Human CuZn superoxide dismutase enzymatic activity in cells is regulated by the length of the mRNA

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Abstract Single functional human CuZnSOD gene encodes two species of mRNA differing in size by 200 nucleotides in the 3′-untranslated region (UTR). We studied the expression of the CuZnSOD cDNA with different 3′- and 5′-UTR. Deletion in the 5′-end does not affect the expression of the enzyme, however, deletion in the 3′-UTR decreases the level of expression of CuZnSOD. The plasmids containing the long CuZnSOD cDNA with all polyadenylation signal sequences utilize primarily the last polyadenylation site and give a long mRNA, which produces three times more enzyme than the short mRNA lacking the last polyadenylation site and the AU-rich region.

Key words: CuZnSOD activity; SOD cDNA; 3′-UTR

1. Introduction

Important components of a defence system against toxic oxygen radicals are superoxide dismutases (SODs) – metalloenzymes, which dismutate superoxide free radicals [1]. Each eukaryotic cell has several types of the superoxide dismutases: mitochondrial SOD (MnSOD), a cytosolic SOD (CuZnSOD) [2,3], and an extracellular CuZnSOD [4].

It has been suggested that SODs are involved in the acute inflammatory response and in the transformation of cells [5–8]. Overproduction of CuZnSOD is believed to be involved in the pathology of Down's syndrome [9,10]. Mutations in the CuZnSOD gene are associated with familial amyotrophic lateral sclerosis [11–14]. The regulation mechanism of SOD activity has not been fully understood yet. It is known that copper takes part in the regulation of CuZnSOD activity at the post-translational level [15]. For rat MnSOD five mRNAs have been isolated and characterized. They all have been derived from a single functional gene, the heterogenity of the MnSOD transcripts resulting from the length variation of the 3′-noncoding sequence [5]. The sequences of MnSOD cDNA of a human tumour and normal cells exhibit differences in both the 5′- and the 3′-untranslated region (UTR), but have no mutations in the translated region [8]. Similary to rat MnSOD, a single functional human CuZnSOD gene gives more than one mRNA: poly(A)-containing RNA from various tissues contains two species of CuZnSOD mRNA, differing in size by 200 nucleotides [16]. These mRNAs differ in the lengths of their 3′-UTR and both of them possess multiple 5′-termini. The short mRNA (0.7 kb) is polyadenylated at the first polyadenylation site and the polyadenylation site of the 0.9 kb SOD mRNA is 249 bp downstream [17].

de Sauvage et al. [18] have studied the alternative polyadenylated amyloid protein precursor mRNA and have demonstrated that the long mRNA, which is created through the usage of the second polyadenylation site, has an ability to produce more protein than the short mRNA. At the same time the long mRNA was degraded more rapidly. The sequence between the two polyadenylation sites in the 3′-UTR of amyloid protein precursor mRNA was also able to increase the production of the chicken lysozyme or the chloramphenicol acetyltransferase after ligation of this 3′-UTR element to the 3′-end of reporter genes. Several works demonstrate that the 3′-UTR of mRNA is involved in the control of the stability of mRNA [19–22].

We cloned and sequenced a full-length human CuZnSOD cDNA from the histiocytic lymphoma cell line U937/B. We studied the effect of 3′- and 5′-UTR on the expression of these cDNAs at the protein level. We demonstrated that compared to cells transfected with a cDNA with truncated 3′-UTR, there is more enzyme synthesized in cells transfected with a long cDNA containing the long 3′-UTR. We did not find any effect of the deletion in 5′-UTR on the expression of the enzyme.

2. Materials and methods

2.1. Construction of U937/B cDNA expression library

Total RNA from the histiocytic lymphoma serum-free cell line (U937/B) was purified on biotinylated oligo(dT) (Promega). cDNA first strand was synthesized from poly(A)$^+$ RNA by MoMuLV reverse transcriptase by using a poly(dT) *Xho*I linker-primer. The second strand was synthesized by DNA polymerase I, protruding ends were filled with T4 DNA polymerase, ligated with *Eco*RI adaptors, phosphorylated at the 5′-end with T4 polynucleotide kinase and restricted with *Xho*I. λ ZAP II vector [23] (Stratagene) was restricted with *Eco*RI/*Xho*I endonucleases, ligated, and packaged. The resulting cDNA library contained about 120,000 clones.

2.2. Analysis of cDNA clones

Complementary DNA were isolated from a library constructed from mRNA of the histiocytic lymphoma cell line U937/B. About 10^5 recombinant phages from the library were screened by using a mutant CuZnSOD cDNA, amplified by the method of PCR [24] from a human liver cDNA library. The cDNA was labelled by multipriming nick-translation (Amersham). From the phage particles of the four hybridizing plaques the plasmid Bluescript SK(M13-) was isolated by using a helper phage R408 [23]. After the restriction analysis two SOD-positive clones were sequenced by using the dideoxynucleotide termination method [25].

2.3. Plasmids and cloning procedures

The basic eukaryotic expression vector pCG [26] was used for the cloning of cDNAs. Standard recombinant DNA techniques were used

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Abbreviations: SOD, superoxide dismutase; UTR, untranslated region; BES, *N*,-*N*-bis(2-hydroxyethyl)-2-aminoethan sulphonic acid; PCR, polymerase chain reaction; PAAG, polyacrylamide gel; dssDNA, denatured salmon sperm DNA.

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in the construction of all plasmids [27]. Briefly, *Xho*I–*Eco*RI fragment from Bluescript(M13-) was cloned into the polycloning site of the plasmid pGEM7, from the latter a *Xba*I–*Bam*HI fragment containing SOD cDNA was cloned into pCG. For deletion in the 5'-end restrictase *Pst*I was used and for deletion in the 3'-end *Bcl*I was used (Fig. 1A).

2.4. Cells and transfection

The monkey cell line COS 1 (cells producing constitutively SV40 T antigen) and the human cell line 293 were grown in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml) at 37°C in a 10% CO_2 incubator. The SOD expression constructs (6 μg) were electroporated into COS 1 cells ($3–6 \times 10^6$) at 180 V and into human 293 cells (10×10^6) at 160 V in 250 μl of DMEM supplemented with 10% FCS, 6 mM BES, 50 μg of dssDNA at room temperature [28].

2.5. Assays of SOD activity

48 h after transfection the cells ($1–2 \times 10^6$) were washed twice with PBS, suspended in 100 μl H_2O and disrupted by four cycles of freezing at −70°C and thawing at +60°C. Lysates were centrifuged for 15 min with 14,000 rpm at 4°C (Eppendorf centrifuge). SOD activity was measured by a pyrogallol method [29]. The reaction of autooxidation of pyrogallol and the inhibition of this reaction by cell extracts was spectrophotometrically monitored. 1 unit of SOD activity equals the amount of enzyme producing a 50% inhibition in the autooxidation of pyrogallol. SOD activity is expressed in units per mg of protein.The total protein in lysates was determined by Coomassie brilliant blue G-250 [30], standardized with bovin serum albumin.

The lysates were analysed on the 15% PAAG under non-denaturating conditions and the SOD activity was determined by nitroblue tetrazolium [31]. SOD activity appears as a transparent band in the blue formazan pigment background. A 15% PAAG can be used to separate MnSOD and CuZnSOD. However, our lysates do not contain any detectable amounts of MnSOD. As a positive control we used CuZnSOD isolated from red blood cells. The cells were lysed for 10 min in one volume of distilled water at 75–80°C, quickly cooled at 40°C and filtrated. The rest of hemoglobin was precipitated by 0.25 vol. of ethanol and 0.15 vol. of chloroform.

2.6. RNA analysis

Cytoplasmic RNA was isolated from cells ($1–2 \times 10^6$) 24 h after transfection by standard method [27]. 20 μg of total RNA was separated on 1.2% agarose/formaldehyde gel, RNA was transferred to a nylon membrane (Nybond N, Amersham) and covalently crosslinked to the membrane with UV light [27]. The membranes were hybridized overnight with a radiolabelled human CuZnSOD cDNA probe (*Xba*I–*Bam*HI fragment, Fig. 1A) in 6× SSC, 5× Denhardt's reagent, 0.1% SDS and 100 μg/ml dssDNA at 68°C, washed 2× SSC, 0.1% SDS at room temperature, 2 × 15 min, and 0.2× SSC, 0.5% SDS at 68°C 2 h (4 × 300 ml) and identified by autoradiography.

3. Results

3.1. Construction of the CuZnSOD expression vectors with differences at the 5'- and 3'-UTR of the cDNA

One SOD-positive clone from a human histiocytic lymphoma cDNA library contained a full-length CuZnSOD cDNA. The long 3'-UTR (345 nucleotides) and the protein coding sequence

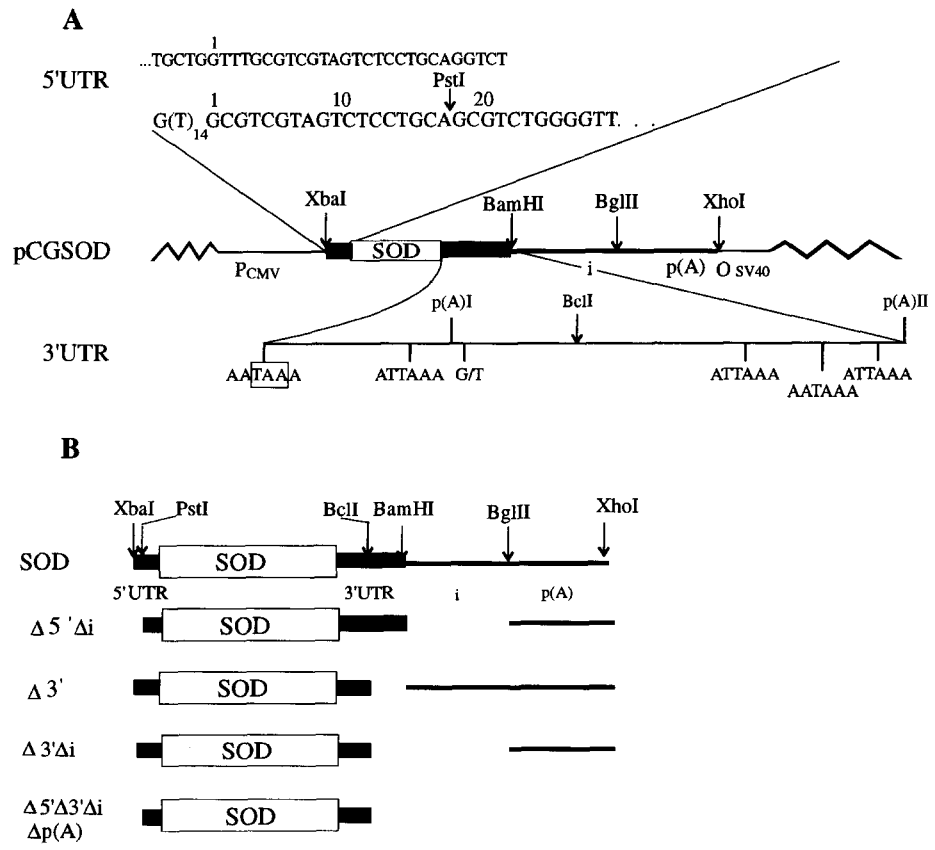

Fig. 1. Schematic structure of plasmid containing the CuZnSOD cDNA and its derivates. The arrows indicate the position of the restriction sites used for cloning. (A) Plasmid pCGSOD contains a bacterial plasmid sequence (∧∧∧), CMV promotor (P), the rabbit genomic sequence for splicing and polyadenylation (i, p(A), thick line), SV40 origin fragment (O) and full-length CuZnSOD cDNA as the *Xba*I–*Bam*HI fragment, open box represents the coding sequence and the filled box, the untranslated regions. 5'-UTR, nucleotide sequence of the transcription initiation region of SOD-1 gene [17] (small type), and 5'-end of the CuZnSOD cDNA in our experiments; 3'-UTR, 3'-region of the CuZnSOD cDNA containing the stop codon TAA, the five polyadenylation signal sequences, two cleavage and polyadenylation sites p(A)I, p(A)II, and the GT-rich box G/T [33] (The sequence is presented in Fig. 4). (B) Deletions in the 5'- and 3'-UTR of SOD cDNA (*Δ5'-Δ3'-*) and in the vector sequence (*ΔiΔ*p(A)).

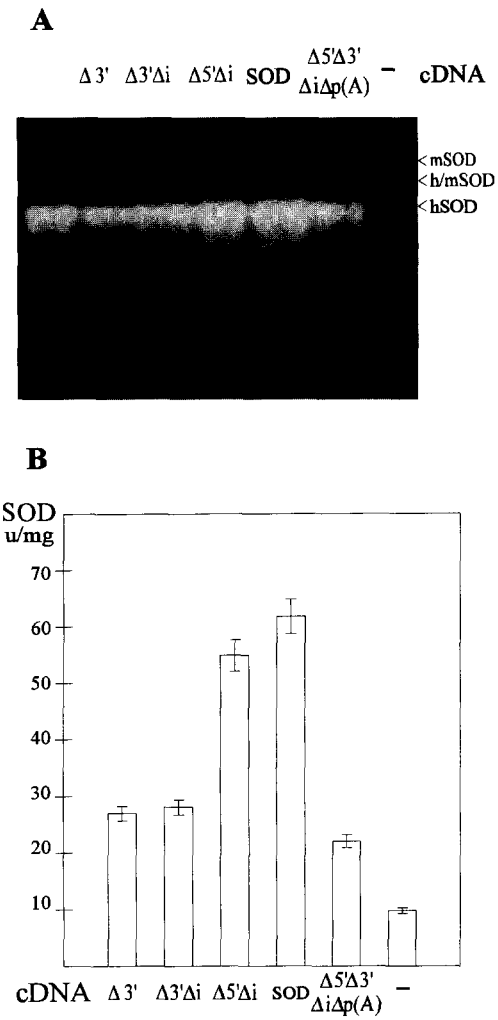

Fig. 2. Analysis of the CuZnSOD enzymatic activity in COS 1 cells transfected with plasmids pCGSODΔ3'-(Δ3'-), pCGSODΔ3'-Δi (Δ3'-Δi), pCGSODΔ5'-Δi (Δ5'-Δi), pCGSOD (SOD), pCGSODΔ5'-Δ3'-ΔiΔp(A) (Δ5'-Δ3'-ΔiΔp(A)) or without SOD plasmid (–). (A) Cell extracts (10 μg of protein) and erytrocyte CuZnSOD (the first left lane) electrophoresed on a native 15% PAAG and stained for SOD activity. The arrows indicate the position of monkey CuZnSOD (mSOD), heterodimer of human and monkey's subunits of CuZnSOD (h/m SOD) and the human CuZnSOD (hSOD). (B) SOD activity (units/mg of cell protein) determined by spectrophotometric pyrogallol method. Each post represents the average of double plates of the same transfection, with error bars included. This experiment was repeated six times with reproducible results, the example shown is repesentative.

are identical to the published one[17,32,33]. The 5'-UTR showed an unusual sequence. While the genomic sequence of the transcription initiation site is ...GCTG GTTTGCG... [17,32], our cDNA has 14 T instead of 3 T: ...G(T)$_{14}$GCG... (Fig. 1A). Another difference is that at position 20 there is a C between two Gs (Fig. 1A). This C is absent in some published genomic sequences [17,32], but it is present in sequence published by Hallewell [33]. The CuZnSOD full-length cDNA, containing in its 3'-end 15 A nucleotide in addition to the genomic sequence, was cloned into the vector pCG as a *Xba*I–*Bam*HI fragment (Fig. 1A, plasmid pCGSOD). The plasmid pCGSODΔ5'-Δi contains neither the oligo(dT)$_{14}$ and 18 first nucleotides at the 5'-end of SOD cDNA up to *Pst*I site nor the rabbit β-globin intron sequence of the vector pCG up to *Bgl*II site (Fig. 1B). The plasmid pCGSODΔ3' lacks the last 185 nucleotides, i.e. the last polyadenylation site of SOD cDNA up to *Bcl*I site, and it contains only the two first polyadenylation signal sequences and G/T box downsrteam p(A)I (Fig. 1A). The plasmid pCGSODΔ3'-Δi is identical to pCGSODΔ3'- with the exeption of lacking the β-globin intron sequence of the vector pCG up to *Bgl*II site (Fig. 1B). The plasmid pCGSODΔ5'-Δ3'-ΔiΔp(A) differs from the others in that it lacks all the rabbit genomic sequence of the vector pCG, the 5'-end and the 3'-end of the SOD cDNA up to *Pst*I and *Bcl*I sites, respectively (Fig. 1B). mRNAs from the plasmids the pCGSODΔ3'-, pCGSODΔ3'-Δi and pCGSODΔ5'-Δ3'-ΔiΔp(A) can be polyadenylated at the first polyadenylation site p(A)I of SOD cDNA (Fig. 1A). The plasmids pCGSOD and pCGSODΔ5'-Δi have the possibility to utilize all polyadenylation sites in CuZnSOD cDNA 3'-UTR (Fig. 1).

3.2. *Expression of the human CuZnSOD cDNAs transfected in monkey cell line COS 1 and in human cell line 293*

The plasmids containing CuZnSOD cDNA or its derivates were transfected into the monkey cell line COS 1 and into the human cell line 293 by electroporation [28]. In transient experiments 48 h after the transfection cells were harvested and lysed. In the lysates the SOD activity was measured by two different methods. (Fig. 2) After the separation of proteins on the 15% PAAG under non-denaturating conditions, the SOD activity was made manifest by the nitroblue tetrazolium method [31]. All SOD cDNA-containing plasmids transfected into COS 1 cells express the human CuZnSOD activity at high level (Fig. 2A). In a control experiment only the carrier DNA was transfected, and only the monkey's own CuZnSOD (mSOD, Fig. 2A, lane –) is expressed there. CuZnSOD is a dimer composed of two identical subunits. Therefore, the active enzyme can also form a heterodimer of monkey's and human subunits, which can be seen in Fig. 2A as the middle band in the extracts of the cell transfected with SOD plasmids. A quantitative evaluation

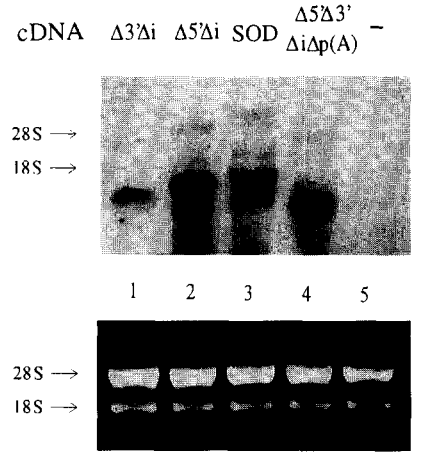

Fig. 3. Northern blot analysis of human CuZnSOD mRNAs. 20 μg of total cytoplasmic RNA of COS 1 cells transfected with the plasmid pCGSODΔ3'-Δi (lane 1), pCGSODΔ5'-Δi (lane 2), pCGSOD (lane 3), pCGSODΔ5'-Δ3'-ΔiΔp(A) (lane 4) or without SOD cDNA (lane 5) electrophoresed on a 1.2% agarose/formaldehyde gel. Hybridization was performed with ^{32}P-labelled human CuZnSOD cDNA. The position of the 18 S and 28 S ribosomal RNA are shown to the left and amounts of RNA are shown on the bottom panel, on the picture of the ethidium bromide-stained agarose gel.

A

ACATTCCCTTGGATGTAGTCTGAGGCCCCTTAACTCATCTGTTATC
CTGCTAGCTGTAGAAATGTATCCTGATAAACATTAAACACTGTAAT
CTTAAAA↓GTGTAATTGTGTGACTTTTTCAGAGTTG**CTTTA**AAGTA
CCTGTAGTGAGAAACTG**ATTTA**T
↓GATCACTTGGAAG**ATTTG**TATA**GTTTTA**TAAAACTCAGTTAAAA
TGTCTGTTTCAATGACCTGT**ATTTT**GCCAGACTTAAATCACAGATG
GGTATTAAACTTGTCAGA**ATTTC**TTTGTCATTCAAGCCTGTGAATA
AAAACCCTGTATGGCACTTATTATGAGGCTATTAAAAGAATCCAA
ATTCAAA↓aaaaaaaaaaaaaaactggagactagttctgtgccgaattcc

B

ATAAATAAATTAAATAAAATAA↓CCCCGGGCAAGACTTTTCTTTGA
AGGATGACTACAGACATTAAATAATCGAAGTA**ATTTT**GGGTGGGG
AGAAGAGGCAGATTCA**ATTTTCTTTA**ACCAGTCTGAAGTTTC**ATT**
TATGATACAAAAGAAGATGAAAATGGAAGTGGCAATATAAGGGG
ATGAGGAAGGCATGCCTGGACAAACCCTTC**TTTTA**AGATGTGTCT
TCA**ATTTG**TATAAAATGGTGTTTTCATGTAAATAAATACATTCTTG
GAGGAGC↓

Fig. 4. Nucleotide sequence of the 3′-UTR of the CuZnSOD cDNA (A) [17] and of the amyloid protein precursor cDNA (B) [18]. The AU motifs are in heavy type, the polyadenylation sites are designated by the arrows, the disruption with an arrow indicates the site of the deletion of the last 185 nucleotide of the 3′-UTR of the CuZnSOD cDNA, and the short 3′-UTR of the SOD mRNA is underlined.The sequence after the last polyadenylation site of CuZnSOD cDNA in our constructs is designated by small type.

was made by a spectrophotometric pyrogallol method (Fig. 2B). The CuZnSOD cDNA lacking the last polyadenylation signal sequences at the 3′-end shows lower enzymatic activity. While comparing the exogeneous SOD activities, i.e. the control level count off, it appears that all plasmids containing Δ3′-cDNA produce three times less enzyme than full-length cDNA (Table 1). Deletion in the 5′-end of SOD cDNA has no significant effect on the expression (Fig. 2). The plasmids pCGSODΔ3′-, pCGSODΔ3′-Δi and pCGSODΔ5′-Δ3′-ΔiΔp(A) give the expression nearly at the same level (Fig. 2). The rabbit genomic sequence for splicing and polyadenylation in the pCG vector does not affect the expression of the SOD cDNA.

The expression of the SV40 T antigen in COS cells allows replication of pCG plasmids to a high copy number, which results in high levels of transcription and enzyme activity. In the human cell line 293 the plasmid pCG does not have a high copy number and the levels of enzyme activity are lower. But like in COS, in cells of the human cell line 293 the expression of full-length or Δ5′-SOD gives three times higher enzyme activity than plasmids containing the short cDNA (Table 1).

3.3. *Analysis of CuZnSOD mRNA in cells transfected CuZnSOD cDNAs*

In our experiments 24 h after transfection pCGSOD plasmids into the COS 1 cells cytoplasmic RNA was isolated and analysed. The results are presented in Figure 3. In the cells COS 1, not transfected with the human CuZnSOD cDNA, no CuZnSOD mRNA could be detected by using a human-specific probe (Fig. 3, lane 5). The CuZnSOD cDNAs lacking the last polyadenylation signal sequences at the 3′-end allow the synthesis of the short mRNA only (Fig. 3, lane 1 and 4). Concerning the transcription of the plasmids pCGSOD and pCGSODΔ5′-Δi, containing all polyadenylation sites and in addition 15 A nucleotide in its 3′-end, the last polyadenylation site p(A)II was preferred. We can primarily see the long mRNA (Fig. 3, lane 2 and 3).

4. Discussion

We studied the effect of 3′- and 5′-UTR on the expression of the CuZnSOD cDNA and observed that, the deletion of the last 185 nucleotides from the 3′-UTR reduces the level of enzyme activity by three times. This deleted fragment contains the last three polyadenylation signal sequences of CuZnSOD cDNA. In human cells normally two CuZnSOD mRNAs of 0.9 and 0.7 kb have been detected [16,17]. For polyadenylation of these mRNAs different polyadenylation sites are used. The short mRNA is polyadenylated at the first polyadenylation site p(A)I and long one at p(A)II (Fig. 1) [17]. Plasmids pCGSOD and pCGSODΔ5′-Δi contain all polyadenylation sites of CuZnSOD mRNA and in addition a $(A)_{15}$-tail (Fig. 4). They allow the production of short and long mRNA. When these plasmids were expressed in the monkey cell line COS 1, mostly the long mRNA was synthesized (Fig. 3). While from the endogenous human CuZnSOD gene shorter mRNA is transcribed four times more efficiently than the longer mRNA [17]. The mechanism, which regulates the ratio between two mRNA species, is unknown. It seems that the ratio is not only regulated by 3′-UTR of cDNA. Evidently others genomic sequences are involved in the splicing of CuZnSOD pre-mRNA. When the long CuZnSOD cDNA containing an oligo(A) in its 3′-end was cloned into the plasmid, the mechanism of the regulation might have been different than that of the chromosomal CuZnSOD gene. Expression of the plasmid giving the long mRNA, produced three times more SOD enzyme than the plasmid with the truncated 3′-UTR of CuZnSOD cDNA (Fig. 2).

An alternative polyadenylation has been shown for some mRNAs: MnSOD mRNA [5], amyloid protein precursor mRNA [18], human PR264/SC35 splicing factor mRNA [21], *Xenopus* α-tubulin mRNA [34]. 3′-UTR can control gene expression through different mechanisms [35–37]. 3′-UTR determines the stability of mRNA in some cases [20–22]. Savant-Bhonsale and Cleveland [20] have shown that granulocyte monocyte colony stimulating factor mRNA contains in its 3′-UTR five AU-rich domains and this region mediates the extreme instability of the mRNA. The most important are AUUUA motifs. For the instability of the mRNA an active translation of this mRNA is required in addition to the AU-rich region in the 3′-UTR. They have proposed that the RNA instability mediated by the AU-motif is achieved through a translation-dependent assembly of a large mRNA-destabilizing complex.

Table 1
Expression of the human CuZnSOD cDNA in monkey cell line COS 1 and in human cell line 293

Transfected plasmid	CuZnSOD activity* (u/mg of cell protein)	
	COS cells	293 cells
pCGSODΔ3′	17 ± 2	2.6 ± 0.3
pCGSODΔ3′Δi	18 ± 2	2.4 ± 0.3
pCGSODΔ5′Δi	45 ± 4	7.9 ± 0.5
pCGSOD	52 ± 4	7.5 ± 0.5
pCGSODΔ5′Δ3′ΔiΔp(A)	12 ± 2	2.4 ± 0.2

*CuZnSOD activity – difference of SOD activity between SOD plasmid transfected cells and control cells.

Sauvage et al. [18] have shown that the alternative polyadenylation regulates translation: two amyloid protein precursor mRNA, differing from 3′-UTR, are translated in *Xenopus* oocytes. It was demonstrated that the long mRNA using the second polyadenylation site produces more protein than the short one. However, the long mRNA is degraded more rapidly. The long 3′-UTR of the amyloid protein precursor mRNA contains the AU-rich region, within it one AUUUA motif (Fig. 4), supposedly mediating the translation-dependent instability of mRNA [20].

We have shown that the plasmid containing a long CuZnSOD cDNA produces more SOD activity than the plasmid with a short cDNA of this gene. In between the first polyadenylation site and the last polyadenylation site of CuZnSOD mRNA contains an AU-rich region, within it one AUUUA motif and five incomplete motifs CUUUA, AUUUG, GUUUUA, AUUUU, AUUUC (Fig. 4). There are no such kind of motifs in the region upstream the first polyadenylation site (Fig. 4). Since the long CuZnSOD mRNA, like amyloid protein precursor mRNA, contains the AUUUA motifs (Fig. 4), we can speculate that the ability of the mRNA to produce more CuZnSOD enzyme may be depend on this sequence in 3′-UTR. In the further experiments we plan the identification of the sequences in the 3′-UTR, which are responsible for enhanced production of the enzyme. The processing of the mRNA of CuZnSOD might be an additional regulatory mechanism for the expression of this important defence enzyme. The post-transcriptional control of the CuZnSOD allows for a fast cellular response in the enzyme level.

References

[1] MacCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[2] Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem. 248, 3582–3592.
[3] Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem. 248, 4783–4796.
[4] Hjalmarsson, K., Marklund, S., Engstrom, A. and Edlund, T. (1987) Proc. Natl. Acad.Sci. USA 84, 6340–6344.
[5] Hurt, J., Hsu, J.-L., Dougall, W.C., Visner, G.A., Burr, I.M. and Nick, H.S. (1992) Nucleic Acids Res. 20, 2985–2990.
[6] St.Clair, D.K., Wan, X.S., Oberley, T.D., Muse, K.E. and St. Clair, W.H. (1992) Mol. Carcinogen. 6, 238–242.
[7] Oberley, L.W., McCormick, M.L., Sierra-Rivera, E., St Clair, D.K. (1989) Free Rad. Biol. Med. 6, 379–384.
[8] St. Clair, D.K. and Holland J.G. (1991) Cancer Res. 51, 939–943.
[9] Elroy-Stein, O., Bernstein, Y. and Groner, Y. (1986) EMBO J. 5, 615–622.
[10] Epstein, C.J., Avraham, K.B., Lovett, M., Smith, S., Elory-Stein, O., Rotman, G., Bry, C. and Groner, Y. (1987) Proc. Natl. Acad. Sci. USA 84, 8044–8048.
[11] Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A. Donaldson, D, Goto, J., O'Regan, J.P., Deng, H.-X. Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S.M., Berger, R., Tanzi, R.E., Halperin, J.J., Herzfeldt, B., Van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D.W., Smyth, C., Laing, N.G., Soriano, E., Pericak-Vance, M.A., Haines, J., Rouleau, G.A., Gusella, J.S., Horvitz, H.R. and Brown Jr., R.H. (1993) Nature 362, 59–62.
[12] Kostrzewa, M., Bruck-Lehmann, U. and Müller, U. (1994) Hum. Mol. Genet. 3, 2261–2262.
[13] Beckman, J.S., Carson, M., Smith, C.D. and Koppenol, W.H. (1993) Nature 364, 584.
[14] Deng, H.X., Hentati, A., Tainer, J.H., Iqbal, Z., Cayabyab, A., Hung, W.-Y., Getzoff, E.D., Hu, P., Herzfeldt, B., Roos, R.P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H.E., Ahmed, A., Roses, A.D., Hallewell,R.A., Pericak-Vance, M.A. and Siddique, T. (1993) Science 261, 1047–1051.
[15] Percival, S.S. and Harris, E.D. (1991) Biochem. J. 274, 153–158.
[16] Sherman, L., Dafni, N., Lieman-Hurwitz, J. and Groner, Y. (1983) Proc. Natl. Acad. Sci. USA 80, 5465–5469.
[17] Sherman, L., Levanon, D., Lieman-Hurwitz, J., Dafni, N., and Groner, Y. (1984) Nucleic Acids Res. 12, 9349–9365.
[18] de Sauvage, F., Kruys, V., Marinx, O., Huez, G. and Octave, J.N. (1992) EMBO J. 11, 3099–3103.
[19] Kruys, V., Kemmer, K., Shakhov, A., Jongeneel, V. and Beutler, B. (1992) Proc. Natl. Acad. Sci. USA 89, 673–677.
[20] Savant-Bhonsale, S. and Cleveland, D.W. (1992) Genes Dev. 6, 1927–1939.
[21] Sureau, A. and Perbal, B. (1994) Proc. Natl. Acad. Sci. USA 91, 932–936.
[22] Chen, C.-Y.A., Chen, T.-M. and Shyu, A.-B. (1994) Mol. Cell. Biol. 14, 416–426.
[23] Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D. (1988) Nucleic Acids Res. 16, 7583–7600.
[24] Saiki, R.K., Gelfond, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487–491.
[25] Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980) J. Mol. Biol. 143, 161–178.
[26] Tanaka, M. and Herr, W. (1990) Cell 60, 375–386
[27] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.-Y.
[28] Ustav, M. and Stenlund, A. (1991) EMBO J. 10, 449–457.
[29] Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474.
[30] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[31] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–278.
[32] Levanon, D., Lieman-Hurwitz, J., Dafni, N., Wigderson, M., Sherman, L., Bernstein, Y., Laver-Rudich, Z., Danciger, E., Stein, O. and Groner, Y. (1985) EMBO J. 4, 77–84.
[33] Hallewell, R.A., Masiarz, F.R., Najarian, R.C., Puma, J.P., Quiroga, M.R., Randolph, A., Sanchez-Pescador, R., Scandella, C.J., Smith, B., Steimer, K.S. and Mullenbach, G.T. (1985) Nucleic Acids Res. 13, 2017–2034.
[34] Rabbitts, K.G. and Morgan, G.T. (1992) Nucleic Acids Res. 20, 2947–2953.
[35] Kruys, V., Marinx, O., Shaw, G., Deschamps, J. and Huez, G. (1989) Science 245, 852–855.
[36] Han, J., Brown, T. and Beutler, B. (1990) J. Exp. Med. 171, 465–476.
[37] Wickens, M. and Takayama, K. (1994) Nature 367, 17–18.