Volume 264, number 1, 125–129 FEBS 08408 May 1990

A putative AP-2 binding site in the 5′ flanking region of the mouse POMC gene

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Received 23 January 1990; revised version received 12 March 1990

Using extracts of AtT-20 cell nuclei, protein binding sites on the POMC gene 5′-flanking region were examined with an exonuclease protection approach. One such binding site, located from −119 to −106 bp upstream from the mouse POMC gene transcription initiation site, which exhibited a close homology to the activator protein-2 (AP-2) site [1]. A double-stranded oligonucleotide containing this site was subsequently used in gel shift assays to demonstrate AP-2 consensus sequence binding activity in extracts of AtT-20 cell nuclei. Gel shift competition experiments using both homologous and heterologous competitor DNA sequences revealed that the AP-2 like factor(s) exhibited specific binding to the mouse AP-2 consensus sequence. Furthermore, AP-2 factor binding was also modulated by a CTF/NF1-like factor. Pretreatment of AtT-20 cell nuclear extracts with alkaline phosphatase prior to inclusion in gel shift assays led to a reduction in the intensities of AP-2 factor-specific bands, indicating a potential involvement of protein phosphorylation in AP-2 factor binding in AtT-20 cells.

Activator protein-2; Proopiomelanocortin; AtT-20 cell; DNA binding protein; Transcription factor

1. INTRODUCTION

The proopiomelanocortin (POMC) gene has proved to be a valuable model of neuropeptide biosynthesis, processing and secretion [2,3]. For instance, several reports ([4,5] for review, see [2]) have established that regulation of POMC biosynthesis occurs primarily at the transcriptional level and this phenomenon is generally applicable to other neuropeptide gene systems [6]. However, transduction mechanisms responsible for alterations in POMC gene transcription rates effected by external stimuli are not well understood. Since most secretagogue-elicited intracellular events can be ascribed to activation of second messenger systems and their consequent actions on phosphorylation-dephosphorylation cascade, second messenger response elements may play a key role in activation of gene transcription. A number of such elements have been described in other neuropeptide gene systems including: a cAMP response element (CRE [7,8]); a phorbol ester (TPA) response element (TRE [9,10]) and elements responding to both of the aforementioned second messenger systems [1,8]. In addition, POMC gene glucocorticoid response elements (GREs) have been characterized [11–13] and a recent report has localized a putative corticotropin releasing factor (CRF) response element on the rat POMC promoter [12].

In order to localize novel *cis*-acting elements in the 5′-flanking region of the mouse POMC gene, we screened approximately 1 kb of this region with an exonuclease protection procedure, using nuclear extracts of mouse corticotrope tumor cells (AtT-20). The locations of exonuclease protection sites were then compared to the published sequence of this region [14] and a putative Activator Protein-2 (AP-2 [1]) site was selected for characterization.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Mouse anterior pituitary tumor cells (AtT-20/D16-16) were maintained in culture as previously described [15]. All cell culture media were obtained from Flow Laboratories, Inc., McLean, VA.

2.2. *Preparation of nuclear extracts*

Crude nuclear extracts of AtT-20 cells were prepared using a modification of the procedure of Dignam et al. [16]. Extracts were stored at −70°C dissolved in a minimal volume of buffer C (containing 20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT).

2.3. *Exonuclease protection assay*

The exonuclease protection assay has been described elsewhere [17]. Briefly, a 2 kb fragment of the mouse POMC promoter region

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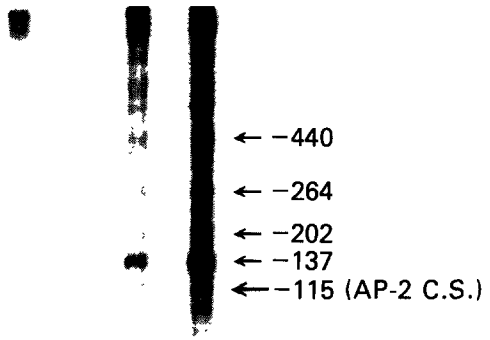


Fig. 1. Localization of a putative AP-2 binding site in the 5′ flanking region of the mouse POMC gene. As described in section 2, increasing concentrations of nuclear extracts of AtT-20 cells were incubated with a 3′-end labeled *Pvu*II fragment of the mouse POMC promoter region (see section 2). This mixture was then digested briefly with a 5′-3′ exonuclease (T7 gene 6 exonuclease) and the resulting ^{32}P-labeled fragments were separated on polyacrylamide gels, dried and autoradiographed. The M_r and associated fragment length of each of the more prominent bands (arrows) was determined by comparison with coelectrophoresed DNA standards. The numerical labels indicate the positions of the exonuclease protection sites (deduced from the fragment lengths) relative to the transcription start site. The putative AP-2 binding site (AP-2 C.S.) selected for further study is also indicated in the text figure in section 3. Data were taken from one of 3 experiments that produced similar results.

(kindly provided by Dr Shosaku Numa, Kyoto, Japan) was subcloned into pGEM (-3zf-), Promega, Madison, WI. The resulting plasmid (pGEM1201-4) was cut with *Pvu*II so that the *lac* operator (adjacent to the multiple cloning site on the vector) was retained on the 3′-end of an approximately 1.4 kb fragment (−1000 to +160 bp of the POMC gene + about 240 bp of vector). The fragment was 3′-end-labeled and incubated with *lac* repressor-β-galactosidase fusion protein and Immunobeads (Bio-Rad, Richmond, CA) precoupled to anti-β-galactosidase to form a precipitable complex. The ^{32}P-DNA-immunobead complex was then incubated with 0–100 μg nuclear extract and digested briefly with T7 gene 6 exonuclease. Finally, ^{32}P-DNA fragments were separated on 6% polyacrylamide sequencing gels and visualized by autoradiography. All of the reagents used in the exonuclease protection assay were purchased from Promega with the exception of the Immunobeads and the T7 gene 6 exonuclease (US Biochemicals, Cleveland, OH).

2.4. *Gel shift assay*

Detection of DNA binding factors in AtT-20 cell nuclear extracts was accomplished using a previously described method [18] with minor modifications. In each treatment condition, 5 μg of nuclear extract was incubated for 15 min at room temperature with a double-stranded, 3′-end labeled oligonucleotide (approximately 0.75 ng per tube) in a buffer containing 20 mM Tris-HCl (pH 8), 3 mM $MgCl_2$, 50 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, 20 μg of BSA and 5 μg of poly[d(I-C)] (Boehringer Mannheim, Indianapolis, IN). Following the incubation period, the mixture was subjected to non-denaturing polyacrylamide gel electrophoresis in 50 mM Tris-borate/EDTA buffer at 10°C and subsequent effects on mobility of the DNA fragment through the gel were visualized by autoradiography. The labeled probe contained 4 copies of the sequence:

5′-AGCCCCCCTCCCGAGG-3′
3′-TCGGGGGGAGGGCTCC-5′

which contains the AP-2 consensus sequence identified by the exonuclease protection assay 5′-CCCCCCTCCC-3′. In some experiments, double stranded competitor DNA was included in various concentrations or nuclear extracts were preincubated with alkaline phosphatase coupled to agarose beads (Sigma, St. Louis, MO) as specified in the figure legends. Competitor DNA sequences were purchased from Stratagene (La Jolla, CA) and homologous consensus sequences (1 × AP-2 and 4 × AP-2) were synthesized in our laboratory using an Applied Biosystems Inc. (Foster City, CA) model 381B oligonucleotide synthesizer. In order to facilitate comparison of data across experiments, competitor DNA concentrations were adjusted so that the number of core sequence copies per unit of competitor DNA was equivalent for all of the competitor DNA species.

3. RESULTS

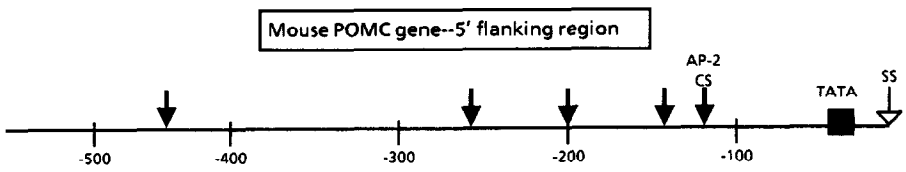
3.1. *Exonuclease protection sites*

Incubation of a ^{32}P-labeled DNA fragment containing approximately 1 kb of the 5′-flanking region of the mouse POMC gene with increasing concentrations of nuclear extracts of AtT-20 cell nuclei produced a number of T7 gene 6 exonuclease protection sites (Fig. 1). In this assay, the location of a particular protection site can be approximated from the relative molecular mass (M_r) of the fragment generated [17]. The approximate positions of the sites labeled in Fig. 1 are depicted in Scheme 1 (filled arrows). In addition, the position of the TATAA box and the transcription start site (SS) are shown. A few other putative hormone and second messenger inducible elements have been described [11–13], but as yet, none have been precisely localized.

The locations of the exonuclease protection sites were compared to the published sequence of the 5′-flanking region of the murine POMC gene [14] and an AP-2 consensus sequence, indicated above, was selected for further study.

3.2. *Characterization of the mouse AP-2 site*

Incubation of ^{32}P-labeled 4 × AP-2 probe with



Scheme 1

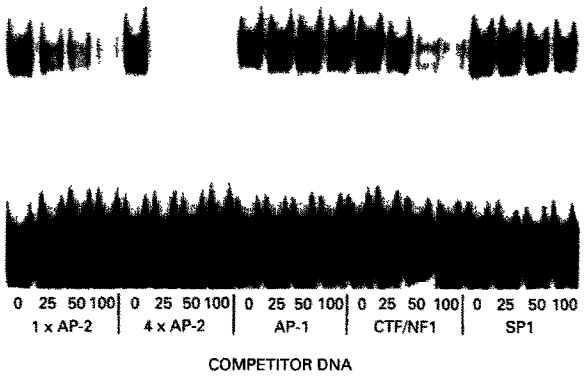


Fig. 2. Characterization of an AP-2-like factor in AtT-20 cell nuclei. Gel shift experiments were performed using the ^{32}P labeled 4 × AP-2 probe described in section 2. Typically, 0.75 ng of probe was incubated for 15 min with 5 μg per assay tube of nuclear extract, 5 μg of poly d(I-C), 20 μg of BSA and various concentrations of double stranded competitor DNA in a final volume of 40 μl. Competitor DNA concentrations are given in consensus sequence units as described in the text. A value of 25 represents 25-fold more consensus sequence units than is contributed by the probe. Protein-DNA complexes were separated on non-denaturing polyacrylamide gels, dried and autoradiographed. Unbound probe can be seen at the very bottom of the lanes. Data were taken from a single representative experiment. Each treatment condition was included in at least 4 experiments.

Fig. 3. Effects of alkaline phosphatase treatment on AP-2 factor binding activity. Duplicate aliquots of AtT-20 cell nuclear extracts were subjected to gel shift analysis as described in section 2, except that the electrophoresis buffer was 100 mM TBE. In addition, parallel aliquots of the extract were pretreated with alkaline phosphatase prior to gel shift analysis. Data were taken from a single representative experiment. (−) = extract preincubated without phosphatase; (+) = extract preincubated with phosphatase.

nuclear extracts of AtT-20 cells resulted in reduced electrophoretic mobility of the probe and this effect could be modified by inclusion of competitor DNA. An example of the results of a typical competition experiment appears in Fig. 2. In general, two bands (see lanes labeled 0 in Fig. 2), moving at a reduced rate as compared to the unbound probe. In addition, these two bands were sometimes accompanied by a third, slower migrating band (data not shown). In competitor experiments, four previously identified consensus DNA sequences were either ineffective (AP-1, AP-3, SP1) or partially effective (CTF/NF1) competitors for nuclear factors binding to the 4 × AP-2 probe. As shown in the figure, both bands were clearly visible in the presence of 100 consensus units of AP-1 or SP1. Addition of AP-3 oligonucleotide to the binding reaction gave results identical to those obtained with AP-1 or SP1 (data not shown). However, addition of CTF/NF1 consensus DNA produced a concentration-related diminution of band intensity. As shown in the lanes marked '1 × AP-2' in the figure, shifted bands were essentially abolished by addition of a 100-fold excess of 1 × AP-2 consensus sequence. Interestingly, a 25-fold excess of unlabeled probe ('4 × AP-2') totally abolished the shifted bands.

Treatment of AtT-20 cell nuclear extracts for 10 min with alkaline phosphatase immobilized on agarose beads essentially abolished shifted bands as compared to identical extracts given the same incubation without the inclusion of phosphatase (Fig. 3). Treatment of ^{32}P-labeled 4 × AP-2 probe with phosphatase without the inclusion of nuclear extract had no effect on the ^{32}P content of the probe (data not shown).

4. DISCUSSION

In order to characterize POMC gene 5'-flanking sequences that might communicate receptor-mediated signals to transcriptional machinery, we attempted to localize possible protein binding sites using an exonuclease protection assay. One such binding site was selected for further characterization. This sequence, located −119 to −106 of the mouse POMC gene, which was used for construction of the gel shift probe, is a permutation of the AP-2 consensus sequence derived by Imagawa et al. [1]. As indicated in the schematic in section 3, several other exonuclease protection sites were routinely seen and are currently under investigation.

The results of the gel shift experiments, represented by Fig. 2, indicate that an AP-2-like factor exists in extracts of AtT-20 cell nuclei. Incubation of nuclear extracts of AtT-20 cells with a ^{32}P-labeled oligonucleotide composed of 4 copies of the AP-2 sequence led to visualization of two bands exhibiting retarded mobility in native gels. The reason that two bands were seen is unknown but might result from binding of AP-2 factor dimers (slower band) vs monomers (faster band) as proposed by Yamamoto et al. [7] in their work on the somatostatin gene CRE. Since a 100-fold excess of 1 × AP-2 sequence was required to abolish shifted bands,

while addition of even the lowest concentration (a 25-fold excess of consensus units) of cold 4 × AP-2 totally abolished both band (Fig. 2), cooperative forces may be operating between molecules of the AP-2-like factor or between the AP-2-like factor and other components of the nuclear extracts. Examples of cooperation between proteins binding to DNA are extensive in the literature [19]. As expected, consensus binding sites for AP-1 [9,10], AP-3 [20] or SP1 [22] were ineffective competitors for nuclear factor(s) binding to the probe at 100-fold excess concentrations (Fig. 2). In fact, these sequences did not abolish binding even when concentrations as high as 200-fold were utilized (data not shown).

However, significant reductions in gel shift band intensities were seen after the addition of a 50- or 100-fold excess of the CTF/NF1 oligonucleotide. These results were suprising because the CTF/NF1 sequence (coding strand = 5′-TGGCTTGAAGCCAA-3′) has practically no similarities to the AP-2 sequence – see [21] for a comparison of CTF/NF1 consensus sequences. Therefore, it is unlikely that this is the result of a direct competition between CTF/NF1 and the 4 × AP-2 probe for the AP-2-like factor and suggests instead that a CTF/NF1-like factor may modulate AP-2 factor binding via protein-protein interactions.

The rapid increase in POMC gene transcription initiation seen after treatment of AtT-20 cells with CRF or other secretagogues which utilize cAMP as second messenger [2,4,5] is independent of ongoing protein synthesis [23] implicating posttranslational mechanisms. Similarly, treatment of these cells with phorbol esters, which activates protein kinase C (PKC), increases POMC mRNA concentrations [24,25]. Since, in HeLa cells, AP-2 mediated transcription can be increased by treatments which increase either cyclic AMP-dependent protein kinase (PKA) activity or PKC activity [1], the AP-2-like protein suggested by the present work may perform a similar function in AtT-20 cells. Thus, activation of either PKA or PKC in AtT-20 cells would lead to specific phosphorylation events crucial to AP-2 binding or to AP-2-driven modulation of POMC gene expression. As shown in Fig. 3. preincubation of nuclear extracts of AtT-20 cells with alkaline phosphatase reduces band intensities in gel shift assays, which is consistent with the first criterion of the above hypothesis. In other neuropeptide gene systems, phosphorylation mechanisms have been implicated in modulation of transcription. For example, Yamamoto et al. [7] have demonstrated that phosphorylation of cellular extracts containing CREB, a nuclear *trans*-acting factor implicated in the regulation of somatostatin gene transcription, dramatically increases cAMP responsive element (CRE)-specific transcriptional activation. It was suggested that the converse (phosphatase treatment) would have a negative effect on CREB mediated transcriptional activation because phosphatase treatment led to a functional alteration in CREB-CRE binding activity [7]. However, alterations in binding were not correlated with transcriptional effects since phosphorylation of extracts containing CREB by addition of PKA did not alter CREB-CRE binding [7].

Since converging actions of PKA and PKC have been demonstrated at several cellular levels [1,15,26], the possible role of protein phosphorylation in the activation of POMC gene transcription merits further investigation. Investigations that are currently in progress in our laboratory will determine if the AP-2-like binding factor described in the present work is identical to the previously described AP-2 [1] or perhaps a functional analog.

Acknowledgements: Dr John Quinn, NCI, NIH, Bethesda, MD, for helpful discussions and instruction concerning the exonuclease protection assay; Dr James Roberts, Mount Sinai School of Medicine, New York, NY, for helpful discussion of the rat AP-2/SP1 binding site.

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