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URE, an Initiator (Inr)-like Site, Suppresses the Promoter of the Rat Dynorphin Gene

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We previously identified a DNA binding element termed the upstream regulatory element (URE) that contains the consensus initiator sequence (Inr) in the upstream promoter of the rat prodynorphin gene. The URE displays specific binding to the upstream regulatory element binding protein (UREB1), a novel transcription regulator. Here, we report that the URE functions as a suppressor element. A series of chloramphenicol acetyltransferase reporters (pCAT) were constructed by subcloning either wildtype or mutated URE sequences into a pCAT reporter plasmid 5′ of bases −135 to +58 of the prodynorphin gene. The basal −135 to +58 dynorphin promoter (pCAT 0.2) has robust transcriptional activity in Chinese hamster ovary (CHO) cells but not in rat pheochromocytoma PC12 cells. This robust transcriptional activity was completely inhibited in the presence of wildtype URE, whereas the mutations of the URE had no effect. Gel mobility shift assays showed that the complex formed by the URE and nuclear protein extracts can be competed by addition of wildtype URE oligonucleotide but not by specific mutations of the URE, defining particular bases required for protein interaction with the URE. The identical URE sequence is also found upstream in the promoter of human macrophage inflammatory protein 1β (hMIP 1β). The suppressive activity of the rat dynorphin URE can be replaced by the hMIP 1β URE. These data suggest that the URE may serve as a suppressor element in the regulation of dynorphin and hMIP 1β gene transcription.

Transcription initiation by RNA polymerase II requires a minimal promoter in mammalian genes. It is generally accepted that the minimal promoter contains a TATA box and a transcription start site, or a TATA box alone (1, 2). A pyrimidine-rich initiator (Inr) sequence overlapping or close to the transcription start site is also implicated in transcription initiation (3-6). The Inr acts together with the nearby upstream TATA element, or independently in TATA-less genes, to coordinate transcription initiation (4). Proteins that bind to Inr consensus sequences, therefore, may play fundamental roles in transcription initiation or selection of alternative transcription start site(s). The motif, 5′ YAYTCYYY 3′ represents the consensus Inr sequence (6). However, the consensus appears weak enough such that the position of the site could be variable (4). The distribution and possible diversity of Inr-like elements among various gene promoters is not yet clear. Some Inr-like elements are found distal to the initiation start site (7) suggesting that the position of the pyrimidine-rich Inr-like elements are not necessarily fixed. An upstream regulatory element (URE) containing a pyrimidine-rich Inr consensus was found at −208 of the dynorphin promoter and characterized with respect to its DNA-protein binding properties and candidate binding proteins (8). However, the function of this element was unclear. Preliminary transient transfection experiments with PC12 cells and HeLa cells showed that the URE neither enhanced transcription of the dynorphin basal promoter nor did it augment cAMP- or phorbol ester-induced stimulation. We report here that the URE is capable of suppressing transcriptional activity of the prodynorphin (hereafter referred to as dynorphin) promoter in CHO cells. The exact same URE sequence is also found at −70 of the human macrophage inflammatory protein (hMIP 1β) promoter. This sequence contains the identical 8 bp core of the rat dynorphin URE and exerts the same suppressive effect when placed upstream of the dynorphin promoter.

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EXPERIMENTAL PROCEDURES

Construction of the URE pCAT reporter vectors. Oligonucleotide inserts of 24 bp containing either the wildtype or mutated rat dynorphin URE sequence (rDyn.URE), or the wildtype (wt) or mutated human MIP 1β URE sequence (hMIP.URE) with 5′ HindIII and 3′ Pst I restriction sites were subcloned immediately 5′ to ~ 200bp (bases −135 to +58) of the dynorphin promoter in pCAT basic vector (pCAT 0.2) (9). Sequences of the wildtype and mutated rat dynorphin URE are shown in Fig. 1A and the human MIP 1β in Fig. 2A.

Cell culture and transient transfection. CHO cells were seeded at 50-60% confluence and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum and 7.5% horse serum. After overnight incubation, medium was removed and 2 ug of each test pCAT plasmid mixed with 10 ul of Lipofectamine (GIBCO/BRL) in 3 ml of serum-free medium were added to the cells in triplicate dishes. Following 5-7 hr of incubation at 37°C, the medium was replaced with the DMEM growth medium. After 40 hr, cells were washed with PBS and harvested. Following three freeze-thaw cycles, cell fragments were pelleted by centrifugation and the supernatant was assayed for CAT enzyme activity as described (9).

Gel mobility shift. The preparation of nuclear extracts from cultured cells, and tyrosine phosphorylated UREB1 protein were conducted as described previously (8, 10). Gel mobility shift experiments were performed as follows: 10 μg of nuclear extract protein or 1 μg of purified protein was incubated with 1-2 ng of ^{32}P-labeled wildtype double stranded URE oligonucleotide in the presence of 2.5 μg of polyd(I-C). The binding reaction was carried out in 1 × C buffer (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) containing 80-100 mM NaCl in a 20 μl final volume. For competition experiments 100-200 ng of each competitor oligonucleotide was added to the assay. After 30 min incubation at room temperature, binding reactions were analyzed by polyacrylamide gel electrophoresis in a 6% precast native gel (Novex).

RESULTS

The pCAT 0.2-rDyn promoter-reporter exhibited a robust transcriptional activity after transfection of the plasmid into CHO cells (Fig. 1B). The transactivation of the dynorphin minimal promoter in CHO cells was completely suppressed in the presence of the wt rDyn URE. In contrast, a high level of transcriptional activity of the dynorphin minimal promoter was not obtained in either PC12 or HeLa cells (9) and no suppressor or enhancer activity of the URE-containing constructs was seen with these cells (data not shown). To determine if the suppression observed in CHO cells was specifically due to the URE sequence, several controls using mutations of the URE sequence were examined. The pCAT 0.2-rDyn.URE Mut1, with the conversion of CC to AA in the URE sequence, produced the same suppression as the wt construct, whereas, the pCAT 0.2-rDyn.URE Mut2, in which the conserved A in the URE sequence is mutated to a G, produced a partial reversal of suppressor activity. A third construct, pCAT 0.2-rDyn.URE Mut3, in which both the conserved A to G and the TC to GA pair were mutated, was completely ineffective in producing suppression (Fig. 1B). The results suggest that the rat dynorphin URE can confer suppressor activity upon the dynorphin minimal promoter in CHO cells and that the A and TC nucleotides in the URE are critical whereas the CC pair is not essential for activity.

Cis-acting elements exert their effects through interaction with sequence-specific DNA binding proteins such that the formation of one or more protein-DNA complexes ultimately alters the efficiency of the basic transcriptional processes. Therefore, the effects of the different mutations on the transcriptional activity of the element(s) also may be reflected by their in vitro protein binding activity. A labeled rat dynorphin wt URE oligonucleotide was incubated with nuclear extracts from CHO cells in the absence or presence of unlabeled wt or mutated URE oligonucleotides, and the results analyzed using the gel mobility shift assay. As shown in Fig. 1C, mutations 2 and 3 of the URE (lanes 4 and 3, respectively) did not compete the binding activity (lane 1), whereas mutation 1 of the URE (lane 5) competed as effectively as the wt URE oligonucleotide (lane 2).

The URE consensus is also found at −70 in the promoter of human macrophage inflammatory protein 1 beta (hMIP 1β), although the sequences flanking the 8 bp that match to the weak Inr consensus are different (Fig. 2A). To test if the hMIP 1β URE functions similarly to the rDyn URE, wt and mutated hMIP 1β URE sequences were inserted immediately 5′ of the promoter fragment in the pCAT 0.2-rDyn plasmid to form wt and mutant pCAT 0.2-rDyn hMIP.URE constructs, respectively, and these constructs were transfected into CHO cells. The pCAT 0.2-rDyn hMIP.URE produced the same, nearly complete suppression of the rat dynorphin minimal promoter as was seen with the rat dynorphin URE (Fig. 2B). Mutation of the hMIP URE eliminated the suppression (Fig. 2B).

The suppression of the rat dynorphin promoter by two identical UREs suggested that they might bind the same nuclear factor(s). To test this idea, competition experiments were performed using gel mobility shifts and CHO cell nuclear extracts (Fig. 3). The hMIP 1β URE formed two specific complexes (Fig. 3A, lane 1) which were competed by itself (lane 2). The rDyn URE competed only the upper complex not the lower complex (lane 3) and the hMIP 1β URE mutation did not compete either of the two complexes (lane 4). The lower complex formed by hMIP 1β URE was not effectively competed by rDyn URE suggesting that differences in the flanking sequence of the hMIP 1β URE may contribute to complex formation. The rDyn URE formed only one specific complex (figure 3B lane 1) which was not competed by either its own mutation or the mutated hMIP 1β URE oligonucleotide (lanes 2 and 5, respectively), but completely competed by itself and the wt hMIP 1β URE (lane 3 and 4, respectively). Previously, we cloned a URE binding protein UREB1 from a rat

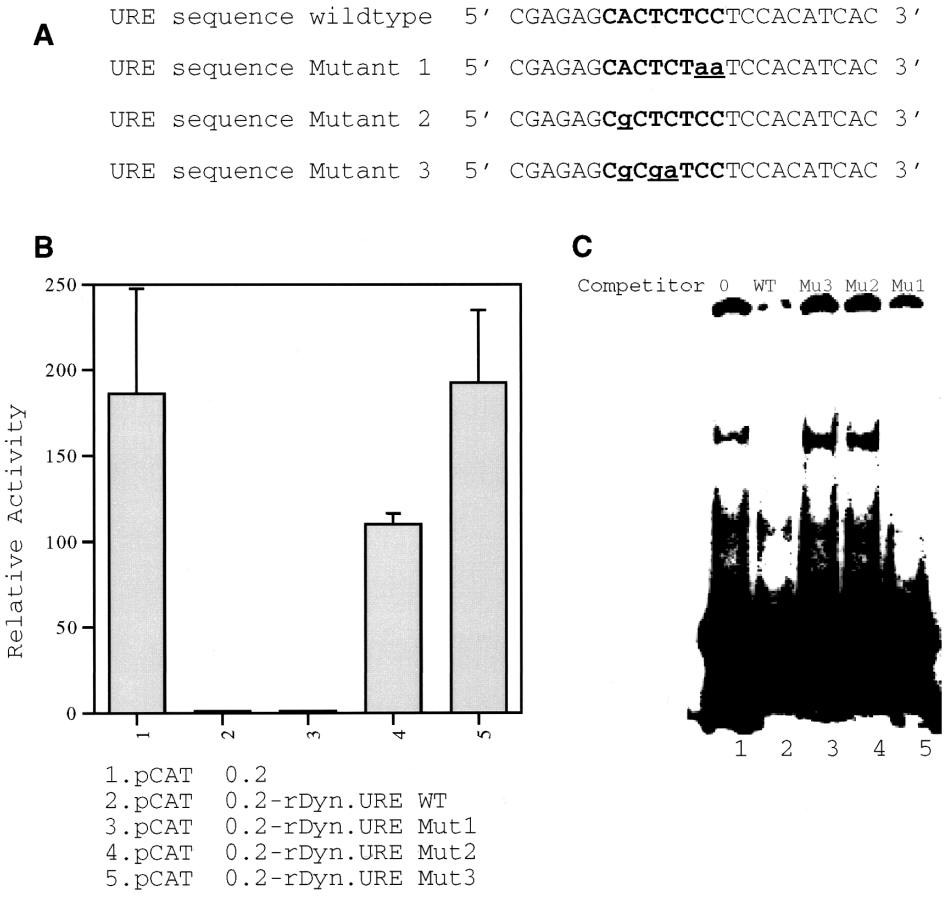

FIG. 1. rDyn.URE suppresses transcription. (A) Wildtype (wt) and mutants of the rDyn.URE sequences. The nucleotides conserved in the consensus Inr sequence that were selected to be mutated are indicated by underlined small letters. (B) Transient transfection. Each bar represents the mean± SD of triplicate determinations. The plasmids were constructed with the wt and mutated UREs placed immediately 5′ of the rat dynorphin promoter driving a chloroamphenicol acetyltransferase reporter gene (pCAT 0.2) and were transfected into Chinese hamster ovary (CHO) cells. CAT activity was determined by counting the butyrylated (^{14}C) chloramphenicol product. The transcriptional activity mediated by the dynorphin promoter (bar 1) is suppressed by the wt URE sequence (bar 2). This suppression can be reversed by URE mutant sequences, mutants 2 and 3 (bars 4 and 5) but not mutation 1 (bar 3). (C) Gel mobility shift using labeled dynorphin URE. The labeled wt URE oligonucleotide was incubated with nuclear extracts of CHO cells in the presence of no competitor or unlabeled wt and mutated (Mu) URE oligonucleotides. The complex formed by labeled URE oligonucleotide (lane 1) is competed by itself (lane 2) and URE mutant 1 (lane 5), but not by URE mutants 2 and 3 (lanes 3 and 4).

brain cDNA library using labeled rDyn URE as a probe (8). Further similarities in the binding properties of the two UREs were tested using gel mobility shift assays and tyrosine phosphorylated UREB1 protein (Fig. 3C). Both the rDyn URE and hMIP 1β URE formed a complex with the UREB1 protein (lanes 1 and 5). The complex formed by hMIP 1β URE and UREB1 was competed by both the hMIP 1β URE and rDyn URE (lane 2 and 3) but not by an unrelated oligonucleotide (the DYNCRE 3 site from the rat dynorhin promoter, see ref. 9) (lane 4). The complex formed by the rDyn URE and UREB1 protein showed the same competition pattern in which the complex is competed by the rDyn and hMIP 1β UREs (lane 6 and 7), but not by the unrelated DYNCRE3 oligonucleotide (lane 8).

DISCUSSION

The suppression of the dynorphin promoter by the rDyn URE and hMIP 1β URE was observed in CHO cells using the rat dynorphin promoter as a basal test promoter. The high level of CAT activity obtained with the basal dynorphin promoter in CHO cells provided the cellular background for examining suppressor activity that was not possible in several other cell lines. The activity of the dynorphin promoter in HeLa cells and PC12 cells was comparitively low, such that transient transfections using the present constructs were uninformative. Thus, the CHO cells provided a cellular context in which the suppressor activity was demonstrable and allowed us to functionally charac-

A

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Dynorphin  URE 5′CGCGAGCACTCTCCTCCACATC 3′
hMIP-1β    URE 5′ CCACTCACTCTCCTGTGCCC  3′
hMIP-1β mutURE 5′ CCACTCCCGTTGGTGTGCCC  3′
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B

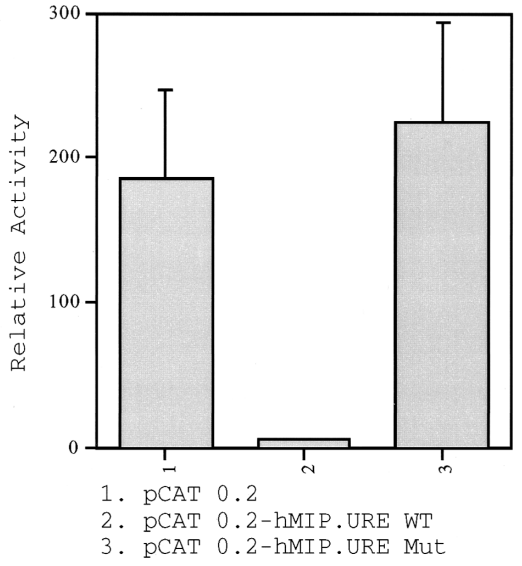

FIG. 2. hMIP 1β URE suppresses transcription. (A) Comparison of rat prodynorphin URE sequence and human macrophage inflammatory protein 1β URE sequence. Identical nucleotides in the two URE sequences are in bold type. The nucleotides mutated in the hMIP 1β URE are underlined. (B) Transient transfection. Bars are the mean± SD of triplicate determinations. The promoter-reporter constructs were as in Fig. 1 but the dynorphin URE was replaced by the wt and mutated hMIP 1β URE oligonucleotides. The transcriptional activity mediated by the dynorphin promoter (lane 1) was suppressed by the wt hMIP 1β URE sequence (bar 2) but not by the mutant sequence (bar 3).

terize the sequcence requirements for suppressor activity. The sequence of the URE (CACTCTCC) conforms to the consensus for the Inr element (YAYTCYYY) (6). The Inr contains several conserved bases and these bases were used as the starting point for mutations of the rat dynorphin URE. The results from the URE mutations in transient transfection assays suggest that the A and TC nucleotides in the URE are critical for conferring suppression of transcriptional activity to the rat dynorphin promoter and nucleotides of the CC pair do not appear to be essential for activity. The identical core sequence from a second gene, the human MIP 1β, could substitute for the URE using the dynorphin basal promoter as the test promoter. Mutation of the hMIP URE core blocked suppression. These data suggest that suppression is mediated by the core URE sequence with little or no functional contribution from the adjacent flanking sequences. In general, the gel mobility shift and oligonucleotide competition data parallel the functional results: the conserved A and TC pair are required for protein-DNA complex formation and the CC pair was not critical. The results from both the transient transfection and binding experiments suggest that the identical sequence within the rDyn URE and hMIP 1β URE mediates the functional effects and supports the majority of the protein-DNA binding activity.

The prototypic Inr usually overlaps the RNA transcription start site and participates in transcription initiation (4, 5, 6). There are also more downstream Inr elements in some genes which exhibit the same functional characteristics (7, 11). Unlike these Inr sequences, several previous studies have indicated that more 5′-located Inr elements appear to act as transcriptional suppressors. A cellular protein, LBP-1 can interact with a long stretch of the HIV-1 promoter that includes a functional TATA element and an initiator element (12, 13, 14). LBP-1 suppressed transcription when bound to an upstream site overlapping the TATA element in the HIV promoter, but enhanced transcription when bound to a region more proximal to the transcription start site. (14). Another protein, YY1 can direct the general transcription machinery to initiate RNA synthesis at its P5 + 1 binding site in the adeno-associated virus type 2 P5 promoter (15, 16). Alternatively, YY1 can suppress transcription when the P5 +1 element is placed upstream of either a synthetic promoter or the SV40 early promoter/enhancer (16). The suppression of transcription by the two Inr-like UREs is consistent with these previous observations. The present data support the idea that Inr-like elements may have dual functions, either initiating transcription or suppressing transcription, depending, in part, upon their spatial location within the promoter. Within or downstream of the transcription start site it has a positive effect on transcription, whereas, when positioned further upstream it suppresses transcription. Initiation factors such as YY1 (15, 16), LBP-1 (12, 13), TF II-I and USF (6) that bind to Inr sequences have been extensively characterized functionally. The function of the UREB1 protein has been difficult to ascertain in transient co-transfection experiments, although we reported that the UREB1 was involved in the regulation of p53 transactivation (10). UREB1 is a member of a family of proteins and recently, a role in transcriptional processes, as a co-activator, also has been proposed for the yeast protein RSP5 and its human homolog hRPF1, both of which are UREB1 homologs (17).

The URE is found in the upstream promoter of both rat dynorphin and human macrophage inflammatory protein 1β and functions as a suppressor of the rat dynorphin promoter. We have not tested if the URE

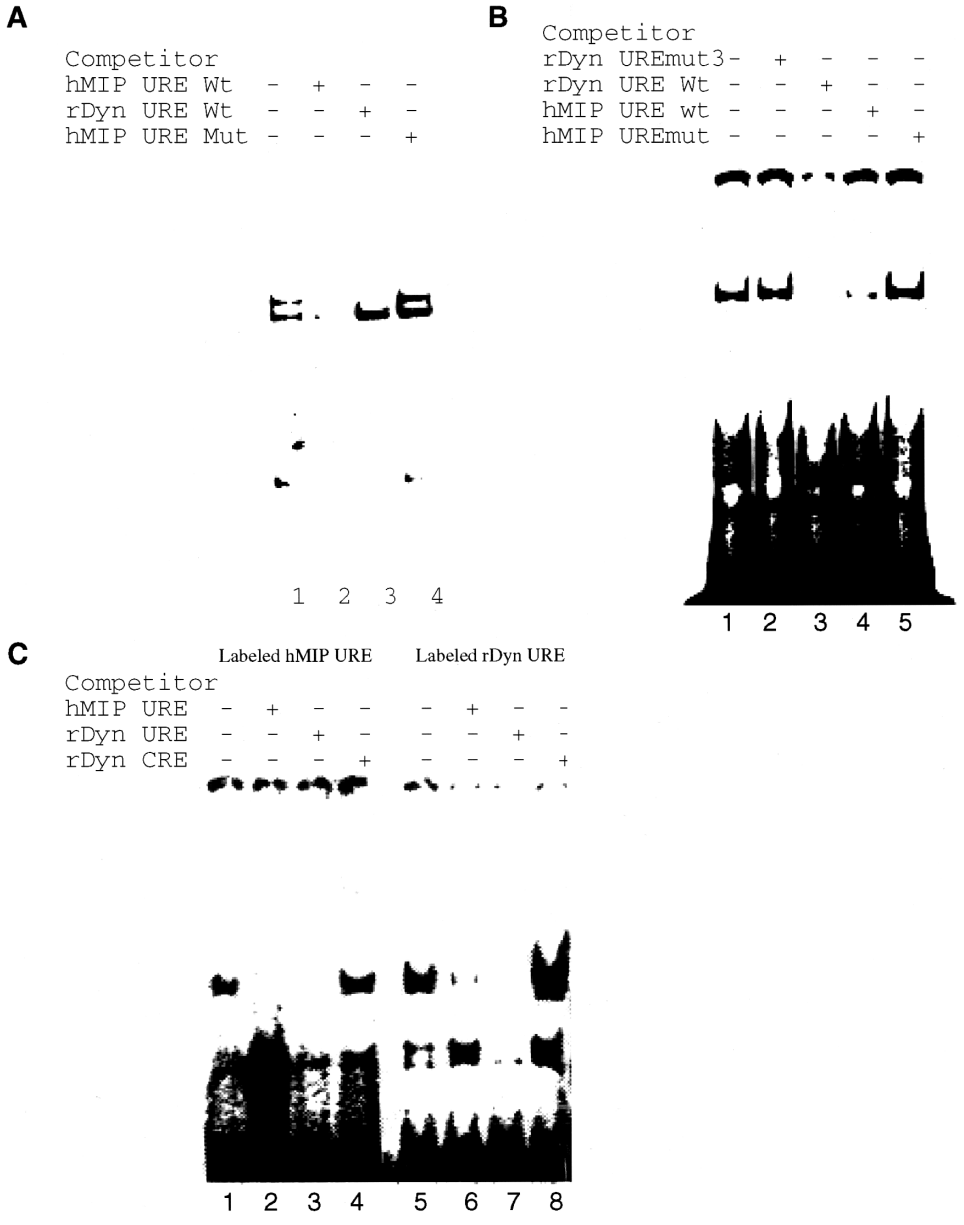

FIG. 3. rDyn URE and hMIP 1β URE recognize nuclear protein(s). (A) Gel mobility shift using labeled hMIP 1β URE oligonucleotide. Labeled hMIP 1β URE oligonucleotide was incubated with CHO cell nuclear extracts in the presence of no competitor or 100 ng of unlabeled competitor as indicated. Two complexes were formed using labeled hMIP URE and nuclear extract (lane 1). Both complexes were competed by the unlabeled wt sequence (lane 2) but not by the mutated sequence (lane 4). The prodynorphin URE competed only the upper complex, suggesting that the flanking sequence of hMIP 1β URE may contribute to the second complex formation. (B) Gel mobility shift assay using labeled rDyn URE oligonucleotide. Reaction conditions were as in panel A. The complex is formed by the labeled rDyn URE and nuclear extract (lane 1), and competed by its wt sequence and wt hMIP 1β URE (lane 3 and 4) and not competed by its mutated sequence and hMIP 1β mutated URE (lane 2 and 5). (C) Comparison of rDyn and hMIP 1β UREs. One μg of UREB1 protein was incubated with either labeled hMIP 1β (lanes 1-4) or labeled rDyn (lanes 5-8) URE oligonucleotides. The complex formed with hMIP 1β URE (lane 1) was competed by itself and rDyn URE (lane 2 and 3), but not by an unrelated CRE containing oligonucleotide (lane 4). The complex formed with rDyn URE (lane 5) was competed by itself and hMIP 1β URE (lanes 6 and 7) but not by the unrelated oligonucleotide (lane 8).

is a gene-specific or a general suppressor. The expression of rat dynorphin is hardly detected in the spinal cord and is transynaptically induced during peripheral inflammation (18). hMIP 1β is also induced by inflammation (19, 20). It is interesting to speculate a similar function in the two genes, such that the URE participates in transcriptional suppression which is released by inflammatory stimuli.

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