGACACTGCC TGTGAGGCCT GTCTTGCTAC AAAAGGGGCA GAGTCACACA GGTGAGGGGC AGTGACTCGG GTTGACTGTT GTCAATGGCT
  1496  CTTGAGTGGG AAGCCCGGGC ACTTAATTAT TGCCGCTGCT GCCACCTGAG CATTGTTTCC TGCAGTTCTA GGGTTGCCCT TAATTGCTGT AGTAACCTGG
                                                                          S  S   R  V  A  L   N  C  C   S  N  L
  1596  GTCCGGGGCC TCGGTGGTGC TCCTGATGTC CCTCACCCAC CCCTGAAGAT CCCAGGTGGG CGAGGGAACA GTCAGCGGGA TCACAGTCTT TCAGCTAGCA
          G  P  G  P   R  W  C   S  *D  V   P  H  P   P  L  K  I   P  G  G   R  G  N   S  Q  R  D   H  S  L   S  A  S
  1696  TCCTGTACTC CGTGAGTACC TGTGGAGAGC CCGGGGGTGC CTGGCACTGG CAAAGTAAGC TGGCCAACCT GACAACCCTG CTCCCCTAGG ACGAGCGGCT
          I  L  Y  S                                                                                      D  E  R  L
  1796  GAATGTGACA GAGGAGCCGA CGTCTAACGA CAAGACCAGG GTCCTGAGCA TCCAGTCCAC GCTCACGGAG GCCAAGCAGG TCACCTGGAG GGCGGTGTGG
          N  V  T   E  E  P   T  S  N  D   K  T  R   V  L  S   I  Q  S  T   L  T  E   A  K  Q   V  T  W  R   A  V  W
  1896  AGTGGTGGTG GCCTCTACAT CGAGCTCCCA GCTGGACCTC TGCCTGAGGG CAGTAAGGAC AGGTAGGCCA CTTCCCCTTA GGGCTGTAGG GGATTTGGGG
          S  G  G   G  L  Y  I   E  L  P   A  G  P   L  P  E  G   S  K  D  S
  1996  CCTGGGCACA GGTGGCTAAT GACTCCATGC TTCATTCAGT TTTGCAGCTC TCCTAGAGTT CGCAGAGGAG CAACTCCAGG CTGACCACGT TTTCATCTGC
                                                  F  A  A   L  L  E  F   A  E  E   Q  L  Q   A  D  H  V   F  I  C
  2096  TTCCCCAAGA ACCGTGAGGA CAGAGGTTGG TATTGCTTGG GAGGGTCCTG GGGAGGGGAG TTCTGGCACC CCAAACTCTC ACCCTATGTT CTCCAAAGCC
          F  P  K   N  R  E  D   R                                                                                A
  2196  GCCCTACTCC GAACCTTCAG CTTTCTTGGC TTTGAGATTG TGAGACCCGG GCATCCCCTT GTCCCCAAGA GACCCGACGC TTGCTTCATG GTCTACACTC
          A  L  L   R  T  F  S   F  L  G   F  E  I   V  R  P  G   H  P  L   V  P  K   R  P  D  A   C  F  M   V  Y  T
  2296  TGGAGAGAGA GGACCCGGGT GAGGAGGACT AGGTGCCAGC CCTGCCCAGT GTCCCTGTGC CCTCTCCTGG GTTAGTCCAC ATGTCGTGAT TGTGCAGAAT
          L  E  R  E   D  P  G   E  E  D   *
  2396  AAACGCTCAC TCCATTAGCG GGGTGCTTCT TCGAGCTGAA TGCTGTGTTT GTCACACTCA AGTGTTGGCT TTAATTCTAA ATAAAGGTTT CTATTTTACT
  2496  TTTTTATTGC TGTTTAAGAT GGTCAGGTGA CCTATGCTAT AGCAGTCTCC TTTGAAGTCT GGAAAAATAG TGTCACCTCC CCTGGCTCAA ATCCAATAAA
  2596  GTGATCTCGT TCATTGGCTG TGTCTTGTGT CCTGCTGCTG GGTCTAGGTT GGGCCCCTGG TAGCTATGGC CCAGAGAGGG TTCAGTCACT CCTGAGTCTA
  2696  TGCTTAAATG GGTGGTAGGG CTGGGGTGTT GTGGACATTT GAGAAGCTA
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Figure 1 Organization and nucleotide sequence of the mouse antizyme gene and deduced amino acid sequence

Upper panel: organization of the gene. Exon and intron sequences are specified by boxes and solid lines respectively, and the protein coding parts in the exons are shaded. The length of the *Eco*RI–*Eco*RI fragment was 5.5 kb; the region depicted by dots in front of the 3′ *Eco*RI site was not sequenced. 5′UTR and 3′UTR refer to 5′- and 3′-untranslated regions respectively. Lower panel: nucleotide sequence of the antizyme gene. Exons and introns are depicted in bold and regular characters respectively. The amino acid sequence is written under the transcribed nucleotide sequence with single-letter codes for amino acids. In the sequence given, the +1 frameshift is predicted to take place and yield the 227-residue antizyme protein. The stop codons at the frameshift site and the end of the protein-coding region are underlined and marked with asterisks. Three potential polyadenylation signals (AATAAA) are underlined with a bold line. Putative DNA motifs for binding of transcriptional regulatory proteins are depicted as follows: c-Myc–Max protein complex (double underlining), Sp1 (single line) and RelA (dotted line).

Samples (1 ml) of diluted cell extracts were incubated overnight at 4 °C with 1 μg of ORF1- or ORF2-specific antibodies and 20 μl of Protein A–Sepharose (Pharmacia Biotech). Resin-bound protein was collected by centrifugation and the matrix was washed once with 2 ml of PBS containing 0.05 % Tween-20. The samples were separated by SDS/PAGE with Tris/glycine [21] or Tris/Tricine [22] buffer systems. Proteins were transferred to Immobilon-P filters (Millipore, Bedford, MA, U.S.A.) and antizyme protein was detected by biotinylated ORF1- or ORF2-specific antibodies (0.5 μg/ml) and avidin–horseradish peroxidase (2.0 μg/ml). The filters were stained with diaminobenzidine (Sigma, St. Louis, MO, U.S.A.) as the substrate.

Other methods

ODC activity was determined essentially as described by Jänne and Williams-Ashman [23]. Extracts from mouse lymphocytic leukaemia cell line L1210 were used as the source of ODC. One unit of ODC activity was defined as the amount of enzyme catalysing the release of 1 nmol of CO_2 from L-ornithine in 60 min at 37 °C. One unit of antizyme activity was defined as the amount of protein inhibiting one unit of ODC activity. Protein concentration was determined with Bio-Rad (Richmond, CA, U.S.A.) protein assay reagents.

RESULTS

Organization of the mouse antizyme gene

Screening a mouse genomic DNA library with antizyme cDNA revealed that, in addition to the active antizyme gene, the mouse genome contains at least one intronless antizyme pseudogene that was, however, not characterized any further. The entire mouse antizyme gene was contained within a 5.5 kb *Eco*RI fragment, all of which was sequenced except the end of the 3′-flanking region (Figure 1). Exon/intron organizations of the mouse and rat antizyme genes were identical (Figure 1, upper panel) [16], and exon 5 of the mouse gene also had three polyadenylation signals. Comparison of the mouse (Figure 1,

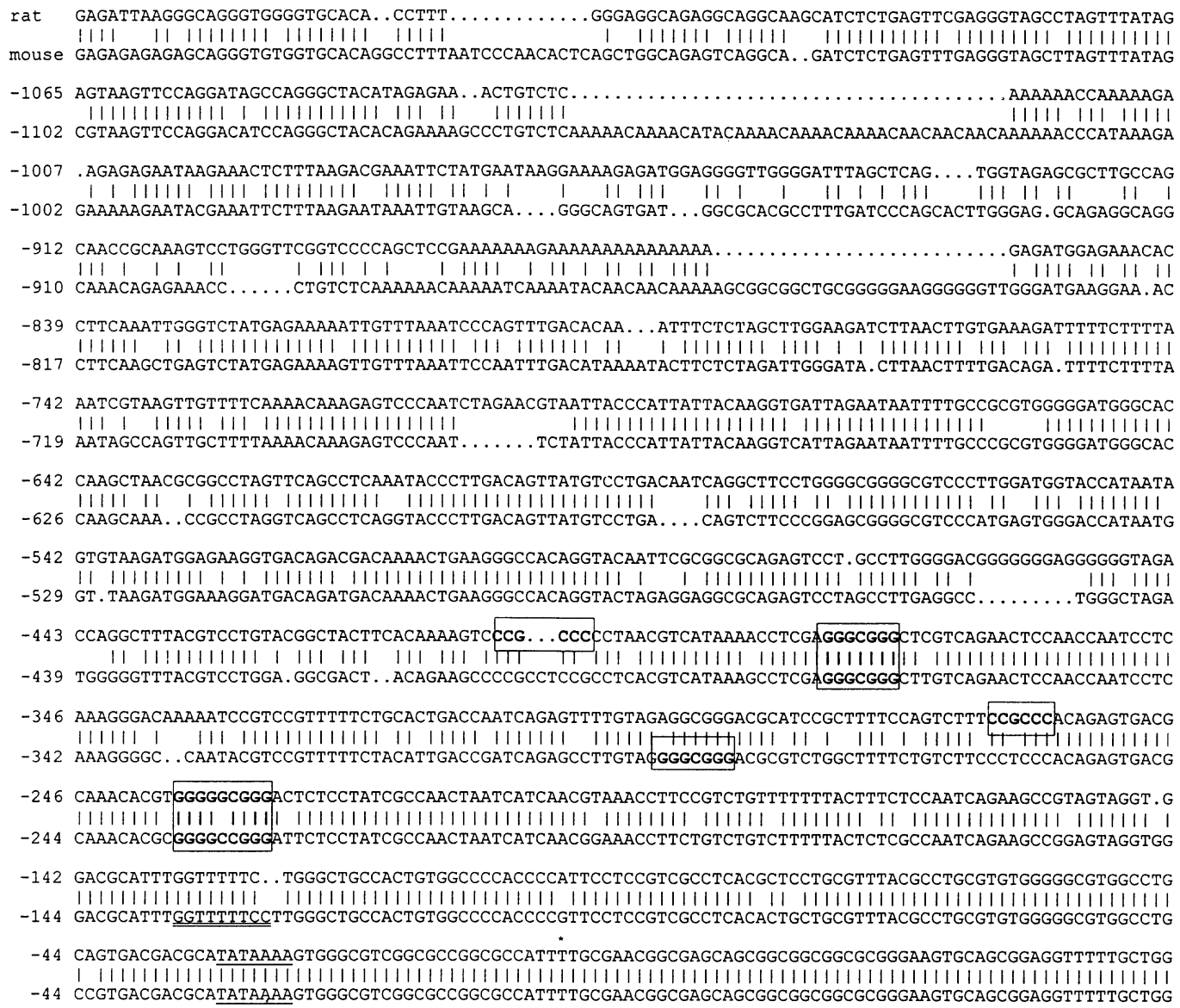

Figure 2 Comparison of promoter regions of the rat and mouse antizyme genes

The BESTFIT program [24] was used to compare rat (upper row, [16]) and mouse (lower row) 5′ flanking sequences. The comparison was made from the transcription start site (depicted by an asterisk) to nt −1200 of the mouse sequence. Potential binding sites for Sp1 transcription factor are framed. The TATA box and the sequence element for a putative RelA-binding site in the mouse sequence are identified by single and double lines respectively.

lower panel) and rat [16] sequences indicated that they are very highly conserved: 89% sequence identity was calculated by the BESTFIT program [24] for the entire transcription unit, from the start site of transcription to the third polyadenylation signal.

Proximal promoters of rodent antizyme genes were also extremely well conserved and exhibited over 90% sequence identity up to nt −400 of the mouse gene, after which there were several dissimilarities, insertions and deletions in both sequences, between the two promoters (Figure 2). The promoter region contained a TATA box sequence (present 24 nt from the transcription start site) along with several DNA motifs, some of which were shared between the mouse and rat promoters, such as those for Sp1 (−226, −284, −366) and RelA (−126) binding. A number of other potential transcription factor-binding sites were also detected. For example, a potential c-Myc/Max-binding element (CACGTG) was found approx. 1.3 kb upstream of the start site of transcription (−1366/−1379; Figure 1, lower panel). A long repetitive element is present in the mouse 5′-flanking region, between nt −1185 and −1292, and it contains 39 GA dinucleotide repeats.

Comparison of nucleotide and deduced amino acid sequences of antizyme between different species

There is a 97% nt sequence identity between mouse and rat antizyme cDNA species (Figure 3). Comparison of mouse (this study), rat [16], human [25] and *Xenopus* [26] antizyme mRNA sequences revealed that nucleotides necessary for frameshifting and the formation of the pseudoknot structure (nt 193–269) were highly conserved (Figure 3), implying that a ribosomal frameshift

Figure 3 Alignment of the cDNA sequences for mouse, rat, human and frog antizyme

The mouse antizyme sequence is used as the reference, and only nucleotides different from those in the mouse sequence are indicated (–, identical nucleotide; dot, a gap in the nucleotide sequence). The initiation (ATG) and stop (TAG) codons are underlined. The conserved pseudoknot structure is highlighted and framed. The arrow identifies the T at nt 205 that was deleted when the expression vector for the in-frame mutant antizyme was assembled.

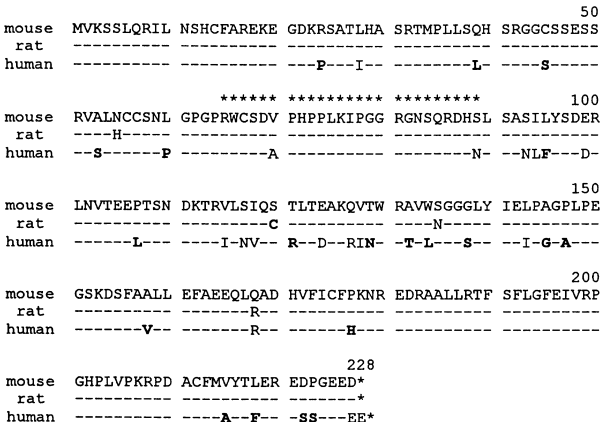

Figure 4 Comparison of the deduced amino acid sequences for mouse, rat and human antizyme proteins

The mouse sequence is used as the reference for comparisons. The deduced amino acid sequence is written with the assumption that +1 frameshift occurs in each species. Only residues different from the mouse sequence are shown, with the conserved and non-conserved changes in regular and bold typefaces respectively (–, identical residue). Amino acids encoded by the pseudoknot structure are identified by the string of asterisks above the mouse sequence.

occurs in the translation of all these antizyme mRNA species. In contrast, comparison with *E. coli* antizyme showed no sequence similarity.

Comparison of mouse and rat antizyme protein sequences showed 98% amino acid sequence identity, with only four residues out of 227 being different (Figure 4). In addition, only one of the four differences was a non-conservative substitution. There were 35 differences between mouse and human antizyme proteins, of which 20 were non-conservative substitutions. Moreover, the human sequence contains 228 amino acid residues, as opposed to 227 residues in the mouse and rat antizyme protein (Figure 4). If this sequence conservation proves to have any bearing on functional characteristics, then the regions 1–120 and 150–210 of antizyme proteins are the most important domains of the molecule.

Expression of mouse antizyme protein in *E. coli*

Antizyme cDNA was generated by reverse transcriptase–PCR with mRNA from a mouse hepatoma cell line (BW-1 cells) as the starting material. The first ORF of mouse antizyme cDNA (ORF1, residues 1–68) terminates at codon 69 (TGA) from the first ATG. To produce a full-length antizyme (molecular mass 29 kDa), the T at nt 205 was deleted, which removed the stop codon from the end of ORF1. In the terminology of this paper, ORF2 stands for the region from the stop codon of ORF1 (nt 206) to nt 685 (the stop codon) and includes amino acid residues 69–227 of antizyme protein.

Mouse antizyme protein with an N-terminal His_6 tag was expressed in *E. coli* by using the frame-shifted mutant antizyme cDNA. The expressed protein was purified to homogeneity by Ni^{2+}-affinity chromatography and appeared as a single band with a molecular mass of 31.5 kDa when analysed by SDS/PAGE (results not shown). The purified protein inhibited ODC activity with a specific activity of 1.1×10^6 units/mg of protein. Recombinant antizyme protein was subsequently used to raise antibodies in rabbits and to establish a specific ELISA for the measurement of antizyme protein concentration from biological samples, as described in detail in the Experimental section.

Expression of the mouse antizyme gene in transfected cells

To test the authenticity of the isolated antizyme gene, CHO, N2A and NIH/3T3 cells were transfected with the pGEM-gAZ

Table 1 Transient expression of the mouse antizyme gene in various cell lines

The indicated cell lines were transfected with pGEM-gAZ (10 μg of DNA) and grown in the absence (−) or presence (+) of 0.1 mM spermidine. The antizyme concentration in the cells was determined by ELISA (see the Experimental section). The results are expressed as means ± S.E.M. for three (N2A cells) independent experiments or as mean values of two (CHO and NIH/3T3 cells) independent experiments, performed and assayed in triplicate.

Cell line	Construct	Spermidine	Antizyme content (pg/mg of protein)	Stimulation by spermidine (% of control)
N2A	pGEM	−	59 ± 5	100
		+	168 ± 27	285
	pGEM-gAZ	−	255 ± 63	100
		+	482 ± 10	189
CHO	pGEM	−	52	100
		+	316	608
	pGEM-gAZ	−	621	100
		+	1460	235
NIH/3T3	pGEM	−	40	100
		+	78	195
	pGEM-gAZ	−	5150	100
		+	5880	114

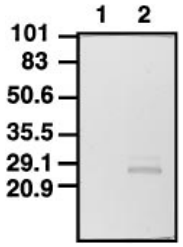

Figure 5 Immunoblot analysis of antizyme protein expressed in NIH/3T3 cells

The cells were transfected with pGEM (lane 1) or pGEM-gAZ (lane 2). Antizyme protein was immunoprecipitated from cell extracts with ORF1- and ORF2-specific antibodies (see the Experimental section). The proteins were separated by SDS/PAGE and transferred to Immobilon-P filters; the antizyme protein was then detected by using biotinylated ORF1- and ORF2-specific antibodies.

construct, along with an empty plasmid as control, and antizyme protein concentration was measured by ELISA specific for antizyme. Antizyme concentration in these cell lines before transfection was in the range of 50 pg/mg of protein (Table 1). Expression of the transfected antizyme gene increased the amount of immunoreactive antizyme protein by 4.3–129-fold, depending on the cell line, with the expression being highest in NIH/3T3 cells (Table 1). The presence of spermidine (0.1 mM) in the culture medium increased antizyme concentration 2–6-fold in non-transfected cells, whereas the increase was somewhat smaller in cells transfected with the antizyme gene (Table 1). In particular, only a marginal increase in response to spermidine was seen in NIH/3T3 cells for reasons currently unknown.

One main band with a molecular mass of 29 kDa was detected on immunoblots from extracts of NIH/3T3 cells transfected with the antizyme gene (Figure 5). There was also another, much weaker, immunoreactive band at approx. 31 kDa. We do not know whether this heterogeneity has any bearing on the use of two different AUG codons in antizyme mRNA as translation initiation codons [9,10]. A mixture of ORF1- and ORF2-specific antibodies was used to generate the results shown in Figure 5.

Table 2 Transient expression of various antizyme cDNA forms in N2A cells

N2A cells were transfected with various antizyme cDNA constructs in pcDNA3.1 vector containing the CMV promoter. The cells were grown in the absence (−) or presence (+) of 0.1 mM spermidine. The antizyme concentration of the cells was determined by ELISA (see the Experimental section). The results are expressed as means ± S.E.M. for three independent experiments performed and assayed in triplicate.

DNA construct	Spermidine	Antizyme concentration (ng/mg of protein)
pcDNA3.1 (vector alone)	−	0.034 ± 0.002
pcDNA3.1-ORF1	−	0.044 ± 0.011
pcDNA3.1-wtAZ	−	0.608 ± 0.231
	+	1.99 ± 0.276
pcDNA3.1-ifmAZ	−	16.2 ± 1.99

Identical results were obtained by using only ORF1- or ORF2-specific antibodies (results not shown).

To gain more insight into the nature of antizyme promoter and the efficacy of frameshifting in transfected cells, N2A cells were also transfected with antizyme cDNA constructs in pcDNA3.1 vector driven by the human cytomegalovirus (CMV) promoter. Transfection with pcDNA3.1-wtAZ yielded an 18-fold increase in antizyme concentration, and provided that transfection efficiencies were similar, the CMV promoter was 2–3-fold stronger than the native antizyme promoter present in pGEM-gAZ construct (compare Tables 1 and 2). Spermidine (0.1 mM) in the culture medium increased antizyme production by 3.3-fold, a magnitude similar to that in N2A cells transfected with antizyme gene sequences (pGEM-gAZ). When the frameshift was introduced into antizyme cDNA (pcDNA3.1-ifmAZ construct), the expression level of antizyme protein was 27- or 8-fold higher than that achieved by transfection with pcDNA3.1-wtAZ in the absence or presence of 0.1 mM spermidine respectively (Table 2).

Immunoreactive antizyme concentration was not significantly increased when a vector encoding only ORF1 was used in these experiments (Table 2). Furthermore no signal over background was detected from these samples even when ELISA was performed by using ORF1-specific antibodies for coating and biotinylated ORF1-specific antibodies for detection (results not shown).

DISCUSSION

The mouse antizyme gene is organized in a fashion identical with the corresponding rat gene, and the encoded proteins are remarkably well conserved. In fact, the conservation of antizyme protein sequence between different species is as good as or better than that of ODC [27] or *S*-adenosylmethionine decarboxylase [28], two key enzymes in polyamine biosynthesis. The proximal promoters of mouse and rat antizyme genes were also highly similar. These findings are interpreted as meaning that the antizyme gene encodes a biologically important protein, the regulation of which at different levels is controlled by conserved mechanisms.

The natural antizyme gene was used for transfections of various cell lines to examine whether its promoter is functional and strong enough to support antizyme production in transfected cells. Antizyme concentration was significantly elevated in all three cell lines tested, indicating that the cloned gene represents the authentic and functional mouse antizyme gene. The fact that

the gene was driven by its own promoter in different cell types of hamster (CHO) and mouse (NIH/3T3 and N2A) origin might reflect the conserved structure of the antizyme promoter. The significance and function of various putative DNA motifs for transcription factor binding and of the long repetitive sequence in the 5′-flanking region remain to be elucidated.

Full-length antizyme with an N-terminal His_6 tag was expressed in *E. coli* by using pcDNA3.1-ifmAZ. The higher molecular mass of the recombinant protein (31.5 kDa) than that of the authentic 29 kDa antizyme protein is explained by the six His residues and eight additional N-terminal residues encoded by the expression vector. The recombinant antizyme inhibited ODC activity; its specific activity was in the same range as that of antizyme extensively purified from rat liver [29] or that expressed in *E. coli* by using rat cDNA [8].

The expression of antizyme in N2A cells by using pcDNA3.1-wtAZ was increased by spermidine in the culture medium, indicating that frameshifting was stimulated. Even in the presence of spermidine, antizyme concentration was only approx. 2 ng/mg of protein, which was still one-eighth of that measured in N2A cells transfected with pcDNA3.1-ifmAZ. In view of these results, the efficacy of frameshifting in N2A cells transfected with the wild-type antizyme expression vector can be calculated to be 10–15% of maximal. This result is consistent with studies evaluating the effect of spermidine on frameshifting efficacy using translation *in vitro* in reticulocyte lysate [9,10].

There is some disagreement about the molecular mass of antizyme. Rom and Kahana [10] have provided evidence that the translation of antizyme mRNA starts at the second AUG codon. In a similar study, Matsufuji et al. [9] found that translation was initiated predominantly at the first AUG in antizyme mRNA, yielding a protein product of 29 kDa, whereas the translation of another, less abundant, form of 23 kDa started at the second AUG codon. In support of the latter finding, Mitchell et al. [30] reported recently that two immunoreactive bands of 29.5 and 24 kDa were detected from cultured cells with AZ-specific antibodies. In the present study, one major band of molecular mass 29 kDa was detected in extracts of NIH/3T3 cells transfected with antizyme gene sequences, suggesting that, at least in these cells, only the first AUG codon is used for translation initiation.

The ORF1 sequence that terminates at codon 69 of antizyme mRNA could potentially encode a 7.4 kDa protein. It is not known whether this protein is translated and/or has any specific biological function. No such low-molecular-mass protein was present among the *in vitro* translation products of antizyme mRNA when reticulocyte lysate was used [9,10]. However, a 7 kDa band was detected when wheat germ lysates were used for translation [9]. In the present study, overexpression of ORF1 sequence under the control of the CMV promoter in N2A cells did not produce any protein detectable by the ELISA specific for ORF1 or by immunoblotting. Furthermore no immunoreactive band of this size was seen on immunoblots from cells transfected with either the antizyme gene or the wild-type antizyme cDNA and grown in the absence of spermidine. Whether the low ORF1 peptide concentration is due to inefficient translation or poor stability remains to be elucidated.

In conclusion, we have cloned, sequenced and expressed the mouse antizyme gene. The genomic sequences will now permit us to generate transgenic animals whose antizyme structure or expression is genetically altered, in order to address several questions pertaining to the physiological functions of antizyme protein.

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REFERENCES

1 Steglich, C. and Scheffler, I. E. (1981) J. Biol. Chem. **257**, 4603–4609
2 Pegg, A. E. (1988) Cancer Res. **48**, 759–774
3 Tabor, C. W. and Tabor, H. (1984) Annu. Rev. Biochem. **53**, 749–790
4 Li, X. and Coffino, P. (1993) Mol. Cell. Biol. **13**, 2377–2383
5 Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K. and Ichihara, A. (1992) Nature (London) **360**, 597–599
6 Mitchell, J. L. A., Judd, G. G., Bareyal-Leyser, A. and Ling, S. Y. (1994) Biochem. J. **299**, 19–22
7 Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S. and Igarashi, K. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8930–8934
8 Matsufuji, S., Miyazaki, Y., Kanamoto, R., Kameji, T., Murakami, Y., Baby, T. G., Fujita, K., Ohno, T. and Hayashi, S. (1990) J. Biochem. **108**, 365–371
9 Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F. and Hayashi, S. (1995) Cell **80**, 51–60
10 Rom, E. and Kahana, C. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3959–3963
11 Hayashi, S. and Murakami, Y. (1995) Biochem. J. **306**, 1–10
12 Murakami, Y., Ichiba, T., Matsufuji, S. and Hayashi, S. (1996) J. Biol. Chem. **271**, 3340–3342
13 Li, X., Stebbins, B., Hoffman, L., Pratt, G., Rechsteiner, M. and Coffino, P. (1996) J. Biol. Chem. **271**, 4441–4446
14 Li, X. and Coffino, P. (1996) J. Biol. Chem. **271**, 4447–4451
15 Salzberg, A., Golden, K., Bodmer, R. and Bellen, H. J. (1996) Genetics (Princeton) **144**, 183–196
16 Miyazaki, Y., Matsufuji, S. and Hayashi, S. (1992) Gene **113**, 191–197
17 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18 Grossberger, D. (1992) Nucleic Acids Res. **15**, 6737
19 Higuchi, R., Krummel, B. and Saiki, R. K. (1988) Nucleic Acids Res. **16**, 7351–7367
20 Pegg, A. E., Borchardt, R. T. and Coward, J. K. (1981) Biochem. J. **194**, 79–89
21 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
22 Schagger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
23 Jänne, J. and Williams-Ashman, H. (1971) J. Biol. Chem. **246**, 1726–1732
24 Smith, T. F. and Waterman, M. S. (1981) Adv. Appl. Math. **2**, 482–489
25 Tewari, D. S., Quian, Y., Thornton, R. D., Pieringer, J., Taub, R., Mochan, E. and Tewari, M. (1994) Biochim. Biophys. Acta **1209**, 293–295
26 Ichiba, T., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1995) Biochim. Biophys. Acta **1262**, 83–86
27 Hickok, N. J., Seppänen, P. J., Gunsalus, G. L. and Jänne, O. A. (1987) DNA **6**, 179–187
28 Pajunen, A., Crozat, A., Jänne, O. A., Ihalainen, R., Laitinen, P. H., Stanley, B., Madhubala, R. and Pegg, A. E. (1988) J. Biol. Chem. **263**, 17040–17049
29 Kitani, T. and Fujisawa, H. (1984) J. Biol. Chem. **259**, 10036–10040
30 Mitchell, J. L. A., Choe, C.-Y., Judd, G. G., Daghfal, D. J., Kurzeja, R. J. and Leyser, A. (1996) Biochem. J. **317**, 811–816

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