Post-transcriptional regulation of tyrosine hydroxylase gene expression by oxygen in PC12 cells

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Post-transcriptional regulation of tyrosine hydroxylase gene expression by oxygen in PC12 cells. Reduced oxygen tension (hypoxia) leads to increased stability of mRNA for tyrosine hydroxylase (TH), the rate limiting enzyme in biosynthesis of catecholamine neurotransmitters. Hypoxia increases the half life of TH mRNA from 10 to 30 hours. The increased stability of TH mRNA during hypoxia results from fast enhanced binding of a cytoplasmic protein (hypoxia inducible protein, HIP) to a pyrimidine-rich sequence within the 3' untranslated region (3'UTR) of TH mRNA. This novel *cis*-element is referred to as hypoxia-inducible protein binding site (HIPBS) and is located between bases 1551 and 1578 of the 3' UTR of TH mRNA. We identified that the (U/C)(C/U)CCCU motif within the HIPBS represents the optimum protein-binding site. Mutations within this region that abolish protein binding prevent also regulation of TH mRNA stability during hypoxia. UV-crosslinking and SDS-PAGE analysis of the HIPBS-protein complexes showed the presence of a major 50 kDa complex. The formation of the complex was augmented when protein extracts were obtained from PC12 cells exposed to 5% O_2. Importantly, formation of the 50 kDa complex was also increased when protein extracts were obtained from carotid bodies or superior cervical ganglia from rats exposed to 10% hypoxia for twenty-four hours.

While mammals thrive in a normobaric atmosphere with 21% oxygen, hypoxia represents a serious challenge to their survival. It is therefore not surprising that oxygen tension (pO_2) is a major factor that regulates function and expression of variety of proteins essential for adaptation to a low O_2 environment. An important example of a protein that is regulated by pO_2 is tyrosine hydroxylase (TH), the rate limiting enzyme in the biosynthesis of catecholamines. Catecholamine neurotransmitters play crucial role in central and peripheral neurohormonal regulation of the cardiovascular and respiratory systems during adaptation to acute and chronic hypoxia. Hypoxia stimulates the activity of TH as well as the synthesis and release of catecholamines in various catecholaminergic cells [1–11]. One well studied example is the hypoxic regulation of TH in O_2-sensitive type I cells of the carotid body that detect reduction of arterial pO_2 and trigger adaptive respiratory response. Stimulation of the carotid body by hypoxia (1 to 48 hr) causes the release of dopamine from type I cells [1, 2], augments dopamine synthesis [3], and results in increased enzymatic activity of TH protein [4, 5] and a several-fold increase in TH mRNA levels [11]. This regulation of TH mRNA during hypoxia is an intrinsic property of type I cells [11]. Neural or hormonal inputs are not required, and the effect is specific for

hypoxia. For example, it is not observed with hypercapnia, which is also a physiological stimulus of carotid body activity [11]. Short-term hypoxia (6 hr) does not stimulate TH gene expression in postganglionic sympathetic neurons or in the adrenal medulla cells [6], although an increase in TH enzyme activity has been reported in these tissues [5].

Because of the scarcity of O_2-sensitive cells in the carotid body, clonal cell lines are needed to study the regulatory mechanisms of hypoxia-mediated TH gene expression. We have identified a dopaminergic, pheochromocytoma derived cell line, PC12 as an excellent model for this purpose. During hypoxia PC12 cells depolarize and release dopamine [12]. In addition, hypoxia leads to augmentation of TH protein content and its enzymatic activity [13], and induces TH gene expression [14]. The sensitivity, magnitude and time course of these responses in PC12 cells are very similar to those measured in the carotid body O_2-sensitive type I cells [14]. The hypoxia-induced increase in the TH mRNA is mediated by a dual mechanism involving an increased rate of TH gene transcription and increased stability of TH mRNA (the half life of TH mRNA increased from 10 hr to 30 hr). During the first hours of hypoxia the increase in the total TH mRNA results primarily from fast transcriptional induction and the contribution of increased mRNA stability is small. During sustained hypoxia the transcriptional rate declines and post-transcriptional regulation contributes primarily to the overall increase in TH mRNA. Thus, an increase in TH mRNA stability is necessary to maintain TH mRNA at the elevated concentration during long-term hypoxia. In the present paper we summarize our studies on the molecular mechanisms involved in regulation of TH mRNA stability during hypoxia.

Methods

Cell culture techniques, exposures of animals to environmental hypoxia, *in vitro* transcriptions, procedures for preparation of protein extracts, gel retardation assays, UV-crosslinking and SDS-PAGE analysis were described previously [11, 14–16].

The wild-type or mutated within HIPBS TH cDNAs were inserted into the *Bam*HI site of the pcDNA3 eukaryotic expression vector (Invitrogen) that contains hypoxia-nonsensitive CMV promoter and neomycin resistance gene. Transfections were performed on 50 to 60% confluent plated PC12 cells using lipofectamine (Gibco, BRL) according to the manufacturer's protocol in the absence of serum or antibiotics. The selection for stably transfected cells was begun 48 hours after transfection by addition of medium containing 400 μg/ml of G418. Pools of stably

transfected cells were used. After exposures to normoxia or hypoxia cells were harvested, RNA was extracted using TRI reagent (MRC, Inc.), digested with the RNase-free DNase (Ambion), phenol-chloroform extracted, precipitated and processed for RNase protection assay (RPA). Ten micrograms of total RNA were hybridized with 200,000 cpm of the TH probe and 100,000 cpm of the 18 S probe in the hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA) at 42°C in a dry oven overnight. Samples were then digested with RNase A/T1 mixture at 37°C for 30 minutes, treated with proteinase K, precipitated and subjected to electrophoresis on a 8% denaturing polyacrylamide. Gels were exposed to X-ray film for 18 to 48 hours.

The TH riboprobe corresponds to *Hin*dIII-*Pst*I fragment of TH cDNA in the pcDNA3 vector. This probe has additional 30 base long sequence derived from the plasmid DNA (*Hin*dIII - *Bam*HI in pcDNA3). These 30 bases differentiate between the endogenous and exogenous TH mRNAs. High specific activity TH probe was transcribed from the template in pSP73 transcription vector (Promega) using SP6 polymerase and gel purified. The 18S probe was transcribed from the T7 18S template (Ambion).

Results

Identification of a novel cis-element in the 3' untranslated region of TH mRNA

An increase in TH mRNA stability correlated with the enhanced binding of a cytoplasmic protein to a sequence in the 3' untranslated region of TH mRNA [15]. Figure 1A shows the pattern of binding of proteins extracted from PC12 cells exposed to 21% O_2 or 5% O_2 to the 3'UTR of TH mRNA, as assessed by RNA gel retardation assays. The formation of this complex was enhanced when proteins were extracted from cells exposed to 5% O_2 from 1 to 18 hours (Fig. 1A). This enhancement in the complex formation was maintained when cells were treated with inhibitors of transcription for up to three hours (the longest time measured), indicating that ongoing transcription is not necessary, at least not for the initial induction in the binding (not shown). Analysis by RNase T1 revealed that the binding region was limited to the 27 base long (1552-1578), cytidine-rich fragment of TH mRNA that we refer to as the hypoxia-inducible protein binding site (HIPBS) [15]. Figure 1B shows a schematic representation of TH mRNA, including the sequence and localization of the HIPBS.

We performed a detailed mutational analysis of the protein binding site within HIPBS [16]. The sequences of important mutants and the quantification of the averaged results of formation of RNA-protein complexes are shown in Figure 1C. As expected, substitution of purines within HIPBS by cytidines [$C_{(6,18,19)}$] and to lesser extent with uridines [$U_{(6,18,19)}$] increased the binding. Importantly, we identified a mutation $A_{(10,11,12)}$ where the substitution of adenines for the CCU sequence completely abolished the binding. This mutated RNA did not compete for the binding of protein to the wild sequence. We further identified the optimal hypoxia-inducible protein binding site that was represented by the motif (U/C)(C/U)CCCU, where the core binding site is indicated by the boldface and underlined cytidines [16]. Substitutions of either one of the cytidines with purine or uridine abolished the protein binding. The HIPBS motif is highly conserved in TH mRNA from different species [16].
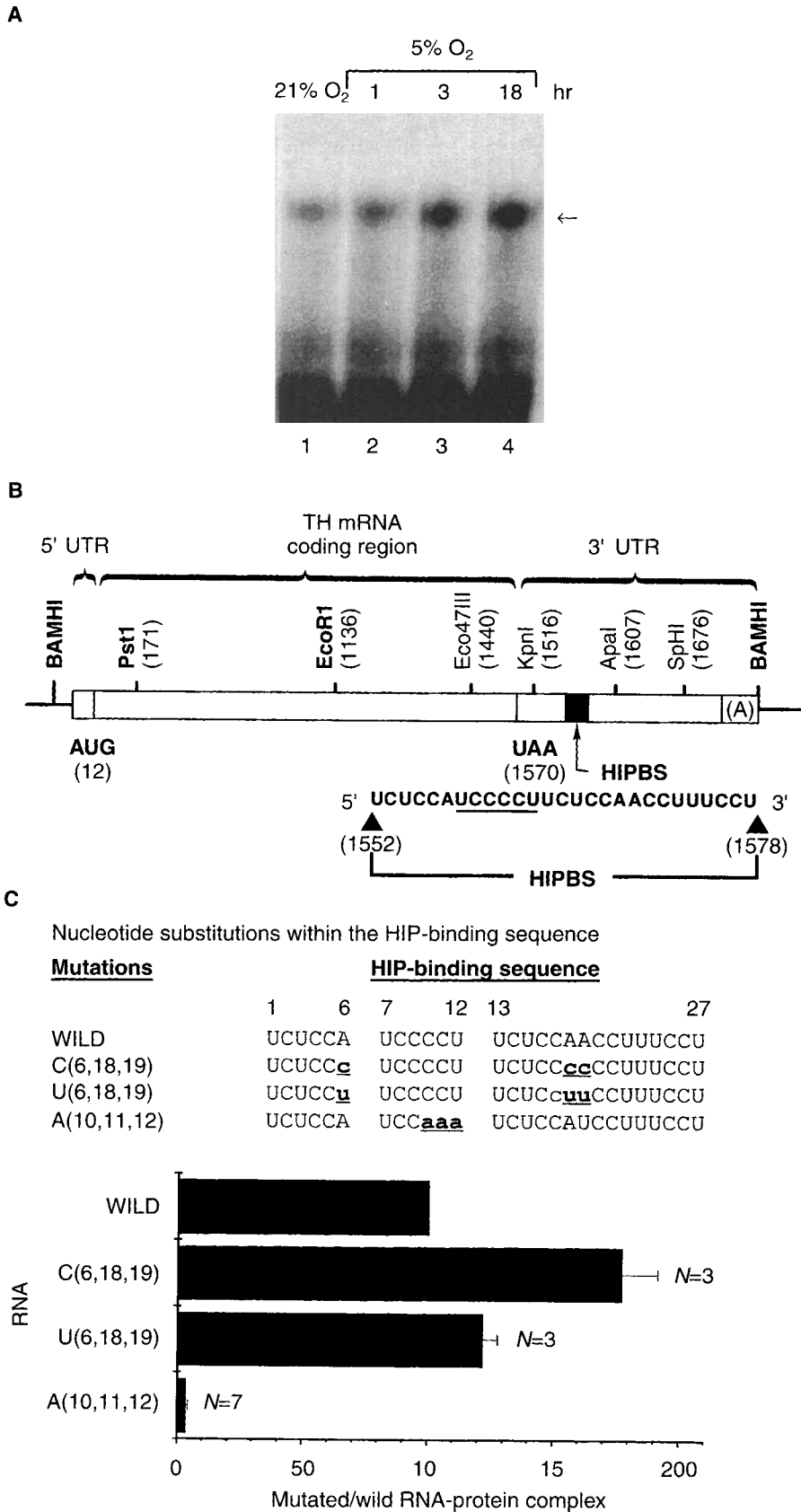
HIPBS-protein interaction is necessary for the regulation of TH mRNA stability during hypoxia

Because hypoxia simultaneously induces both an increase in the rate of TH gene transcription and an increase in TH mRNA stability, it is difficult to isolate the stimulant's effect on stability alone. We have developed a system, however, that focuses exclusively on the post-transcriptional regulation of TH gene expression during hypoxia. Our system uses stably transfected PC12 cell lines that, in addition to the endogenous TH gene, express wild-type (WT tTH mRNA) (UCCCCUU) or mutated within HIPBS (MUT$_{(10-13)}$ tTH mRNA) (UCC$AGAG$) TH mRNAs under control of a viral, hypoxia non-inducible cytomegalovirus (CMV) promoter in pcDNA3 eukaryotic expression vector. Both endogenous and transfected TH mRNAs are detected simultaneously using an RNase protection assay, with a probe that differentiates transfected mRNAs by an additional 30 bases located at the 5' end of the 5' untranslated region. Gel retardation assays in Figure 2A show that the MUT$_{(10-13)}$ transcript does not complex with PC12-cell cytoplasmic proteins. The degradation rates of transfected TH mRNAs were measured during normoxia (C) and hypoxia (H) 16 hours after the inhibition of transcription with actinomycin D. Figure 2B shows the result of such experiment. The samples collected before actinomycin D was added are represented by C_0. It is clear that while hypoxia stabilizes the WT tTH mRNA (open arrow, lanes 1 to 3), similarly to the endogenous eTH mRNA (solid arrow; compare lanes 3 and 2 or 5 and 6); the MUT$_{(10-13)}$ tTH mRNA (lanes 4 to 6) is degraded after 16 hours of transcriptional inhibition in both normoxic and hypoxic cells and is not stabilized by hypoxia during even shorter exposures to hypoxia.
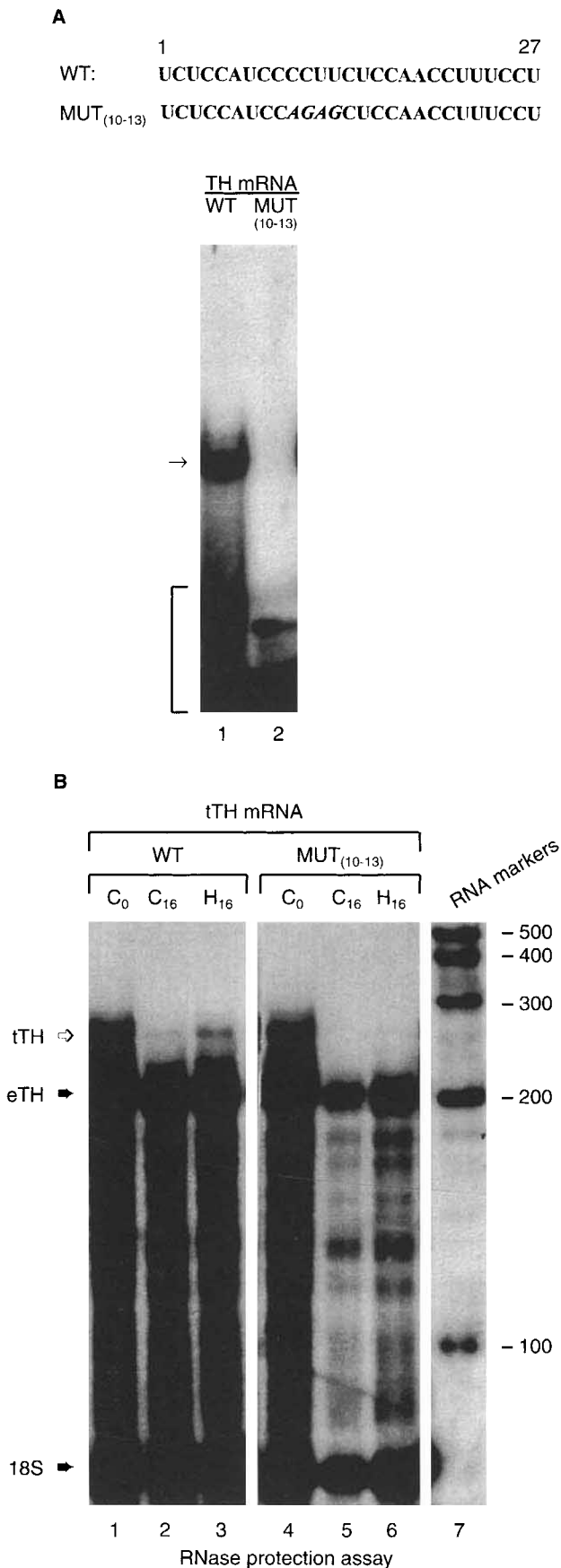
Trans-acting elements involved in regulation of TH mRNA stability during hypoxia

HIPBS binding protein were analyzed on SDS-PAGE following UV-crosslinking of the RNA-protein complexes (Fig. 3). The binding reactions were performed in the presence of poly(U) homopolymer that was used as a nonspecific competitor. A major 50 kDa and a minor 80 kDa bands were observed (arrows). Formation of both complexes was completely abolished when unlabeled 162 base TH transcript was added to the binding reaction, and neither band was formed when MUT$_{(10-13)}$ TH mRNA (Fig. 2A) was used in the binding reaction (not shown). Poly(C) RNA was a strong competitor of both complexes. Based on the above results, and on the fact that the protein core binding site is the UCCCCU motif, we concluded that the protein factors in both complexes show preferential affinity for the C-rich sequences, and that the C-rich binding proteins are necessary for the formation of the complex with the TH mRNA. Most importantly, we found an enhancement of the 50 kDa complex when proteins were extracted from hypoxic cells (Fig. 3A, lanes 2 and 4) as compared to normoxic cells (Fig. 3A, lanes 1 and 3). The formation of the 80 kDa complex also increased.

Figure 3B shows the formation of UV-crosslinked complexes from crude protein extracts of either carotid bodies (Fig. 3B, lanes 1 and 2), superior cervical ganglia (lanes 3 and 4) or adrenal glands (lanes 5 and 6). The carotid body is an extremely small structure (ca. 10,000 cells per carotid body), but 10 carotid bodies (from 5 rats) were sufficient to obtain approximately 80 μg of crude protein extract. The formation of the 50 kDa complex was

A

5% O$_2$

21% O$_2$ | 1 | 3 | 18 | hr

1 2 3 4

B

TH mRNA
coding region

5' UTR   3' UTR

BAMHI
Pst1 (171)
EcoR1 (1136)
Eco47III (1440)
KpnI (1516)
ApaI (1607)
SpHI (1676)
BAMHI

(A)

AUG (12)

UAA (1570) — HIPBS

5' UCUCCAUCCCCUUCUCCAACCUUUCCU 3'

▲ (1552)   ▲ (1578)

HIPBS

C

Nucleotide substitutions within the HIP-binding sequence

Mutations	HIP-binding sequence		
	1 6	7 12	13 27
WILD	UCUCCA	UCCCCU	UCUCCAACCUUUCCU
C(6,18,19)	UCUCC**c**	UCCCCU	UCUCC**cc**CCUUUCCU
U(6,18,19)	UCUCC**u**	UCCCCU	UCUCc**uu**CCUUUCCU
A(10,11,12)	UCUCCA	UCC**aaa**	UCUCCAUCCUUUCCU

RNA

WILD

C(6,18,19) — N=3

U(6,18,19) — N=3

A(10,11,12) N=7

0   50   10   15   200

Mutated/wild RNA-protein complex

Fig. 1. *Identification of a novel cis-element in the 3' untranslated region of TH mRNA.* **A.** Gel retardation assay showing that protein binding to HIPBS is increased when protein extracts are obtained from PC12 cells exposed to hypoxia (1 to 18 hr). **B.** Schematic representation of TH mRNA and position and sequence of HIPBS. **C.** Average results showing effects of mutations within HIPBS (table) on the formation of HIPBS-protein complex.

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A

1   27
WT: UCUCCAUCCCCUUCUCCAACCUUUCCU

MUT$_{(10-13)}$ UCUCCAUCC*AGAG*CUCCAACCUUUCCU

TH mRNA
WT MUT
(10-13)

1   2

B

tTH mRNA

WT   MUT$_{(10-13)}$

C$_0$ C$_{16}$ H$_{16}$ C$_0$ C$_{16}$ H$_{16}$ RNA markers

— 500
— 400

— 300

tTH ⟹

— 200

eTH ➡

— 100

18S ➡

1   2   3   4   5   6   7
RNase protection assay

Fig. 2. *Mutations within HIPBS that abolish protein binding prevent regulation of TH mRNA stability during hypoxia.* **A.** Gel retardation assay showing that mutation MUT$_{(10-13)}$ prevents formation of the RNA-protein complex. **B.** RNase protection assay showing regulation of mRNA stability for transfected wild-type (WT, open arrow, lanes 1 to 3) or transfected mutated (MUT$_{(10-13)}$, lanes 4 to 6) TH mRNAs. Endogenous TH mRNA is indicated with a solid arrow. Cells expressing transfected TH mRNAs were treated with actinomycin D (5 µg/ml) and exposed to normoxia (C) or hypoxia (H) for 16 hours.

particularly enhanced when protein extracts were obtained from carotid bodies and superior cervical ganglia, but not adrenal glands, from rats exposed to 10% hypoxia for twenty-four hours.

Discussion

The results presented in this paper indicate that the interaction between the pyrimidine-rich HIPBS element and the hypoxia-inducible, poly(C) binding protein(s) is necessary for regulation of TH mRNA stability during hypoxia. The pyrimidine-rich sequences are primarily known to regulate splicing or translation [17]; however, cytidine-rich elements have also been shown to mediate the erythroid cell-specific stability of α$_2$-globin mRNA [18, 19]. These data are consistent with our hypothesis that cytidine-rich HIPBS and its binding protein regulate TH mRNA stability. The HIPBS-like elements are present in 3' UTRs from other mRNAs that are likely to be regulated by hypoxia. We found HIPBS-like elements in the 3' UTR of erythropoietin (EPO), inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNFα), myoglobin (MYO), tryptophan hydroxylase (TPH) and vascular endothelial growth factor (VEGF) mRNAs. In the case of EPO mRNA, binding of cytoplasmic protein to the HIPBS-like region was reported [20]. On the other hand, VEGF mRNA that is also stabilized during hypoxia is implicated to be regulated by a different mechanism although the HIPBS-like elements are also present in the 3' UTR of VEGF mRNA [21]. In other cases there is only indirect evidence for post-transcriptional regulation.

We have previously reported that the HIPBS-protein complex was approximately 74 kDa [15]. Currently we see the 50 kDa complex as the major one because we have used poly(U) RNA as a nonspecific competitor. When UV-crosslinked complexes are formed without poly(U) RNA, a complex migrating at approximately 80 kDa is dominant. After poly(U) is added, however, most of this complex disappears and the 50 kDa complex becomes dominant. Since both 50 kDa and 80 kDa complexes are induced by hypoxia, both can be involved in binding to TH mRNA. The currently known poly(C) binding proteins include the 66 and 64 kDa nuclear proteins K and J [22], the 43 kDa cytoplasmic poly(C)-binding protein that binds to α$_2$ globin mRNA (α-PCBP) [23, 24], and the 48 kDa LOX mRNA binding protein that binds to the multiple repeats of pyrimidine rich motifs C$_4$A/GC$_3$UCUUC$_4$AAG, and inhibits 15 lipoxygenase mRNA translation [25]. Based on our studies with the antibodies against the K protein (3C2 [22], gift from Dr. G. Dreyfuss) we found that protein K was not involved in the formation of the TH mRNA-protein complex, although it was present in the cytoplasmic extracts (data not shown). The 43 kDa α-CP1 and α-CP2 proteins bind preferably to the CCUCCC sequences and are involved in regulation of the α$_2$ globin mRNA stability [18, 19, 23, 24]. These

A

B
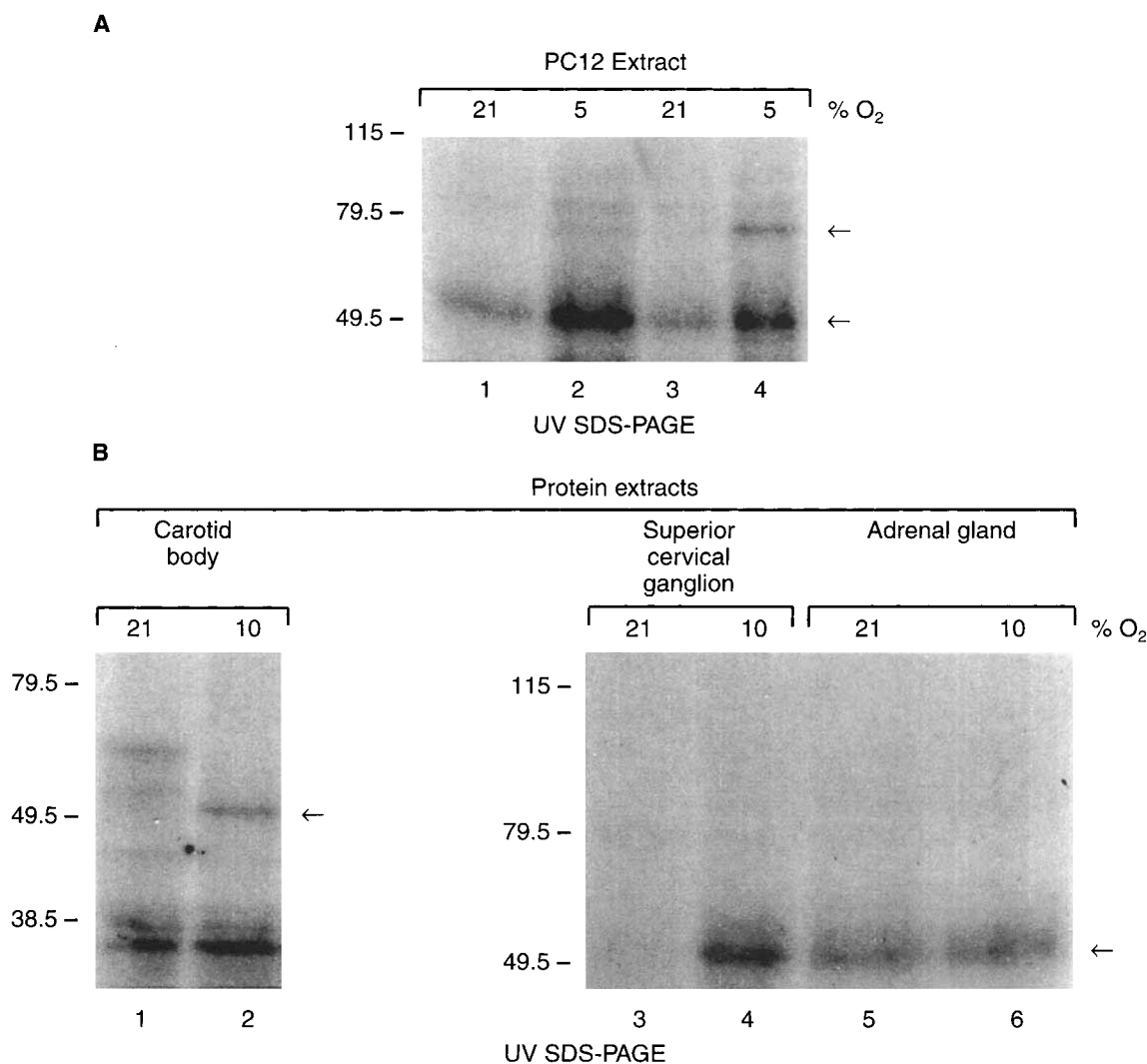
Fig. 3. *Identification of the HIPBS-protein complexes using UV-crosslinking.* The major 50 kDa and minor 80 kDa complexes are indicated with arrows. A. Formation of the 50 kDa and 80 kDa complex is induced when protein extracts are prepared from PC12 cells exposed to hypoxia (lanes 2 and 4) as compared to normoxia (lanes 1 and 3). Two independent experiments are shown. B. Formation of the 50 kDa, but not the 80 kDa complex is induced when protein extracts are prepared from carotid bodies (lanes 1 and 2) or superior cervical sympathetic ganglia (lanes 3 and 4) obtained from rats exposed to 10% hypoxia for four hours. The formation of the 50 kDa complex is not induced by hypoxia in adrenal gland (lanes 5 and 6).

proteins show similarities with the TH mRNA binding protein that form the 50 kDa complex as far as molecular weight and affinity for the C-rich sequences. We have recently initiated purification of the HIP using poly(C) agarose affinity chromatography and found that we have enriched for protein of approximate molecular weight of 46 kDa. We are presently continuing identification of this protein factor.

Regulation of gene expression at the post-transcriptional level may be particularly important during energy deprivation, such as hypoxia or ischemia, because it allows for increases in the steady-state levels or maintenance of mRNA that is already available in the cell. Very little is known about regulation of mRNA stability that physiologically occurs in the whole animal. Our data showing that binding of protein to HIPBS is increased when proteins are extracted from carotid bodies or superior cervical ganglia obtained from hypoxic animals indicate that TH

mRNA may be regulated at the post-transcriptional level in these tissues during hypoxia. Therefore, the induction of TH mRNA during hypoxia in type I cells of carotid body [11] may involve post-transcriptional regulation. On the other hand, we previously reported that the TH mRNA is not increased in the superior cervical ganglion during short-term, mild hypoxia [11]. The present observation showing increased binding of protein to HIPBS during hypoxia indicates that if TH mRNA stability is increased in the sympathetic neurons during hypoxia, it may help to maintain the existing concentration of TH mRNA rather then to augment it.

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