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Hypoxia Stimulates Binding of a Cytoplasmic Protein to a Pyrimidine-rich Sequence in the 3'-Untranslated Region of Rat Tyrosine Hydroxylase mRNA*

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Reduced oxygen tension (hypoxia) induces a 3-fold increase in stability of mRNA for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, in the pheochromocytoma (PC12) clonal cell line. To investigate the possibility that RNA-protein interactions are involved in mediating this increase in stability, RNA gel shift assays were performed using different fragments of labeled TH mRNA and the S-100 fraction of PC12 cytoplasmic protein extracts. We identified a sequence within the 3'-untranslated region of TH mRNA that binds cytoplasmic protein. RNase T1 mapping revealed that the protein was bound to a 28 nucleotide long sequence that is located between bases 1551-1579 of TH mRNA. Moreover, protein binding to this fragment was prevented with an antisense oligonucleotide directed against bases 1551-1579 and subsequent RNase H digestion. This fragment of the 3'-untranslated region of TH mRNA is rich in pyrimidine nucleotides, and the binding of cytoplasmic protein to this fragment was reduced by competition with other polypyrimidine sequences including poly(C) but not poly(U) polymers. The binding of the protein to TH mRNA was increased when cytoplasmic proteins were extracted from PC12 cells exposed to hypoxia (5% O_2) for 24 h. Electrophoresis of the UV cross-linked RNA-protein complex on SDS-polyacrylamide gel electrophoresis revealed a complex of 74 kDa. The potential role of this protein-TH mRNA interaction in regulation of TH mRNA stability during hypoxia is discussed.

Tyrosine hydroxylase (TH)[1] (EC 1.14.16.2) hydroxylates L-tyrosine to L-dopa (1, 2) and is the rate-limiting enzyme in catecholamine synthesis. TH activity (V_{max}) is enhanced in a number of neural and neuroendocrine tissues by reduced oxygen tension (hypoxia) (3-5). We reported recently that TH gene expression is stimulated by hypoxia in the O_2-sensitive (type I) cells of the carotid body but not in other catecholamine tissues such as the adrenal gland or sympathetic ganglia (6). We also found that TH gene expression is enhanced by hypoxia in pheochromocytoma (PC12) cells, which are biochemically and morphologically similar to carotid body type I cells (7). Further studies in PC12 cells revealed that reduced O_2 tension led to increases in both the rate of TH gene transcription and stability of TH mRNA (9). The present study was undertaken to identify potential molecular mechanisms that might mediate the increase in TH mRNA stability during hypoxia.

Messenger RNA stability (*i.e.* rate of degradation) is an important mechanism for regulation of gene expression (for review, see Refs. 8-10). The turnover of mRNAs is regulated by interactions among *trans*-acting factors (regulatory proteins) and specific sequences located mainly within the 3'-untranslated regions (3'-UTR) of some mRNAs. In recent years a number of unique *cis*-acting elements, such as the iron-responsive elements (11) and adenosine/uridine(AU)-rich sequences (12, 13), have been shown to bind cytosplamic proteins. These elements have been implicated in regulation of mRNA stability in eukaryotic cells. We hypothesized that increased stability of TH mRNA during hypoxia is mediated by the interaction of a cytoplasmic protein(s) with a unique *cis*-element on the 3'-UTR of TH mRNA. Computer analysis of the 3'-UTR of TH mRNA revealed that it does not contain any of the identified consensus elements (IRE or AU-rich sequences) normally associated with mRNA stability. In the present study we report that a 28-b pyrimidine-rich sequence within the 3'-UTR of TH mRNA binds cytoplasmic protein(s). Importantly, this interaction is enhanced when proteins are extracted from PC12 cells exposed to hypoxia.

MATERIALS AND METHODS

Cell Culture and Preparation of Cytoplasmic Extracts—Rat PC12 cells were grown as described previously (7). When cells reached 90% confluency they were exposed to either normoxia (21% O_2, 5% CO_2, remainder N_2) or hypoxia (5% O_2, 5% CO_2, remainder N_2) in an O_2-regulated incubator (Forma Sci., Inc.) for 18 h.

The S-100 fraction of cytosolic proteins was obtained as described (14). Following exposure to either normoxia or hypoxia, cells were collected and washed in ice-cold PBS (pH 7.5) and then in 5 volumes of hypotonic buffer containing 15 mM Tris (pH 7.9), 10 mM KCl, 0.2 mM EDTA (pH 8), 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Cells were then centrifuged and lysed in 3 volumes of the same hypotonic buffer for 10 min. The cells were homogenized with 20 strokes of Dounce homogenizer (pestle B), the nuclei were separated by centrifugation, and the remaining cytoplasmic lysates were extracted with 0.11 volume of 10 × cytoplasmic buffer (0.3 M Hepes, pH 7.9, 1.4 M KCl, 0.03 M $MgCl_2$). The extracts were then centrifuged at 100,000 × *g* at 4 °C for 60 min and dialyzed for 5 h in the 2 × binding buffer (see below) at 4 °C. Protein yield (Bio-Rad assay) was normally in the range of 5-15 μg/μl. We did not observe differences in the protein yield obtained from PC12 cells exposed to normoxia and hypoxia. The extracts were aliquoted and stored frozen at -80 °C.

Plasmid Constructs and RNA Transcripts—A TH cDNA in SP64 plasmid (pSPTH-3) (Fig. 1*A*) was used to generate fragments of TH mRNA.

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[1] The abbreviations used are: TH, tyrosine hydroxylase; 3'-UTR, 3'-untranslated region(s); PBS, phosphate-buffered saline; b, base(s); bp, base pair(s); EPO, erythropoietin; TAE, Tris acetate-EDTA buffer.

A 600-base pair (bp) *Eco*RI/*Bam*HI fragment of TH cDNA that contains the last 300 bp of the coding region and the entire 3′-UTR was cut from the pSPTH-3 vector and subcloned into *Eco*RI-*Bam*HI sites of the transcription vector PBS+/− phagemid (Stratagene). We refer to this vector as PBS-TH600. A 162-bp *Kpn*I/*Sph*I (1521–1682) fragment of TH cDNA that encodes the region in the 3′-UTR of TH mRNA was cut from the pSPTH-3 vector and subcloned into the same restriction sites of vector SP73 (Promega Corp.). This clone (pSP73-TH162) was sequenced and found to be identical with the published sequence of TH cDNA (15). Our cloning strategy allowed us to generate three different transcripts for use in RNA-protein-binding reactions (Fig. 1*A*). A 1200-b long fragment of TH mRNA, which contained most of the coding region for TH mRNA, was obtained by transcription from the original pSPTH-3 plasmid linearized with *Eco*RI. A 600-b fragment of TH mRNA that contained the remainder of the coding region and the entire 3′-UTR was generated by transcription from plasmid PBS-TH600 linearized with *Bam*HI. The 162-b fragment from the 3′-UTR of TH mRNA was obtained by transcription from plasmid pSP73-TH162 linearized with *Hin*dIII, which is located 2-bp 3′ from the *Sph*I site. In addition, a nonspecific mRNA of 138-b was obtained from the SP73 vector DNA linearized with *Hpa*I.

In vitro transcriptions were performed using SP6 polymerase (Promega Corp.) for the SP64 and SP73 vectors and T7 polymerase (Promega Corp.) for the PBS vector. *In vitro* transcription of the test fragments was performed in the presence of 50 μCi of [α-^{32}P]UTP (Amersham Corp.; 3000 > Ci/mmol), 2.5 mM of all four unlabeled nucleotides, 20 mM dithiothreitol, 2 mg/ml of bovine serum albumin, RNasin, and the appropriate polymerase at 40 °C for 60 min. Unlabeled RNA that was synthesized as described above but without radioactive nucleotide was used in competition experiments. Following *in vitro* transcription, the template DNA was digested with RNase-free DNase (Boehringer Mannheim), and the RNA was purified on a RNase-free G-50 column (Boehringer Mannheim). In all cases a single RNA band of the appropriate size was resolved on a denaturing polyacrylamide gel.

In some RNA binding experiments competitor RNA that contained a polypyrimidine (poly(Y)$^+$) sequence was transcribed from clone DUP33Y5 linearized with *Nco*I (16). The same competitor RNA that did not contain the poly(Y) sequence (poly(Y)$^-$) was transcribed from the same vector cut with *Mbo*II. In other competition experiments, RNA homopolymers of poly(C) or poly(U) (Pharmacia LKB Biotechnology Inc.) were used.

The antisense oligodeoxyribonucleotides that were complementary to sequences (1551–1579 and 1633–1659) located within the 3′-UTR of TH mRNA were used in the binding reactions. The sequences of these oligonucleotides were 5′-AGAGGTAGGGGAAGAGGTTGGAAAGGAC-3′ and 5′-TGGGGGTAGGAGGGTTGTGACGAAGAG-3′. Oligonucleotides were synthesized in our laboratory on a DNA synthesizer (Millipore).

RNA-Protein-binding Reaction and Electrophoresis of Complexes—Binding reactions were performed essentially as described by others (17–18). Cytoplasmic proteins (5–40 μg/reaction) were incubated in 10 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 200 ng/μl of *Escherichia coli* tRNA, 10% glycerol, and 1 mM dithiothreitol at 30 °C for 20 min. Specific RNA transcripts (40,000–100,000 cpm/reaction) were added, and the binding reaction in total volume of 30 μl was incubated for 30 min at 30 °C. For the competition experiments, proteins were incubated first (15 min) with the indicated amounts of competitor RNA and then with the labeled RNA for an additional 15 min. The indicated amounts of antisense oligonucleotides were added simultaneously with the labeled TH mRNA fragment. After completion of the binding reaction, RNase T1 (20 units, RNase T1, Calbiochem) and heparin sulfate (to the final concentration of 5 mg/ml) were added sequentially to the reaction mixture for 10 min each at 30 °C. In some experiments the binding reaction was treated with Proteinase K (Boehringer Mannheim; final concentration, 2 mg/ml, 30 min, 37 °C). Electrophoresis of RNA-protein complexes was carried out on 7% native polyacrylamide gel (acrylamide/methylene bisacrylamide ratio, 60:1) with 0.5 × Tris borate-EDTA buffer. The gels were preelectrophoresed for 1 h at 8 V/cm followed by electrophoresis of RNA-protein complexes for 2 h at 35 A. Gels were dried and exposed to x-ray film (Kodak). The quantitative assessment of RNA-protein bands was performed by optical density measurements (Image ProII Plus) and Student's *t* test for statistical analysis.

RNase T1 Mapping—The binding reaction between the 162-b fragment of the 3′-UTR of TH mRNA and cytoplasmic proteins was performed as described above. Following electrophoresis of the RNA-protein complex, the gel was exposed to x-ray film at 4 °C overnight, and the location of specific RNA-protein bands was determined. The identified bands were cut out of the gel and transferred to a dialysis bag, and the RNA was electroeluted into 1 × TAE buffer using 150 V for 2 h at 4 °C. This procedure allowed for isolation of the RNA fragments that were bound to proteins and thus protected from RNase T1 digestion. The isolated RNA was phenol/chloroform-extracted and ethanol-precipitated using yeast tRNA as a carrier. 600–1500 cpm of labeled RNA were usually recovered. The entire 162 TH mRNA fragment (not bound to the protein) as well as half of the eluted RNA were digested with RNase T1 (10 units, 37 °C, 30 min). The RNase T1-digested entire 162-b fragment as well as the nondigested and the RNase T1-digested fragment of RNA, which was eluted from the RNA-protein complex, were electrophoresed on a 12% denaturing polyacrylamide gel. The gel was dried and exposed to x-ray film overnight at −80 °C. A comparison of the sequences of the RNase T1-digested fragments from the eluted RNA and the 162-b fragment not incubated with the proteins allowed for identification of sequences in the 162-b fragment of the 3′-UTR of TH mRNA, which were protected from RNase T1 digest by bound proteins (17).

UV-Cross-linking and Electrophoresis of RNA-Protein Complexes—RNA-protein-binding reactions were performed as described above. Open microcentrifuge tubes containing the binding reactions were irradiated with a total of 4 × 10^6 J/cm^2 of UV light (UV cross-linker FB UVXL-1000; Fisher Sci., Inc.), boiled in SDS sample buffer without β-mercaptoethanol, and electrophoresed on an 8% SDS-polyacrylamide gel along with prestained protein molecular weight markers (Bio-Rad). The gels were dried and then exposed overnight to x-ray film at −80 °C. The size of RNA-protein complex was determined using RFLP molecular weight software (DNA ProScan Inc.).

RESULTS

RNA gel retardation assays were performed to determine the binding pattern of cytoplasmic proteins to TH mRNA. Binding reactions were performed using three separate fragments of rat TH mRNA, which encompassed the entire length of this mRNA (Fig. 1*A*). Fig. 1*B* shows that the addition of the S-100 fraction of cytoplasmic protein extract (*CPE*) retarded migration of RNA in the form of smear (*lane 2*), which was resolved into a single band after addition of RNase T1 (*RT1*) and heparin (*H*) (*lane 3*) to the binding reaction. This band was abolished by treatment of the binding reaction with Proteinase K (*PK*, *lane 4*). This result indicates that the observed band (*lane 3*) was due to interaction of a protein(s) with the labeled RNA. Fig. 1*C* shows that a band at the same level was obtained when either the 162- or 600-b TH mRNA fragments were used (compare *lanes 3* with *2*). Note, however, that this band was not observed when the 1200-b fragment of TH mRNA (*lane 1*) was used. A nonspecific band was detected when the plasmid SP73 RNA (*lane 4*) was used. The similar binding pattern obtained with both the 162- and 600-b fragments of TH mRNA is due to inclusion of the 162-b fragment within the 600-b fragment (Fig. 1*A*). The binding of protein to the 162-b fragment was proportional to the amount of protein extract used (Fig. 2*A*) and also to the amount of probe used at a constant concentration of cytoplasmic extract (not shown).

To determine if the observed RNA-protein complex is specific, competition experiments were performed using nonlabeled, specific (162-b fragment of 3′-UTR) and nonspecific plasmid mRNA. Fig. 2*B* shows the results from one such experiment. The binding of protein to the 162-b fragment of the 3′-UTR of TH mRNA (*lane 2*) was reduced or abolished after addition of 10 × and 50 × molar excess of nonlabeled, specific mRNA to the binding reaction (*lanes 3–6*). In contrast, competition with the same concentration of nonspecific plasmid RNA had no effect on the binding activity (*lanes 7–10*).

To determine if exposure of PC12 cells to hypoxia affects the binding of protein to the 162-b fragment, gel retardation assays were performed using the S-100 fraction of cytoplasmic protein extracted from PC12 cells exposed for 18 h to either 5% O_2 (hypoxia) or 21% O_2 (normoxia). Fig. 3*A* shows that the formation of the 162-b RNA fragment-protein complex was substantially increased when extracts from hypoxic cells were used (*lane 2*) as compared with extracts taken from normoxic cells (*lane 1*). This difference was approximately 2.5-fold and was

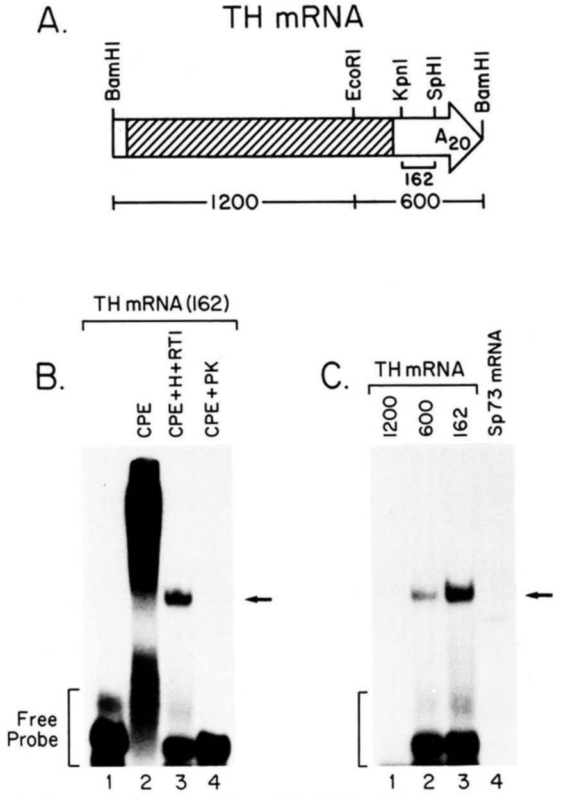


FIG. 1. *A*, restriction map of rat TH cDNA. *Open areas* represent the 5′- and 3′-UTR, and the *dashed area* represents the coding region. 1200, 600, and 162 represent fragments of TH mRNA obtained from *in vitro* transcription as described under "Materials and Methods," which were used in the gel retardation assays. *B* and *C*, formation of specific TH mRNA-protein complexes (*arrows*). Addition of cytoplasmic protein extracts (*CPE*) (40 μg) to the labeled RNA resulted in a smear-like shift (*panel B, lane 2*), which resolved into a single band after addition of heparin (*H*) and RNase T1 (*RT1*) (*panel B, lane 3*). Treatment with proteinase K (*PK*) abolished the band (*panel B, lane 4*). The specific band was present only when the 600 or 162-b TH mRNA fragments were used (*panel C, lanes 2* and *3*) but not when the 1200-b TH or plasmid Sp73 mRNAs were used (*panel C, lanes 1* and *4*).

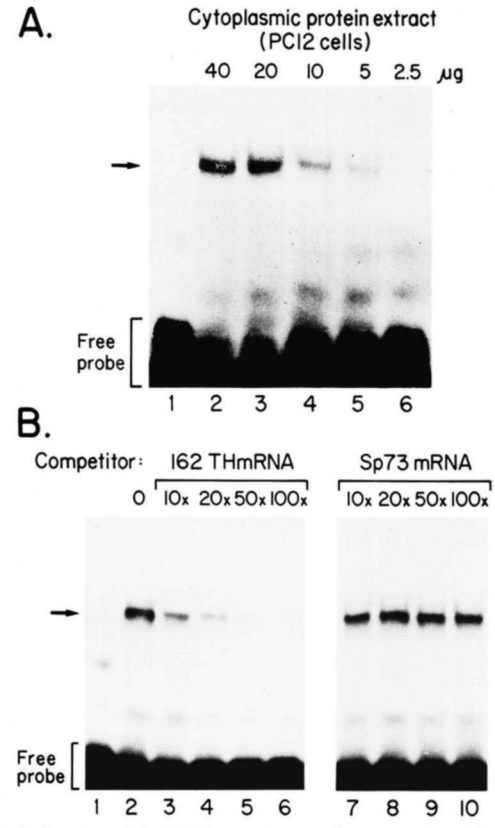


FIG. 2. *A*, the size of the RNA-protein complex was proportional to the amount of the protein used (indicated amounts of cytoplasmic protein extract are shown) (*lanes 2–6*). *B*, the binding of protein to TH mRNA was reduced and abolished with nonlabeled 162-b TH mRNA (*left*) but not with nonlabeled plasmid Sp73 mRNA (*right*).

statistically significant ($p < 0.001$, $n = 13$, Fig. 3*B*).

To identify the sequence within the 162-b fragment that forms the RNA-protein complex, RNase T1 mapping assays were performed. RNase T1 digests RNA after each G residue. Results from these experiments are shown in Fig. 4. The RNase T1 digest of the entire 162-b TH mRNA fragment, which was not incubated with proteins, is presented in *lane 1*, and its sequence is given in Fig. 4*B*. The RNase T1 digestion of the 162-b fragment gives two relatively long G-free regions of 28 and 21 b (*lane 1*). Fig. 4*A*, *lane 2*, shows the TH mRNA fragment that was eluted from the gel band following gel retardation assay, and *lane 3* shows the eluted RNA after additional digestion with RNase T1. The RNA fragment that was eluted from the gel following the gel retardation assay migrates as a 28-b fragment (compare *lanes 2* and *1*), which is not digested further by RNase T1 (*lane 3*). The sequence of this 28-b fragment is indicated with *bold* and *underlined letters*, and the sequence of the 21-b, G-free fragment is *underlined* in Fig. 4*B*. The results from this experiment indicate that the 28-b G-free fragment located between 1551–1579-b of 3′-UTR of TH mRNA binds to cytoplasmic protein. Identical results were obtained when either normoxic or hypoxic S-100 extracts were used for the RNase T1 mapping study.

To confirm that formation of the TH mRNA-protein complex involves the 28-b fragment, we performed selective RNase H cleavage of this fragment by addition of an antisense oligodeoxyribonucleotide that was complementary to the 28-b fragment. Following hybridization of the antisense oligonucleotide to the 28-b fragment of RNA, the RNA moiety was digested by RNase H present in the protein extracts (19). If the 28-b fragment is involved this should prevent protein binding. Results from this experiment show that this is indeed the case (Fig. 5).

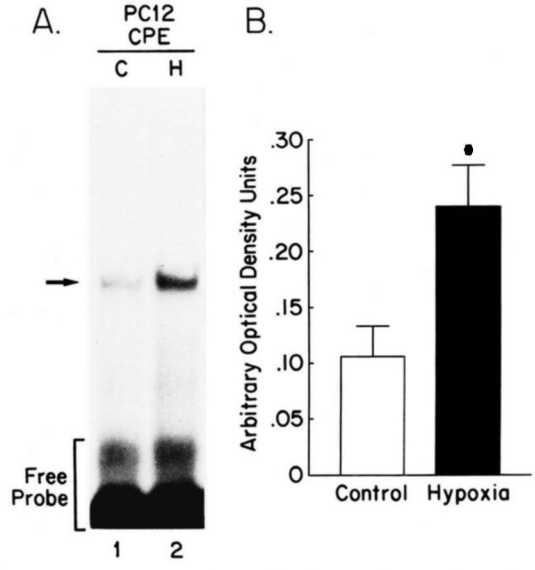
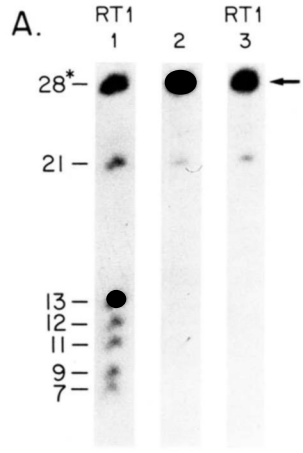


FIG. 3. **Effects of hypoxia on the formation of TH mRNA protein complex.** *A*, RNA gel retardation assay using cytoplasmic protein extracts (*CPE*) (20 μg) from PC12 cells exposed to normoxia (*C*) (*lane 1*) or hypoxia (*H*) (*lane 1*). *B*, averaged results (means ± S.E.) were obtained from optical density measurements in 13 independent experiments (normoxia, *white bar*; hypoxia, *black bar*). Hypoxia significantly increased formation of RNA-protein complex by 2.5-fold ($p < 0.001$).



B.

(1519) 5' UACCACCCACggUgCCAgggCCUUUCCCAAAg

UCUCCAUCCCCUUCUCCAACCUUUCCUggCCCAgAggCUUUCCCAUgUgUgU

ggCUgggCCCUUUgAUgggCUCCUCUUggACCCCCAUCCUCCCAACACUgCUUC

UCAACCAUgUCUUACUACUgCAUg 3' (1681)

FIG. 4. **Identification of the sequence on TH mRNA that binds protein using RNase T1 mapping.** *A*, *lane 1*, RNase T1 digest of the 162-b fragment of TH mRNA; *lane 2*, fragment of protected TH mRNA eluted from the RNA-protein complex; *lane 3*, eluted, protected RNA as in *lane 2* was further digested with RNase T1. RNA size markers are indicated in nucleotides. *Asterisk* indicates the protected sequence. *B*, the sequence of the 162-b fragment of TH mRNA from base 1519 to 1681. The protected sequence is *bold* and *underlined*. The 21 nucleotide G-free fragment is *underlined*.

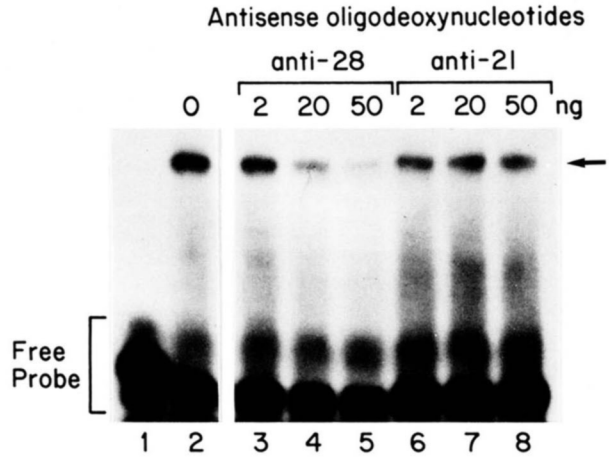


FIG. 5. **Inhibition of the TH mRNA-protein complex formation with oligonucleotide antisense to the 28-b fragment of TH mRNA.** Addition of indicated amounts (*lanes 3–5*) of the 28-b antisense oligonucleotide prevented formation of the complex. Addition of an antisense oligonucleotide that was complementary to the 27 b of TH mRNA (1633–1659 b) did not prevent formation of specific complexes (*lanes 6–8*).

Addition of 2–50 ng of the antisense oligonucleotide resulted in a substantial reduction in formation of the specific complex (*lanes 3–4*). However, addition of an antisense oligonucleotide complementary to bases 1633–1659 of TH mRNA (this region contains the 21-b long G-free fragment that sometimes yields a weak band in RNase T1 assay) did not cause reduction of the RNA-protein complex formation (*lanes 5–7*). This result is further evidence that the 28-b fragment identified by RNase T1 digestion is the protein-binding site in TH mRNA.

Analysis of this 28-b fragment of TH mRNA revealed that it is rich in pyrimidine ribonucleotides (86%). Recent findings have shown that pyrimidine-rich sequences bind proteins involved in RNA processing (for review, see Ref. 20). We therefore

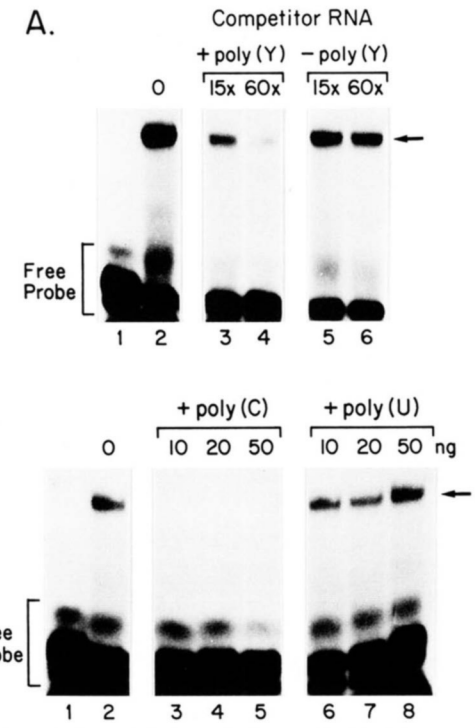


FIG. 6. **Competition of TH mRNA-protein binding with polypyrimidine RNA.** *A*, formation of the specific TH mRNA protein complex was competed with the RNA containing a string of 28 pyrimidines (*lanes 3* and *4*, *poly(Y)*$^+$) but not with the same RNA that did not contain a polypyrimidine sequence (*lanes 5* and *6*, *poly(Y)*$^-$). *B*, the binding was competed with poly(C) homoribopolymer (*lanes 3–5*) but not with poly(U) homoribopolymer. The amounts of competitior RNA are indicated.

performed experiments to determine if protein interactions with the 162-b fragment is reduced by competition with other pyrimidine-rich sequences. Results from these experiments are shown in Fig. 6. Protein binding was reduced when nonlabeled RNA that contained a fragment of 28 pyrimidines (poly(Y)$^+$; Fig. 6*A*, *lanes 3* and *4*,) but not with the same RNA lacking the poly(Y) sequence (poly(Y)$^-$; *lanes 5* and *6*) was added to the binding reaction. Interestingly, the binding was also competed by poly(C) but not by poly(U) homopolymers (Fig. 6*B*). This finding indicates that the TH mRNA-binding protein shows a high affinity for poly(C) sequences.

To determine the size of TH mRNA-protein complexes, the UV-cross-linking was performed, and the products were analyzed on an SDS-8% polyacrylamide gel. As shown in Fig. 7 one major RNA-protein complex of approximately 74 kDa was resolved. This complex occasionally appeared as a doublet. The complex was not observed after the binding reaction was treated with Proteinase K (*lane 8*). Moreover, the complex was abolished when excess (150 and 250 ng) of nonlabeled specific 162-b TH mRNA was used as competitor (*lanes 2* and *3*). Addition of the same concentration of nonlabeled, nonspecific plasmid RNA did not interfere with UV cross-linking (*lanes 4* and *5*). The formation of the 74-kDa complex was substantially increased when protein extracts used for UV cross-linking were from PC12 cells that had been exposed to hypoxia (compare *lanes 6* with *7*). This finding corresponds with the increased binding observed in the RNA gel retardation assays when protein was extracted from PC12 cells that had been exposed to hypoxia (see Fig. 3). The 74-kDa complex contains 28 nucleotides of RNA, which accounts for approximately 8 kDa of the complex. Thus the approximate size of the binding protein is approximately 66 kDa. We have also occasionally observed a smaller band representing an RNA-protein complex of 53 kDa (not shown). This complex was not regulated by hypoxia.

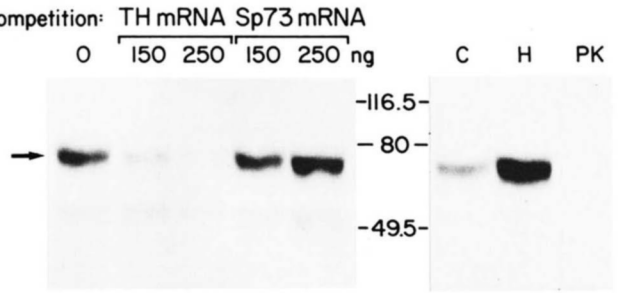


FIG. 7. **Identification of TH mRNA-protein complexes using UV cross-linking.** A major band of 74 kDa was identified (*arrow*). The appearance of the 74-kDa band was abolished by addition of nonlabeled 162-b TH mRNA (*lanes 2* and *3*) but not by nonlabeled plasmid Sp73 mRNA (*lanes 4* and *5*). The UV cross-linked complex was substantially increased when cytoplasmic protein extracts were used from PC12 cells that had been exposed to hypoxia (*H*) rather then normoxia (*C*) (compare *lanes 6* with *7*). Treatment with Proteinase K (*PK*) (*lane 8*) prevented appearance of the TH mRNA-protein complex.

DISCUSSION

In the present study we showed that the 3′-UTR of tyrosine hydroxylase mRNA contains a novel *cis*-element that interacts with cytoplasmic protein(s). Importantly, we showed that this interaction is augmented by hypoxia. We propose that this RNA-protein interaction is involved in regulation of TH mRNA turnover during normoxia and hypoxia (7).

We have identified a 28-b fragment located within the 3′-UTR of TH mRNA that is the site of protein binding. The exact sequence within the 28-b fragment that is required for protein binding was not determined in the present study. However, this sequence is pyrimidine-rich (86%) and contains an uninterrupted sequence of 11 pyrimidines, and the protein-binding activity can be reduced substantially by competition with sequences that contain long stretches of pyrimidines. These data indicate that the pyrimidine-rich feature of this fragment may be directly involved in protein binding. Computer analysis of the 3′-UTR TH mRNA shows that this 28-b sequence may be located within a stem-loop secondary structure, which is a common feature of RNA binding sequences.

The 28-b protein-binding fragment of the 3′-UTR of TH mRNA is highly homologous with a fragment of 3′-UTR of erythropoietin (EPO) mRNA that binds a cytoplasmic protein (ERBP) (18). It has been proposed that this sequence is involved in regulation of EPO mRNA stability in Hep3B cells (18). The region of EPO mRNA that binds ERBP contains a fragment (782–811 b) that is pyrimidine-rich (66%), with an uninterrupted sequence of 9 pyrimidines (bases 794–801). The sequence from base 789 to 811 is highly homologous (72%) with the identified protein-binding sequence in the 3′-UTR of TH mRNA.

Long polypyrimidine tracts are usually present within introns, immediately upstream from the 3′ splicing site. It has been shown that these polypyrimidine tracts participate in the splicing of various pre-mRNAs (21, 22). In addition, 5′ transcript leaders of ribosomal proteins mRNAs and several other mRNAs under translational control contain polypyrimidine tracts (23, 24). The identified pyrimidine-rich fragment of 3′-UTR of TH mRNA is located within the terminal exon (exon 13) of TH pre-mRNA, which to date has not been shown to be involved in alternative splicing (25, 26). We therefore suggest that the identified pyrimidine-rich sequence in the 3′-UTR of TH mRNA represents a novel element that regulates the turnover of TH mRNA.

Results from UV cross-linking experiments revealed that a 66-kDa cytosplamic proteins binds to the identified sequence of TH mRNA. It does not exclude the possibility that other proteins are involved in the TH mRNA-protein complex via a protein-protein type of interaction. Interestingly, the 66-kDa protein corresponds approximately in size to the 70-kDa protein, which was shown to bind to the 3′-UTR of EPO mRNA (18). However, in contrast to our results, no increase in the formation of the EPO mRNA-protein complex was observed during hypoxia (18).

Several polypyrimidine tract-binding proteins have recently been identified and characterized (for review, see Ref. 20). There is evidence that these proteins are located in the nucleus and are part of the RNA splicing apparatus (pPTB) (21, 22) or a member of family of proteins referred to as heterogenous nuclear ribonucleoprotein particles (I (27); C1 and C2 (28); K and J (29)). In this regard, C proteins show high affinity for the poly(U) (30), whereas the K and J proteins show high affinity for the poly(C) tracts (29). In the cytoplasm a 56-kDa protein has been identified that binds to the polypyrimidine sequence of the mRNA, which encodes ribosomal r-protein (24). It is difficult to determine if the TH mRNA-binding protein represents one of the earlier characterized polypyrimidine tract binding proteins. It should be noted that there is similarity in the size of the TH mRNA-binding protein (66 kDa) and the 68-kDa K or J heterogenous nuclear ribonucleoprotein particles (29) and to a lesser extent with 57–62-kDa pPTB (21, 22, 27). However, in contrast to the pPTB that can be competed from poly(Y) binding by poly(U) homopolymers (22), the binding to the TH mRNA can only be prevented by poly(C) homopolymers. Therefore, the binding properties of the TH mRNA-binding protein appears to be more similar to the K or J proteins (29). Because these proteins are found in the nucleus rather than in the cytoplasm (29), the TH mRNA-binding protein may be different and may represent a novel class cytoplasmic polypyrimidine-binding proteins.

The functional significance of the TH mRNA-protein binding was not elucidated in the present study. However, a major finding in the present study was that the intensity of TH mRNA 66-kDa protein-binding complex was increased during hypoxia. In view of the increased stability of TH mRNA and the increased binding of the 66-kDa protein to the 3′-UTR of TH mRNA measured during hypoxia, it is tempting to hypothesize that the binding of the 66-kDa protein is involved in regulation of TH mRNA stability. Interestingly, it was reported recently that the polypyrimidine tract-binding protein, heterogenous nuclear ribonucleoprotein particles I, binds to nuclease hypersensitive regions of the heterogeneous nuclear RNA-protein complexes (27). Therefore, it might be that the pyrimidine-rich tracts are nuclease hypersensitive and participate in the degradation of RNA. The binding of protein(s) may protect these sensitive regions from nucleases and thus promote stability of mRNAs that contain these binding sequences.

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