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Research Report

Cloning of a DNA binding protein that is a tyrosine kinase substrate and recognizes an upstream initiator-like sequence in the promoter of the preprodynorphin gene

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Abstract

A 90 bp fragment prepared from the promoter region of the rat preprodynorphin gene formed a complex with rat brain nuclear extracts as assessed by gel mobility shift assays. An 8 base pair sequence, CACTCTCC, termed upstream regulatory element (URE), was identified within this fragment as a binding site by DNase 1 footprint analysis and gel mobility shift assays with synthetic oligonucleotides. The URE is a consensus sequence for a transcription initiator (Inr) element although in the preprodynorphin promoter it is located upstream at −208 and overlaps a region conserved between rat and human promoters. A unique 310 amino acid protein (UreB1) that specifically bound the URE was cloned from a rat brain cDNA library using the URE-containing oligonucleotide. Recombinantly expressed, affinity purified UreB1 protein retains specific binding to the URE oligonucleotide. UreB1 contains a tyrosine kinase phosphorylation consensus and binding is enhanced following phosphorylation with the $p43^{v\text{-}abl}$ tyrosine kinase. The UreB1 tyrosine phosphoprotein increases transcription in vitro, consistent with a positive transcriptional regulatory function. UreB1 transcripts are well expressed in subsets of neurons in multiple brain areas suggesting that, in addition to regulation of the preprodynorphin gene, it may have a more generalized role in gene transcription.

Key words: Transcription factor; Proto-oncogenes; Tyrosine kinase; Phosphotyrosine; Dynorphin

1. Introduction

The preprodynorphin gene is expressed in neural and non-neural cells and codes for a precursor protein from which a family of structurally-related opioid peptides are enzymatically cleaved [5,10,16,18,25,31,32] (reviewed in ref. 1). Dynorphin peptides have been suggested to modulate a variety of responses, for instance, nociception [22,26,45,48], posterior pituitary secretion [3,39,59], intestinal peristalsis [36], feeding [46], and reproduction [6,11,35,43]. Pharmacological or physiological stimuli that mediate changes in preprodynorphin gene expression have been characterized in several CNS circuits [12,24,29,39,42,50,55]. These in vivo studies have shown that profound and sustained changes in preprodynorphin mRNA levels can accompany alterations in neural function such as activation of nociceptive inputs to spinal cord [24,28,29] or seizure activity in hippocampal formation [12,27,37,42]. Thus, dynorphin synthesizing neurons have been postulated to participate in long-term adaptive (or maladaptive) changes in neuronal excitability associated with persistent pain, stress and epileptic processes. While in many dynorphin-containing circuits detailed analyses of biochemical and neuroanatomical parameters have been conducted, the molecular biological mechanisms governing preprodynorphin gene expression are less well established.

The increase in preprodynorphin mRNA levels following neuronal activation, in part, reflects induction of preprodynorphin gene transcription. Hereafter, we will refer to this gene and its promoter as the dynorphin gene or dynorphin promoter. One mechanism through which increases in transcription are effected is via formation or modulation of protein-DNA complexes at distinct DNA sequences called enhancers or

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cis-acting elements. The dynorphin gene and a portion of its promoter region have been cloned and a 510 bp fragment of 5′-flanking sequence was functionally characterized for *cis*-acting elements in transient expression assays [10]. The sequence between bases + 58 and −122 confers constitutive expression on a CAT reporter gene and both positive and negative elements, as well as a minor alternative promoter, appear to be present between −122 and −510 in the 5′ flanking region [10]. Characterization of larger fragments of the 5′ flanking region indicates the presence of a cAMP responsive element farther upstream from the transcription initiation site [33,34,44]. These transient expression studies in cell lines broadly indicate regions of DNA that contain enhancer or *cis*-acting elements but do not identify the proteins that utilize these DNA elements as sequence-specific recognition sites.

A variety of strategies can be employed to identify the proteins that bind to regulatory DNA sequences in the promoter. In the present studies, we examined small restriction fragments obtained from approximately 1.9 kb of 5′-flanking region for potential protein binding sites with gel mobility shift assays using extracts of cell nuclei isolated from whole rat brain. Information generated from these experiments and subsequent footprinting was used to synthesize a double stranded oligonucleotide probe with which to screen a rat brain cDNA expression library. A unique protein, UreB1, was obtained which, when highly purified, retains binding to the nucleotide recognition sequence. In situ hybridization shows that the transcript of this gene has a more widespread distribution in brain than that for dynorphin peptide-expressing neurons. The UreB1 protein contains a consensus domain for tyrosine phosphorylation and, once phosphorylated, increases transcription of a reporter gene in vitro. These data suggest that the protein may be involved in coupling tyrosine kinase signalling pathways to transcriptional control process.

2. Materials and methods

2.1. Isolation and evaluation of restriction fragments from the dynorphin promoter

An approximately 1.9 kb fragment of plasmid pRDP16 which contains 1859 bp of upstream sequence was gel purified and digested by *HpaI* I or *Dde*I. The digest was electrophoresed in a 1.1% agarose gel and the individual bands were cut out, electroeluted into dialysis bags and the fragments ethanol precipitated. For gel shift analysis, each restriction fragment was labeled by filling in the recessed 3′ ends with [^{32}P]deoxynucleotides and Klenow fragment of DNA polymerase 1.

2.2. Preparation of brain nuclear extracts

Brain nuclear extracts were prepared by a modification of a previously published method [56]. Tissue was collected into ice-cold sucrose-magnesium-HEPES buffer (SMH: 0.32 M sucrose, 3 mM $MgCl_2$, 10 mM HEPES, pH 6.8), rinsed in the same buffer, weighed quickly, and homogenized in 6–8 volumes (wt:vol) of ice cold SMH buffer with 10 to 12 strokes in a motor driven teflon glass homogenizer. When several brains were processed, the homogenate was filtered through a nylon mesh prior to centrifugation at 2,500× *g* for 10 min. The pellet was resuspended in 2.1 M sucrose, 1 mM $MgCl_2$, 10 mM Hepes, pH 6.8, mixed well and centrifuged at 48,000× *g* for 1–1.5 h. The pellet was recovered in approximately 0.5 to 10 ml of SMH (i.e. an excess in relationship to the pellet) and repelleted in a swinging bucket micro or floor model centrifuge at 2,500× *g*. The volume of the pellet was estimated, and 2 to 3× the pellet volume of 1× Buffer C (420 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.25 mM dithiothreitol, 0.5 mM phenylmethysulfonylfluoride, 1.5 mM $MgCl_2$ and 20 mM Hepes, pH 7.5) [9] was added followed by a volume of 2× Buffer C equal to the pellet volume, making the final NaCl concentration 420 mM. This mixture was homogenized with a Dounce homogenizer or, if the pellet was small, directly with a pestle made of dental acrylic to fit a 1.5 ml centrifuge tube. The 1.5 ml centrifuge tube was spun at 48,000×g in a Beckman J-21 centrifuge and JM 18.1 rotor. The protein content of the supernatant was measured with the BioRad protein assay reagent [2].

2.3. Gel mobility shift assay and footprinting

Binding studies with the various restriction fragments and whole rat brain nuclear extract were performed as described [9,23,53]. Ten μg of nuclear protein, 2.5 μg of poly dI-dC and different amounts of *Hpa*II-*Hpa*II restriction fragment as competitor were used in the gel mobility shift assay. Binding assays were carried out in 1× Buffer C (see above section) but the final NaCl concentration in the assay was adjusted to be between 80 and 100 mM. Binding reactions were analyzed by polyacrylamide gel electrophoresis (PAGE) in a 4% native gel (29:1 acrylamide:bisacrylamide) after 30 minutes incubation of the binding reaction at room temperature. DNase I footprinting of the *Hpa*II-*Hpa*II fragment was carried out as described [38,40]. The *Hpa*II-*Hpa*II fragment was blunted by cutting a small fragment off the 3′ end with *Sma*I and then 5′ end-labeled with α-[^{32}P]deoxynucleotides by filling in with Klenow fragment of DNA polymerase I. 50,000 cpm of single strand labeled fragment was incubated with 10 μg of rat brain nuclear extract. Each reaction was run in duplicate and analyzed by electrophoresis through a 5% long ranger gel (J.T. Baker, Philipsburg, NJ). A double-stranded oligonucleotide (+strand: 5′ cgagagcactctcctccacatcac 3′) was synthesized and used in subsequent gel shift analyses, screening of a rat brain cDNA library, and for characterizing the binding of recombinant proteins.

2.4. Cloning and sequencing UreB1

A directionally-cloned rat brain cDNA expression library was constructed in the Uni-ZAP XR lambda bacteriophage vector (Stratagene, La Jolla, CA). Inserts of cDNA are cloned in the sense orientation, in frame, within the N-terminal region of the LacZ gene. Expression of a LacZ-fusion protein can be induced by incubation of the plates of phage plaques with nitrocellulose filters soaked in 100 mM isopropyl-*thio*-β-galactoside (IPTG). The filters, with the expressed proteins bound, were screened with the above double-stranded 24mer oligonucleotide labeled with [^{32}P]ATP with T_4 polynucleotide kinase. We followed the protocol of Singh et al. [53], which does not utilize an initial denaturation step and successive renaturation [58]. One positive colony was found among 2×10^6 recombinants which retained oligonucleotide binding activity upon plaque purification and rescreening. After plasmid rescue, the cDNA insert was sequenced by the dideoxynucleotide chain termination method using the Sequenase kit from United States Biochemicals (Cleveland, OH). The clone was designated as upstream regulatory

element binding protein 1 (UreB1). Sequencing of the clone showed that the cDNA insert began at an EcoRI site that was within the coding sequence (Fig. 3). Consequently, the bacteriophage library was rescreened with the UreB1 insert, at low stringency (1×SSC, 42°C) to obtain additional clones with which to further characterize the original UreB1 clone. Twenty-seven additional colonies were obtained and 10 μl of overnight culture from these colonies were spotted onto a nitrocellulose membrane. This membrane was subjected to a final stringent wash (0.1×SSC at 68°C for 1 h) and six colonies were obtained. The one with the largest insert (2.4 kb) was sequenced which showed that the extra bases mainly represented a long stretch of 5′ non-coding region. Based on this result and binding studies with bacterial extracts, all of the of the subsequent characterization and subcloning was performed with the original UreB1 clone.

2.5. Preparation of bacterial cell extracts

Crude E. coli cell extracts from the UreB1 clone were examined for specific gel shift activity. Ten ul of an overnight culture was transferred into 10 ml of fresh LB ampicillin (50 μg/ml) broth, grown at 37°C until the O.D. at 600 nm was close to 0.9. IPTG was then added to a final concentration of 1–2 mM and the cells were grown for another 6 h at 37°C. Cell pellets were collected by centrifugation at 4,000 rpm at 4°C, resuspended and washed once in 1×C buffer (see above). The cells were then subjected to 3 freeze-thaw cycles and debris were pelleted by centrifugation at 50,000× *g*. Ten μg of protein from the bacterial lysate supernatant was used for the gel mobility shift assays which were analyzed in a 6% non-denaturing polyacrylamide gel. Analysis of the protein extract was done under denaturing conditions with a 10% polyacrylamide-SDS gel. Gels and molecular weight markers were obtained from Novex Inc. (San Diego, CA).

2.6. Expression of UreB1 in central and peripheral nervous systems

RNA blots were prepared as described [13,14]. Total RNA was isolated by the guanidinium-$CsCl_2$ centrifugation method [4]. Blots were probed with either an *Xho*I-*Xho*I restriction fragment of UreB1 labeled by random priming or a gel purified synthetic 48 mer oligonucleotide (5′ tagctgaggtggttggggttacagtgggaagatggattgatcgtgtag 3′) that was end-labeled by polynucleotide kinase. The oligonucleotide probe was complementary to bases 1015 to 1072 which are located at the 5′ end of the open reading frame of the UreB1 cDNA. The cDNA insert and the oligonucleotide probe gave identical results. In situ hybridization was performed as described [48] on tissue obtained from rats that had been fixed via intracardiac perfusion with a 4% paraformaldehyde, phosphate buffered saline solution. Tissue sections (18 μm thick) were thaw-mounted onto gelatin-coated slides and hybridized with the oligonucleotide probe overnight, washed, and coated with Kodak NTB-3 emulsion. Exposure time was 5 weeks.

2.7. Production and purification of recombinant UreB1

The pQE11 vector was obtained from Qiagen (Chatsworth, CA). An *Xho*I-*Xho*I fragment from the UreB1 cDNA insert was subcloned in-frame into the appropriate pQE11 vector; the orientation was confirmed by DNA sequencing. The pQE11-UreB1 construct contained nearly the entire UreB1 coding sequence (less 5 amino acids, MIISR, of the N-terminus) and the 3′ untranslated region. The protein was produced by transforming E. coli strain M15 with the pQE11-UreB1 fusion vector. From an overnight culture, 0.3 mls were transferred to 300 ml of fresh superbroth containing 50 μg/ml ampicillin and 20 μg/ml kanamycin. IPTG was added to a final concentration of 2 mM when the O.D. at 600 nm of the culture reached 0.7–0.9. The bacteria were harvested after 6 h further growth at 37°C. Preparation of the bacterial lysate and purification of recombinant protein were performed according to the protocol provided by the manufacturer (Qiagen). The bacterially expressed UreB1 recombinant protein was stored in the elution buffer of 8M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 5.9 at 4°C.

2.8. Phosphorylation of recombinant UreB1

One μg of the UreB1 recombinant protein was incubated with the $p43^{v\text{-}abl}$ tyrosine kinase according to the manufacturer's protocol (Oncogene Science, Uniondale, NY). Phosphorylation reactions were analyzed by SDS-PAGE and autoradiography. The presence of a tyrosine phosphate following reaction with the v-*abl* kinase was determined by SDS-PAGE and western blotting with an antiphosphotyrosine-HRP conjugated antibody (ICN, Irvine, CA) and the enhanced chemiluminescence reaction using reagents from Amersham (Rockford, IL). UreB1 that had been phosphorylated with non-radioactive ATP was used in gel shift assays or for in vitro transcription reactions.

2.9. In vitro transcription

The basic in vitro transcription assay was performed as described [38]. The template used was constructed from a dynorphin minimal promoter-CAT reporter construct cloned into pCAT basic plasmid [10,44]. The minimal promoter encompassed bases −135 to +65 and the URE oligonucleotide ligated into the *Hin*dIII-*Pst*I site of the pCAT basic polylinker immediately upstream from base −135 of the minimal promoter. The specific transcripts were detected by solution hybridization and electrophoresis. Briefly, RNA pellets from the transcription reaction were resuspended in 10 μl of hybridization buffer (400 mM KCl, 500 mM Tris HCl, pH 8.3) containing a 5′ end-labeled oligonucleotide antisense to bases 233 to 252 of pCAT basic. These bases are near the 5′ end of the chloramphenicol acetyltransferase transcript that is run off from the URE/dynorphin minimal promoter/CAT template (Fig. 6). The hybridization mixture was heated to 70°C for 5 min, cooled until reaching 32°C or less and the total reaction was loaded on a 6% non-denaturing polyacrylamide gel and electrophoresed with 0.5×TBE as the running buffer. The results were visualized by exposing the dried gel to a phosphoimager plate and reading out the stored signal from radioactive decay with a Molecular Dynamics (Sunnyvale, CA) phosphoimager device.

3. Results

The initial gel mobility shift analysis was performed on several small DNA restriction fragments of between 90 and 200 bp in length. Two of them clearly exhibited protein-DNA complex formation with the brain nuclear extract. One of these active fragments, a 90 bp *Hpa*II-*Hpa*II restriction fragment located at −223 to −133, was examined further (Fig. 1A,B). Complex formation with the labeled 90 bp *Hpa*II fragment was specifically competed by addition of increasing amounts of unlabeled 90 bp restriction fragment. Complex formation, as determined by DNase I footprinting with whole brain nuclear extracts, could be attributed to an 8–15 bp region within the 5′ portion of the 90 bp *Hpa*II fragment (Fig. 1C). Two aspects of this sequence were of note: first, the sequence, CACTCTCC, matches

the degenerate consensus for an initiator (Inr) element [15,49] and, second, the Inr-like sequence overlaps at its 3′ end the sequence, CTCCTCCACATC, which is one of several long sequences that are fully conserved between rat and human [10,31]. The sequence also has homology with the recognition element for some members of the c-*ets* family of cellular protooncogenes [23]. The sequence was named upstream regulatory element (URE) to distinguish it from the Inr elements which are usually nearer to the site of transcription initiation [49,54].

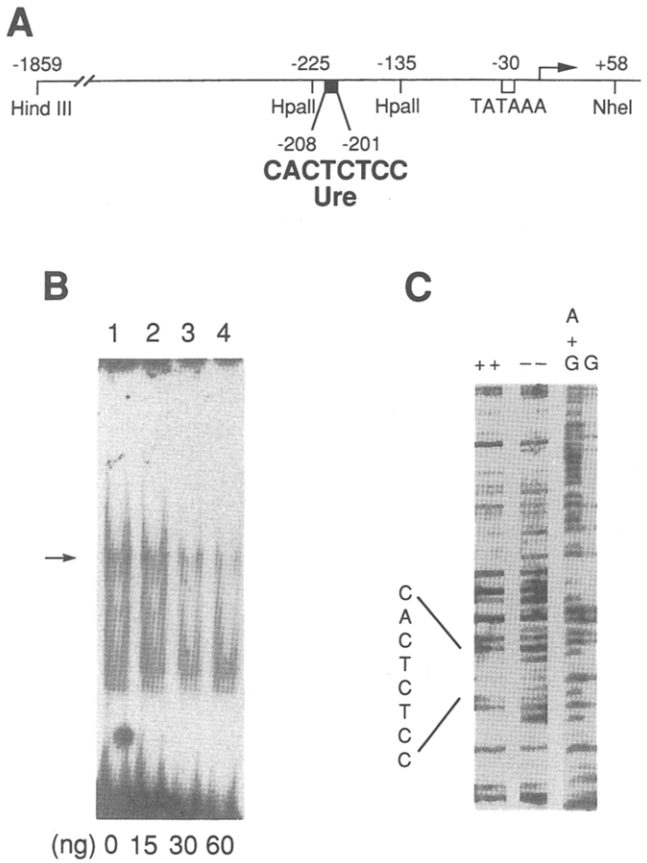

Fig. 1. Identification of the URE site. A: map of ~1.9 kb of *Hin*dIII-*Nhe*I restriction fragment spanning nucleotides −1859 to+ 58 of the rat dynorphin gene and promoter region which was used as the starting material for further digestion. The URE site is depicted as a filled square with sequence underneath; TATA box as an open square, and the transcription initiation start site is at the rightward directed arrow. B: gel mobility shift using the 90 bp *Hpa*II-*Hpa*II restriction fragment and extract of isolated nuclei prepared from whole rat brain. Different amounts (ng) of *Hpa*II-*Hpa*II restriction fragment as competitor were used in the binding reactions as indicated at the bottom of the figure. C: DNase I footprint of the *Hpa*II-*Hpa*II fragment. Each reaction was run in duplicate; (+) and (−) represent lanes from reactions with and without nuclear extract, respectively. The right lanes contain the adenosine-guanine specific Maxam-Gilbert chemical sequencing reactions [39]. The sequence corresponding to the strongest footprint is shown to the left and is what we have designated the upstream regulatory element (URE).

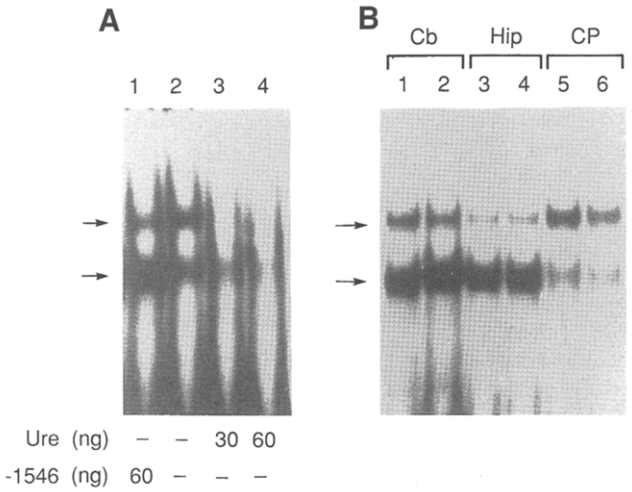

Fig. 2. A: a 24 nucleotide synthetic double-stranded oligonucleotide centered on the URE sequence formed two specific complexes (arrows) when incubated with whole brain nuclear extract. Complex formation was competed with increasing amounts (ng) of unlabeled URE oligonucleotide but not with a 60-fold excess of a dissimilar oligonucleotide from the −1546 region of the dynorphin promoter. B: regional variation in composition of URE-nuclear protein complexes. Gel mobility shift assays using 10 μg of nuclear extract prepared from 3 different brain regions were analyzed in duplicate: cerebellum (Cb, lanes 1, 2), hippocampus (Hp, lanes 3, 4) and caudate-putamen (CP, lanes 5, 6). The lower complex is very abundant in cerebellum and hippocampus compared to caudate-putamen whereas the upper complex is barely present in hippocampus but readily detectable in cerebellum and caudate-putamen.

The region of the footprint centered on the 8 bp URE sequence was tested for complex formation using a synthetic 22 bp double-stranded oligonucleotide. With whole brain nuclear extract (Fig. 2A), complex formation was observed which was competed by unlabeled URE oligonucleotide. Lack of competition with an unrelated oligonucleotide, 5′ GGCTGCTGCGTCAGAGCATG 3′, from the −1546 region of the dynorphin promoter [33,34,44] (which is similar to the ENKCreII enhancer [7,8]) showed that complex formation was specific for the URE sequence. With whole brain nuclear extract and the URE oligonucleotide as probe, we observed *two* complexes of approximately equal abundance. Additional analysis of nuclear extracts from three brain regions, caudate-putamen, hippocampus and cerebellum, showed that each region contained differing amounts of the two complexes (Fig. 2B). One interpretation consistent with these data is that complex formation with the URE involves more than one protein whose concentrations vary between different regions of the nervous system. Alternatively, a biochemical process that regulates binding of a single protein (e.g. homodimerization) may be different between the different regions.

Using the ^{32}P-labeled URE oligonucleotide, a rat cDNA expression library was screened. One clone,

designated UreB1 for upstream regulatory element binding protein, retained binding activity after plaque purification and further screening. The sequence is shown in Fig. 3. Data from two overlapping clones are shown. The first clone, whose 5′ end was an *Eco*RI site (underlined), was nearly full length when compared to the size of the major brain transcript of approximately 1.4 kb (see Fig. 7A). Re-screening of the cDNA library with the excised cDNA insert from the UreB1 clone revealed 6 colonies that hybridized under stringent washing conditions. Sequencing of the one with the largest insert (2.4 kb) showed that it contained the sequence of the entire UreB1 clone and a longer 5′ non-coding sequence of 944 bp. This 5′ sequence is included in the UreB1 sequence depicted in Fig. 3. These data suggest the presence of larger UreB1 transcripts and indeed we have observed additional larger bands in some Northern blots although they do not appear to be as abundant as the main transcript (see Fig. 7A). The open reading frame of the UreB1 clone codes for a unique 310 amino acid protein. The nucleotide sequence and the encoded UreB1 protein do not appear to be related to nucleic acid and protein sequences identified to date. The UreB1 protein also does not contain protein motifs that resemble known DNA binding proteins. Further examination of the sequence for other protein motifs indicated a sequence at amino acids 197–205 (KSNTEYHKY) which conforms to a tyrosine kinase consensus (K, X_3, E, X_3, Y).

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   1 gtggaattcttagagccaaatgtgaatgatagcaataatctatacattctagtagccagg
  61 gaggacaagttttgccatgaaagatccaggttacaaaactctaatgcagttcaaactcag
 121 aatgatgacacattccaacattcaatcaggaagggctacaaagaccctatctaaaaaaac
 181 aagaaacaaaaaccaaatcaagtcaataaaccaaagtaccactataggaagatttgtaaa
 241 tgttaaattattttaccaacgatctttatttttaaaatgaagcccaacttcaatcaaagtg
 301 aagtttttaatgttaattctttatacaaatcaatctttagagtgacagttctgagtttct
 361 ctgtgtcaccttggctgtcctccaacagaggttgacctgcctctgcctgcccgagtgcta
 421 cgattgaagaccgtgccaccaacgctcgaggccttaaagatgataacctacaaaattctg
 481 ctttaaagaatattctattgtgacgtaagcctgtaaatcttaccacttgagaggtctctg
 541 atataattagatcacaagtttaaggtcagtttgtgtcacacagttcaaggctagactgaa
 601 cttcttcacagcaagaccttatctcaaaaacaagcaaaaacataaacaaaaaaggaaaaa
 661 aagaaaaaagaaaaaaacccacccaactacaataagatcacaactcaagaattttaagtt
 721 taacaaacatacttaagacttctcccaaaaaaaaaaaagtatattcccagaaatatagaa
 781 actctctatttcaatgattttgtatatatccttagagaagaaacattttcttggcacaga
 841 ggcagagctgaaacaatgtcttactatgtaagacattctctcgtgccgaattcggcacga
 901 gagaagaagggcaggatgctgggggtctactgcgagagtggtacatgatcatctctcgag
                                                   M  I  S  R  E
 961 agatgttcaacccctatgtatgccttgttccgtacctcacctggtgaccgggtcactacac
       M  F  N  P  M  Y  A  L  F  R  T  S  P  G  D  R  V  T  T  R
1021 gatcaatccatcttcccactgtaaccccaaccacctcagctacttcaagtttagctggac
       S  I  H  L  P  T  V  T  P  T  T  S  A  T  S  S  L  A  G  R
1081 gatatgtagccaaagctgtaatgacaaccgccctcctggagtgctactttactaggtctt
       Y  V  A  K  A  V  M  T  T  A  L  L  E  C  Y  F  T  R  S  F
1141 tctacaaacacatcttgggcaaatctgtcaggtatacagatatggagagtgaggattacc
       Y  K  H  I  L  G  K  S  V  R  Y  T  D  M  E  S  E  D  Y  H
1201 acttctaccagggtctagtttatctgcttgaaaatgatgtctctacattaggttatgacc
       F  Y  Q  G  L  V  Y  L  L  E  N  D  V  S  T  L  G  Y  D  L
1261 tcaccttcagcactgaggttcaagaatttggagtatgtgaggttcgtgacctcaaaccca
       T  F  S  T  E  V  Q  E  F  G  V  C  E  V  R  D  L  K  P  N
1321 atggagccaacatcttagtaacagaggagaacaagaaggaatatgtacacctggtttgcc
       G  A  N  I  L  V  T  E  E  N  K  K  E  Y  V  H  L  V  C  Q
1381 agatgagaatgacaggagccatccgcaaacagctggcagccttcttagaaggcttctatg
       M  R  M  T  G  A  I  R  K  Q  L  A  A  F  L  E  G  F  Y  E
1441 aaatcattccaaagcgcctcatatccatctttactgagcaggagttagagctgctctata
       I  I  P  K  R  L  I  S  I  F  T  E  Q  E  L  E  L  L  Y  T
1501 cagggctgcctaccatcgacatagatgacctaaaatctaacactgagtaccacaagtacc
       G  L  P  T  I  D  I  D  D  L  K  S  N  T  E  Y  H  K  Y  Q
1561 agtccaactccattcagatccaatggttctggagagcattgcgttcctttgaccaagcag
       S  N  S  I  Q  I  Q  W  F  W  R  A  L  R  S  F  D  Q  A  D
1621 accgtgccaagttcctccagtttgtcacaggtacttccaaggtgcccctgcaaggatttg
       R  A  K  F  L  Q  F  V  T  G  T  S  K  V  P  L  Q  G  F  A
1681 ctgcccttgaaggcatgaatggcattcagaagtttcagattcatcgagacgacagatcca
       A  L  E  G  M  N  G  I  Q  K  F  Q  I  H  R  D  D  R  S  T
1741 cagatcgcctgccttcagctcacacatgttttaatcaactggacctgcctgcctatgaga
       D  R  L  P  S  A  H  T  C  F  N  Q  L  D  L  P  A  Y  E  S
1801 gctttgagaagctccgccacatgctactattggccatccaggagtgctctgaaggctttg
       F  E  K  L  R  H  M  L  L  L  A  I  Q  E  C  S  E  G  F  G
1861 ggctggctaataaaataaggccctgcctacttctgtggggtttttctaccatttttggacc
       L  A  N  K
1921 tggggaggggggggggtttaaaagatccagaaagaaaatgtcaaaatccaataaatgaaa
1981 ttcaccaactcaccatgtgtgtgtccagctgcccatcttcctagtgcatacctgttcgct
2041 tctcattctctttcctctaccctttccttgcctttccctttccatgccgtccatgatcc
2101 ccaccccatgtgttaagaaggcagttgctttgcagggacctgtttgtccaactgaacagt
2161 gtgctcctcagattctgtgttcagaaggatttgctgcattgagacttgaaaccttggta
2221 aggggaaaattatatatatatatatttttttgttctgtttgcatttcttaatttgtgctt
2281 ggaatgtgttgatgtgcacagctaatgattcaatgcgagacaagattggcatctgtgttg
2341 tggaggttcaaataaagagcactcttcataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 3. Sequence of UreB1 clone. To clone UreB1, a rat brain cDNA expression library was screened with a labeled double-stranded 24 mer oligonucleotide (5′ cgagagcactctcctccacatcac 3′) that contains the URE sequence. One clone (UreB1) was isolated and the insert extended from the *Eco*R I site (underlined) to the poly-A tail and contained an open reading frame coding for 310 amino acids. Translation is started from the first in-frame methionine although there is not a Kozak consensus sequence at this residue [35]. Six more colonies were obtained upon rescreening with the UreB1 insert, one of which had a longer 5′ non-coding region of 887 nucleotides. The sequence of the composite is shown.

Bacterial lysates prepared from the UreB1 clone induced with IPTG specifically bound the URE-containing oligonucleotide (Fig. 4A). In contrast to brain nuclear extract, with the bacterial extract, generally one band was seen on the gel retardation autoradiograms. A 60- or 120-fold excess of unlabeled URE oligonucleotide competed the binding whereas a 120-fold excess of the oligonucleotide centered on the −1546 site of the dynorphin promoter was ineffective in blocking complex formation.

To further verify the ability of the protein coded by the UreB1 clone to bind to its recognition sequence, we evaluated a purified preparation of recombinantly expressed UreB1 protein. An *Xho*I-*Xho*I fragment of the insert, which contains nearly the entire coding sequence less 5 amino acids at the putative N-terminus, was subcloned into the pQE 11 expression vector to form a pQE11-UreB1 fusion expression construct. The expressed fusion protein contains 6 histidine residues at the N-terminus which provide a means for affinity purification on an immobilized nickel-chelate matrix. With this system large quantities of recombinant fusion protein can be obtained as a highly purified preparation (Fig. 4B). While the pQE11-UreB1 fusion protein adds 6 histidines to the N-terminus, this produces little change in the molecular weight since they replace the 5 N-terminal amino acids lost from the *Xho*I-*Xho*I fragment used for subcloning. Incubation of the labeled URE oligonucleotide with 0.5 to 2 μg of affinity purified UreB1 again yielded a gel shift band of approximately the same mobility as that seen with crude bacterial lysate. This complex displayed specific binding in that binding was competed by unlabled URE oligonucleotide and not by the unrelated −1546 oligonucleotide (Fig. 4C).

UreB1 contains a tyrosine kinase consensus (Fig. 5A) and the influence of tyrosine phosphorylation was

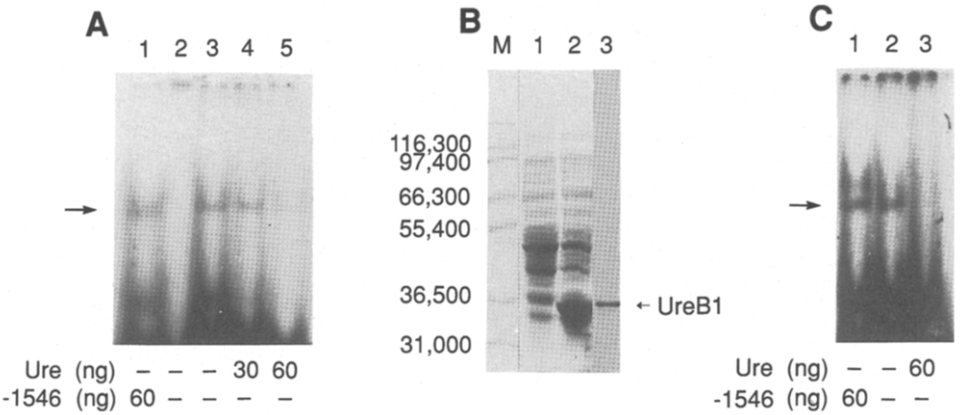


Fig. 4. A: bacterial cell extract from the UreB1 clone forms a complex with the URE oligonucleotide. This complex was specifically competed by ascending concentrations (ng) of unlabeled URE oligonucleotide but not by an excess of unrelated oligonucleotide. Lane 1, UreB1 extract plus URE probe and 60 ng of -1546 competitor; lane 2, URE probe alone, no extract; lane 3, URE probe + extract; lanes 4 and 5, competition with unlabeled URE oligonucleotide. B: induction and purification of recombinantly expressed UreB1. A 10% polyacrylamide denaturing gel stained with Coomassie blue shows in lane M, molecular weight markers; lane 1, bacterial lysate without induction; lane 2, bacterial lysate after 6 h of induction with IPTG; lane 3, purified recombinant UreB1 from the final eluate of the NTA-Ni resin. The size (~ 36 kDa) of the induced or purified proteins corresponds to the predicted molecular weight of the protein encoded by the UreB1 open reading frame. C: binding activity of the purified recombinant protein. Two μg of the purified recombinant protein were used in a gel mobility shift assay with the labeled URE oligonucleotide. All lanes contained labeled URE oligonucleotide and purified protein to which was added: lane 1, 60 ng of non-specific oligonucleotide as competitor; lane 2, no competitor; lane 3, 60 ng unlabeled URE as competitor.

explored using gel mobility shift and in vitro transcription assays with a URE-CAT template. Recombinantly expressed, purified UreB1 protein functioned as a substrate for the p43$^{v\text{-}abl}$ tyrosine kinase. Phosphorylation was demonstrated by kinasing the protein using [γ-^{32}P]ATP or unlabeled ATP. Tyrosine phosphorylation was determined by electrophoresis and blotting of phosphorylated and non-phosphorylated UreB1 preparations and detection by either (a) autoradiography or (b) probing of the blot with an anti-phosphotyrosine antibody. In the autoradiogram, a single labeled band of approximately 36 kDa was detected in the lane containing the ^{32}P-labeled phosphoprotein, which co-migrated with the recombinantly expressed UreB1 protein (Fig. 5B). Similarly, the anti-phosphotyrosine antibody detected an approximately 36 kDa phosphorylated protein and not the non-phosphorylated protein (Fig. 5C). These data are consistent with the idea that UreB1 is phosphorylated on a tyrosine residue. Preparations of phosphorylated UreB1 produced a more intense gel shift than non-phosphorylated UreB1 (Fig. 5D). The specificity of this effect was determined with UreB1 that had been phosphorylated by protein kinase C (PKC). UreB1 contains 4 potential PKC phosphorylation motifs. Although phosphorylation with PKC and [γ-^{32}P]ATP was demonstrable, no binding was detected with PKC-phosphorylated UreB1 (not shown).

The tyrosine phosphorylated form of UreB1 increased transcription of a URE-dynorphin minimal promoter-CAT template in an in vitro assay with brain

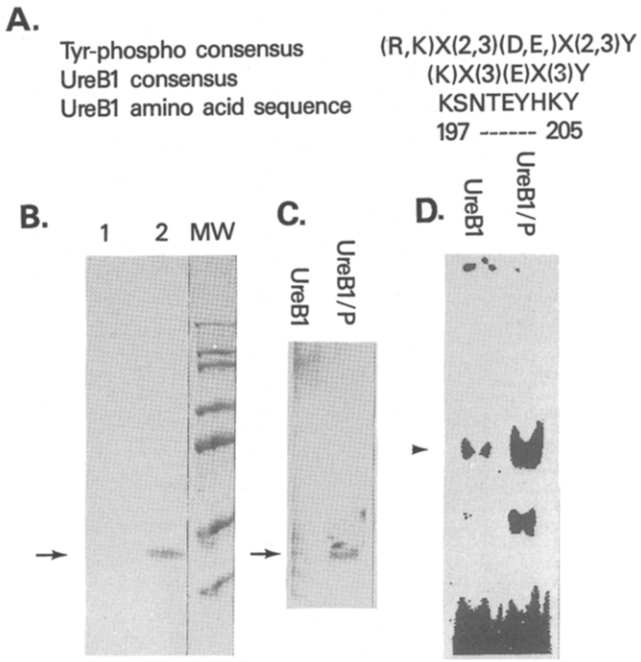


Fig. 5. Tyrosine phosphorylation and binding of UreB1. A: consensus amino acid sequence for tyrosine kinase and the conforming sequence in UreB1. B: affinity-purified, recombinant UreB1 is labeled in vitro by the tyrosine kinase p43$^{v\text{-}abl}$ and [γ-^{32}P]ATP (lane 2) and co-migrates with unlabeled recombinant UreB1 (lane 1) which was visualized by staining (not shown). Lane 3, molecular weight markers. C: phosphorylation with p43$^{v\text{-}abl}$ produces a protein that reacts with an anti-phosphotyrosine antiserum. UreB1 was phosphorylated with the v-*abl* kinase and non-radioactive ATP, electrophoresed and transferred to an immobilon membrane. Only the lane containing UreB1 that had been incubated with ATP and the kinase was immunoreactive. D: a more intense gel shift is obtained with the phosphorylated (UreB1/P) preparation in comparison to the non-phosphorylated preparation (UreB1).

nuclear extract. The template was linearized at the *Eco*R I site within the CAT coding sequence to yield a 214 nucleotide transcript. Fig. 6 shows that the amount of in vitro transcript is elevated in the presence of UreB1 in comparison to addition of bovine serum albumin or no added protein. Increased amount of transcript was also observed upon addition of phosphorylated UreB1 and not with addition of the v-*abl* kinase alone to the reaction mixture. Activation of transcription by tyrosine phosphorylated UreB1 increased with increasing concentrations of phosphorylated UreB1 (Fig. 6B–D). These data are consistent with the hypothesis that UreB1 is a positive transcription regulatory factor.

RNA blots prepared from discrete brain regions showed that the UreB1 gene is expressed at approximately equal levels in frontal cortex, caudate-putamen and hippocampus (Fig. 7A). While not shown, a band of the same size was also seen in 8 other regions (cerebellum, olfactory bulb, medulla pons, mesencephalon, thalamus, hypothalamus and dorsal and ventral spinal cord). In situ hybridization revealed that the expression varied greatly between neuronal populations within any one grossly dissected region. For example, in a single section through the medulla, expression was particularly dense in neurons of the raphe obscurus (Fig. 7B), whereas the nearby facial nucleus expressed an intermediate level of transcript (Fig. 7C). In the peripheral nervous system, dorsal root ganglion neurons clearly expressed the UreB1 transcripts. (Fig. 7D). The montage of rat forebrain shows the diffuse expression in frontal cortex, nucleus accumbens and olfactory tubercle (Fig. 8). The apparent enrichment in the pyramidal layer of the olfactory tubercle, in part, is due to the tight packing of the cells but is also a function of the sparse or absent expression within the plexiform layer of the olfactory tubercle. Other regions rich in white matter also showed low levels of expression such as the forceps minor of the corpus callosum in Fig. 8.

4. Discussion

The present data identify a protein binding site at −208 in the dynorphin promoter termed URE. The URE site is pyrimidine rich, contains the consensus for an Inr element, and overlaps a region that is conserved between the rat and human promoters. A 310 amino acid URE binding protein (UreB1) was cloned and crude bacterial lysates from the clone, as well as recombinantly expressed, affinity purified UreB1, display specific binding to the URE. Binding, phosphorylation and in vitro transcription data obtained with the purified recombinant protein suggest that UreB1 is a unique sequence-specific DNA binding protein which may be regulated by a tyrosine phosphorylation-dependent mechanism.

The clone obtained contained a long open reading frame with several potential ATG translation start codons located at the N-terminus. None of these ATG codons were contained within a sequence that matched the Kozak rule [35]. Consequently, we chose the first in frame ATG from which to start translation. This yielded

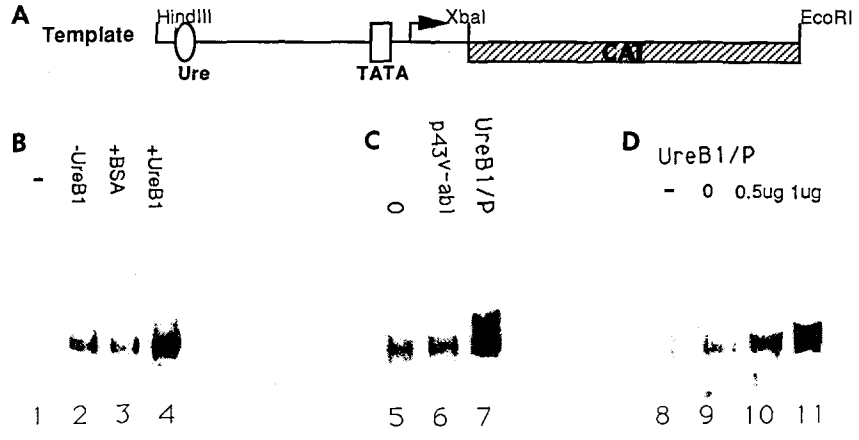


Fig. 6. Activation of in vitro transcription by UreB1 in vitro. A: diagram of Ure-dynorphin minimal promoter-CAT template. A synthetic 21 bp Ure-containing oligonucleotide was ligated directly to minimal promoter and the CAT containing vector was linearized at the *Eco*RI site at + 243 in the CAT coding sequence. B: effect of unphosphorylated UreB1 on transcription. Template was used at 200 ng in lanes 2 to 7. Lane 1, control without template; lane 2, no UreB1 recombinant protein; Lane 3, 2 μg of BSA; lane 4, addition of 2 μg of unphosphorylated UreB1 recombinant protein. C: effect of phosphorylated UreB1 recombinant protein on transcription. Lane 5, template only; lane 6, with v-*abl* kinase but without UreB1 recombinant protein; lane 7, with v-*abl* kinase and phosphorylated UreB1 recombinant protein. D: ascending concentrations of phosphorylated UreB1 recombinant protein increase transcription. In lanes 9–11, 100 ng of template were used. Lane 8, control without template; lane 9, no UreB1 recombinant protein; lane 10, 0.5 μg of phosphorylated UreB1 recombinant protein; lane 11, 1 μg of phosphorylated UreB1 recombinant protein.

a 310 amino acid protein of approximately 36 kDa molecular weight. It is recognized that this is arbitrary and that there are two other ATG codons just 3′ to the first which could also function as translation start codons. The 5′ untranslated region was obtained from a second longer, but overlapping, clone identified by rescreening the cDNA library. This clone contained multiple nonsense codons in all three reading frames 5′

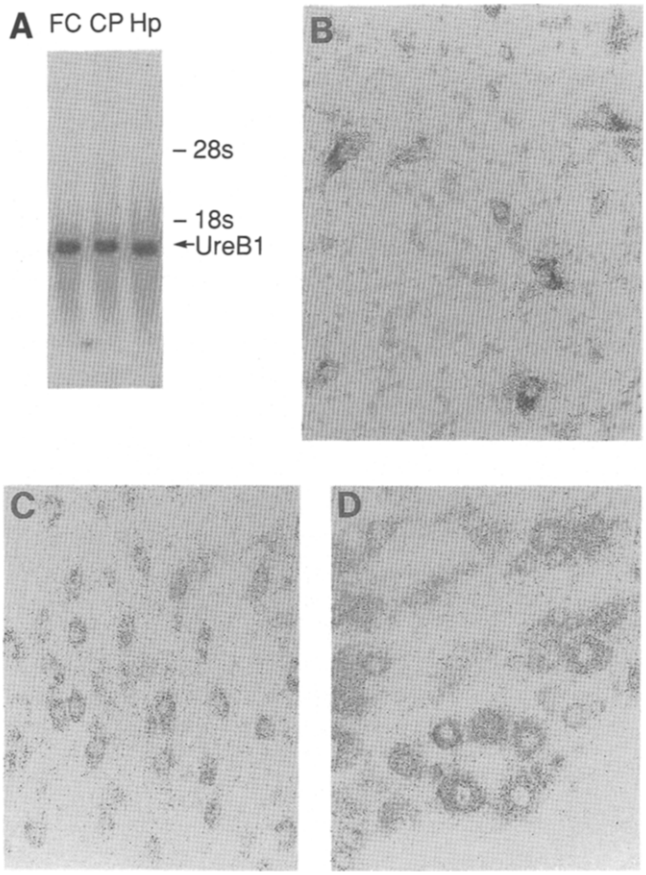


Fig. 7. Expression of UreB1 in central and peripheral nervous systems. A: RNA blot of UreB1 mRNA in frontal cortex (Fc), caudate-putamen (CP) and hippocampus (Hp). Each lane contains 20 μg of total RNA. The blot was probed with a 1,440 bp *Xho*I-*Xho*I fragment of the UreB1 clone labeled by random priming. The major species detected migrated between 1.4 and 1.2 kb, which was similar to the size of the original UreB1 insert. B–D: localization of UreB1 transcripts by in situ hybridization. B and C are 200× magnification, bright-field photomicrographs from two regions of the same section of rat brainstem showing differential expression in (C) the facial nucleus and (B) nucleus raphe obscurus. Raphe obscurus was one of the regions surveyed with the highest expression of UreB1 and labeled cells are nearly completely filled in with silver grains. Immediately adjacent is the facial nucleus which displays an intermediate level of expression. D: UreB1 expression in dorsal root ganglion neurons. Abundant grain accumulation is clearly visible over the dorsal root ganglion neurons. The sections were hybridized with a gel purified synthetic 48 base oligonucleotide complementary to bases 1015 to 1072 in the open reading frame of the UreB1 cDNA. Exposure time was 5 weeks. Northern blots of brain regional total RNA using this oligonucleotide gave the same pattern and size transcript as that obtained with the labeled cDNA insert shown in A.

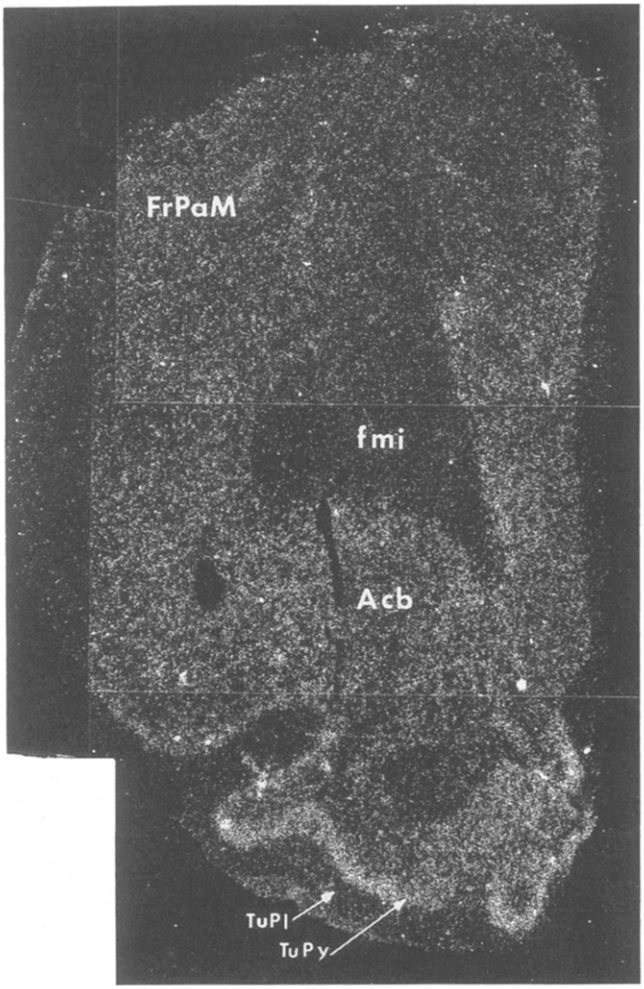


Fig. 8. UreB1 expression in forebrain. The montage shows a section through the nucleus accumbens/anterior olfactory tubercle in dark-field illumination. Note the low level of expression in the forceps minor of the corpus callosum compared to surrounding cortex. A similar low level is seen in the plexiform layer (predominantly white matter) in comparison to the tightly packed cells of the pyramidal layer. FrPaM, frontoparieto-motor cortex; fmi, forceps minor of internal capsule; Acb, nucleus accumbens; TuPl, plexiform layer of olfactory tubercle; TuPy, pyramidal layer of olfactory tubercle.

to the *Eco*RI site indicated in Fig. 3 suggesting that translation starts in the vicinity of the designated ATG. The choice of translation start is supported by preliminary data using an antibody to the recombinantly expressed UreB1 protein which detected an approximately 36 kDa protein in brain nuclear extracts [20]. The encoded UreB1 protein was unique and did not contain a previously identified DNA binding motif or long stretches of basic amino acids typical of several classes of DNA binding proteins and we are presently characterizing the region of the protein responsible for DNA binding. However, a consensus amino acid sequence for tyrosine phosphorylation was present and is discussed below.

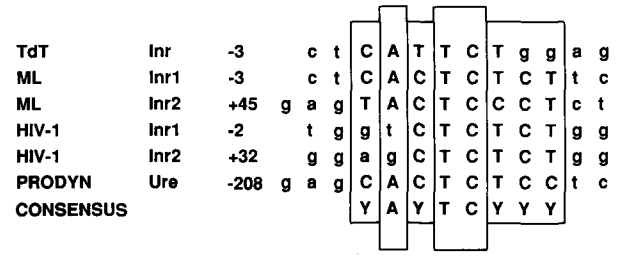
UreB1 contains one consensus for tyrosine phosphorylation at amino acids 197–205 which prompted us to investigate whether UreB1 could be tyrosine-phosphorylated and if phosphorylation influenced the activity of UreB1 in vitro. The importance of tyrosine phosphorylation for the function of UreB1 is reflected in two ways. First, the binding of the protein to the URE is enhanced relative to the non-phosphorylated protein. Second, the activity of $p43^{v\text{-}abl}$ phosphorylated UreB1 in an in vitro transcription assay is increased relative to non-phosphorylated UreB1. The $p43^{v\text{-}abl}$ phosphorylated preparations of UreB1 yielded a protein that was immunoreactive with an anti-phosphotyrosine antibody. This reactivity reinforces the idea that the observed effects in the gel shift assay and on in vitro transcription are due to phosphorylation at a tyrosine residue. These data, however, do not allow us to rule out effects of phosphorylations by other kinases at other sites to produce positive or negative regulation of UreB1 functions.

Regulation of transcription factors by tyrosine phosphorylation is not a feature commonly found in this class of proteins. Protein tyrosine kinase activity has been suggested to be an important step in activation of transcription by interferon alpha/beta signalling [17, 57]. Genes regulated by interferon have an enhancer element in their promoters called interferon-stimulated response element (ISRE). The ISRE is a binding site for a multi-protein transcription factor complex called interferon-stimulated gene factor 3 (ISGF3). The 113 and 91/84 kDa proteins which are members of the ISGF3 complex have been shown to be tyrosine phosphorylated following immunoprecipitation from interferon-treated cells. Furthermore, phosphorylation of the 113 kDa protein was shown to increase binding of the ISGF3 complex to oligonucleotides containing ISREs relative to the non-phosphorylated proteins [17]. The latter finding is similar to our observation that tyrosine phosphorylation of UreB1 increases its binding to the URE relative to non-phosphorylated UreB1. While we have not conducted experiments to show that UreB1 is tyrosine phosphorylated in vivo, the increase in in vitro transcription following tyrosine phosphorylation suggests that this step is important for full realization of transcriptional activity.

The binding data suggest that UreB1 is capable of binding to the URE as either a monomer or a homodimer. One major band was generally obtained with recombinant UreB1 in the gel shift assay. However, in extracts of isolated brain cell nuclei, gel shifts with the synthetic Ure site showed two distinct bands, both of which display specific competition. Furthermore, within specific brain regions the amount of each of the two complexes displayed considerable variation. These data demonstrate that at least two types of complexes may form at the URE. Several possibilities can account for these observations: (a) different proteins are present in the two complexes, (b) a post-translational regulatory mechanism (e.g. phosphorylation) modulates the amount of complex forming in the different regions or (c) a combination of both. UreB1 could fulfill any of these conditions assuming it can form a homodimer or heterodimerize with another protein.

Expression of the UreB1 gene occurs throughout the CNS. Expression appears to be enriched in neurons, since grain accumulation over the corpus callosum, white matter tracts of the spinal cord, and in non-neural cells of the dorsal root ganglion was comparatively lower than adjacent neuronal populations. The multiplicity of neuronal cells in which UreB1 transcripts are localized indicates that it is not solely involved in dynorphin gene expression which has a more restricted distribution [1,5]. The widespread distribution of UreB1 transcripts is consistent with the presence of URE binding activity in multiple brain regions, including the cerebellum which does not express appreciable amounts of dynorphin transcripts [5]. Since the distribution of the expression of UreB1 does not precisely match that of the dynorphin gene it suggests that UreB1 is not sufficient for tissue-specific expression of the dynorphin gene. Rather, a broader role in gene regulation is suggested especially in relationship to tyrosine kinase regulated processes.

The present paper is one of the first reports of a protein that binds to an upstream Inr consensus sequence. UreB1 has positive transcriptional modulatory activity, but we do not know if UreB1 can also function in transcription initiation. As a *cis*-acting element the URE has several features in common with an Inr element. The URE sequence is represented in the degenerate Inr consensus, YAYTCYYY (Y = T or C). This pyrimidine-rich sequence is common to the minimal promoter of many genes transcribed by RNA polymerase II [49]. The Inr element generally is very near the transcription start site and it can act together with the nearby upstream TATA element, or independently in genes without TATA elements [54], to coordinate transcription initiation. Proteins that bind to Inr consensus sequences, therefore, may play fundamental roles in transcription initiation or selection of alternative transcription start sites. Comparison of Inr elements from several genes is depicted in Fig. 9, which shows the Inr sequences and their location near the transcription initiation site. In contrast to Inr elements, the URE is located more than 200 base pairs upstream of the start site in the dynorphin promoter, hence the name upstream regulatory element (URE) to distinguish it from the Inr elements nearer the transcription start site. While we make this distinction, it is important to note that the dynorphin gene appears to contain an alternative transcription start site in the approximate region (−208) of the URE. A potential



TdT	Inr	-3		c	t	C	A	T	T	C	T	g	g	a	g
ML	Inr1	-3		c	t	C	A	C	T	C	T	C	T	t	c
ML	Inr2	+45	g	a	g	T	A	C	T	C	C	C	T	c	t
HIV-1	Inr1	-2		t	g	g	t	C	T	C	T	C	T	g	g
HIV-1	Inr2	+32		g	g	a	g	C	T	C	T	C	T	g	g
PRODYN	Ure	-208	g	a	g	C	A	C	T	C	T	C	C	t	c
CONSENSUS						Y	A	Y	T	C	Y	Y	Y		

Fig. 9. Comparison of the URE sequence at −208 in the dynorphin gene promoter with nucleotide sequences of known Inr elements from several genes. Capital letters denote bases which match the degenerate consensus shown in the bottom entry. The numbers represent the location of the Inr element with respect to the transcription start site. The figure was adapted from Roy et al. [49]. TdT, human terminal deoxynucleotidyl transferase [54]; ML, major late promoter of adenovirus; HIV, human immunodeficiency virus.

TATA box is located at −266 and a cDNA clone has been isolated that begins 70 bases upstream from the major cap site (i.e. at −70) [10]. Thus, despite its upstream location, the potential for the URE to function as an Inr within the context of the dynorphin gene requires additional consideration. Furthermore, the possibility of coordinate gene regulation is raised by examination of the promoter of carboxypeptidase E. This enzyme participates in posttranslational processing of neuropeptides to their final neuroactive form and the promoter for the carboxypeptidase E gene lacks a TATA box motif but does contain an Inr-like sequence [30]. Thus, there is the potential for URE or Inr-binding proteins to co-regulate both genes.

We have not tested the binding of UreB1 to known Inr sites but, given the nucleotide sequence similarity, it is possible that the URE may function in both transcriptional control and initiation. In this regard, multiple roles or functions at upstream *cis*-acting elements have been suggested for two previously identified Inr-binding proteins. The protein YY1, a 68 kDa cellular protein [21,47,51] can function as an initiator protein for the adeno-associated virus P-5 promoter and can also modify transcription by binding to an upstream site between −50 and −60, where it acts as a transcriptional repressor [52]. A multi-functional role also has been suggested for another initiator protein TFII-I [49]. This basic helix-loop-helix transcription factor is a 120 kDa protein which recognizes the consensus Inr-element and functions as a transcriptional initiator. TFII-I is immunologically similar to USF, another cellular helix-loop-helix factor that binds to an upstream E-box enhancer element [15,19]. TFII-I can also bind as a heteromeric complex with USF to the upstream element, suggesting roles in both transcription initiation and modulation [49]. A dual role for UreB1 may also be considered.

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