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Characterization of Phorbolester-Inducible Human Neuronal Factors Involved in *Trans*-Activation of the Galanin Gene

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The expression of the neuropeptide galanin (GAL) is elevated *in vivo* upon nerve stimulation, injury, and *in vitro* by phorbol 12-myristate-13-acetate (PMA), suggesting that a signal pathway involving protein kinase C activation may be involved in GAL-gene activation. When plasmids containing a different length of the bovine GAL-promoter fused to luciferase were transfected into the human neuroblastoma cell line (SK-N-SH subclone SH-SY5Y), a PMA-responsive element was identified in the promoter-region −68 to −46 base pairs (bp). Co-transfection experiments with plasmids expressing cJun and cFos revealed that they could act alone, as well as synergistically with PMA to induce luciferase activity. Electrical mobility shift assays revealed that a cAMP response element (CRE)-like sequence (TGACGCGG; −59 to −52 bp) bound PMA-inducible nuclear proteins present in SH-SY5Y cells. These proteins appear to bind mainly as CRE-binding-protein/activating-transcription-factor (CREB/ATF) and Jun/ATF heterodimers. In addition, an apparent PMA-inducible protein(s) not recognized by CREB/ATF and Jun antibodies bound to the CRE-like containing probe.

Galanin (GAL) is a neuroendocrine peptide [1] which is found in specific neuronal systems in the brain and spinal cord, as well as in the peripheral nervous system, like in the gastrointestinal tract, pancreas, adrenal gland and uro-genital tract (for review, see [2, 3]). GAL has been implicated in diseases such as diabetes (for review, see [3, 4]) and dementia of the Alzheimer's type (for review see [5]). In addition, GAL has been shown to be a potent elevator of plasma growth hormone and prolactin levels, to be involved in nociception, as well as in the control of gastrointestinal motility [2, 6, 7] and food intake [8, 9]. Hence, in view of the data above, the possible physiological and pathophysiological implications of GAL, it is of great interest to obtain information on the processes governing GAL-gene regulation.

GAL-expression can be regulated by nerve stimulation (depolarization, damage or injury), by hormones (estrogen and glucocorticoids), by growth factors like nerve growth factor (NGF) and by pharmacological activation of different second messenger systems [10-13]. Thus, in reference to the latter, GAL-expression is increased in primary cultures of bovine adrenal chromaffin cells, as well as in the neural crest-derived human neuroblastoma cell line (SK-N-SH subclone SH-SY5Y) cells, following elevation of intracellular calcium, activation of the protein kinase A (PKA) and protein kinase C (PKC) pathways [11, 14].

The partial organization of the of the bovine GAL gene has been established, i.e. the promoter region and the three first exons and introns [14, 15]. Our analysis [14] of the bovine GAL-promoter in human neuroblastoma SH-SY5Y cells demonstrated that 0.131 kilobases (kb) of the promoter was sufficient for basal expression, and that an apparent silencer of the gene was located between 5 and 0.9 kb upstream from the transcriptional start site. Despite the presence of several consensus-binding-sequence in the proximal GAL-promoter that are known to mediate phorbol 12-myristate-13-acetate (PMA)-induced responses, it appeared that none of our GAL-constructs, using between 5 kb and 131 base pairs (bp) of the promoter and the first exon, could be clearly *trans*-activated by PMA [14], suggesting that such a PMA-responsive element(s) may reside elsewhere on the GAL-gene or may not have been clearly revealed in the SH-SY5Y cells under the conditions used.

In this study, we identified a PMA-responsive ele-

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ment in the GAL-gene and characterized the human *trans*-activating protein(s) in the neural crest-derived cell line (SH-SY5Y) involved in this response. Moreover, we identified regions and/or sequence(s) on the GAL-gene that are necessary and sufficient for maximal basal activity in this human cell line.

MATERIALS AND METHODS

Plasmids. The construction of the 131GAL-luc plasmid [14], and the promoterless plasmid (pXP2) [16] which served as control, have been described elsewhere. The 46GAL-luc plasmid was constructed by cleaving a promoter-exon fragment (XhoI-SacI; −451 to +215) [14] with the blunt-end generating enzyme BsrBI. A 227 bp fragment, containing 46 bp of the promoter, including the TATA-box, and essentially the entire first exon down to +181 was subcloned into the pXP2 plasmid which had been digested with SmaI. The orientation of the insert was checked by PstI digestion. The 68GAL-luc plasmid was constructed by isolating the insert from the 131GAL-luc plasmid (excised by SmaI and KpnI), followed by partial digestion with BsrBI which generates a fragment between −68 and +181. This fragment was recloned into the pXP2 plasmid which had been digested with SmaI. The 91GAL-luc plasmid was constructed by digestion of the 131GAL-luc [14] using SmaI and KpnI, thus liberating the insert from the pXP2 plasmid. The purified insert was partially digested with Cac8I which removes 37 bp from the 5′-end and the remainder of the insert was religated into the empty 131GAL-luc vector.

Other plasmids. A plasmid driven by the thymidine kinase (tk) promoter containing three AP1-binding-sites driving the expression of the chloramphenicol acetyltransferase, PK3-tk-CAT [17], expression plasmids driven by the Raous Sarcoma Virus (RSV) promoter driving the expression of the enzyme β-galactosidase (pRSV-βGal) [18], the cJun (RSV-cJun) [19] and cFos (RSV-cFos) [20]. The plasmids pBS and/or Bluescript (Stratagene) were used as "inert" DNA to keep the amount of total DNA constant in those experiments where various plasmids were combined.

Cell-culture, -transfection, and enzyme assays. Human neuroblastoma (SK-N-SH subclone SH-SY5Y) cells [21] were cultured in Dulbecco's Modified Eagles Medium supplemented with 8% heat-inactivated fetal bovine serum, L-glutamine and antibiotics, in 5% CO_2 atmosphere. Experiments were carried out on cells between the 50th and 70th passage. For experiments, cells were plated in 6-well plates at a density of 0.5×10^6/well approximately 48 hours before transfection. Transfection was performed with the calcium-phosphate-method using 2 μg of the different GAL-luc plasmids, the promoterless pXP2-plasmid, or the PK3-tk-CAT plasmid, together with 2 μg pRSV-βGal (control for transfection efficiency); in one serie of experiments 1.2 μg RSV-cJun and 0.6 μg RSV-cFos or 1.8 μg Bluescript or pBS were co-transfected.

After 24 hours the old medium was removed, and the cells were washed twice with phosphate buffered saline (PBS) before the addition of medium with 10 nM PMA diluted in dimethylsulfoid (DMSO) or the same amount of medium with only DMSO (for controls). After 18 hours of drug-treatment, the cells were washed twice with PBS, harvested and assayed for luciferase activity. β-galactosidase and chloramphenicol acetyltransferase (CAT) were performed with commercial kits essentially according to the manufacturer (Promega Corporation, US). Protein concentration was measured according to the manufacturer using the BCA protein-assay kit (Pierce, US).

Preparation of nuclear extracts and electrical mobility shift assay (EMSA). Nuclear extracts were prepared essentially as described previously [22]. For the EMSA, nuclear extracts (2-4 μg of protein) were preincubated in the presence or absence of Jun (3-10 μl), cAMP responsive element (CRE) binding-protein (CREB; 1-5 μl) or activating-transcription-factor (ATF; 1-2 μl) antibodies (see below) on ice for 60 min with 20 μl of 10 mM Hepes-buffer (pH 8.0) containing glycerol (17 %), NaCl (10 mM), EDTA (0.1 mM), $MgCl_2$ (4 mM), DTT (2 mM), BSA (100 μg/ml), spermidine (4 mM), poly d[I-C] (60 ng/μl; Pharmacia) and sonicated salmon sperm DNA (50 ng/μl). The labelled probe was added and the samples were incubated at room temperature for an additional 15 min. The complexes were separated on a polyacrylamide gel (5%) and visualized by autoradiography The sense strands of the annealed oligonucleotides used were as follows: TPA (identical to PMA)-response element (TRE)-consensus (CGCTTGA**TGACTCA**GCCGGAA), CRE-consensus (GATTGCC**TGACGTCA**GAGAGCTAG), TRE-like (GATCCGGGGAC**TGAGTTC**CGGTCACTCC). CRE-like (GATCCGAGCCCG**TGACGCGG**CCGAGCGGC; Fig. 1). The latter two contain the bovine GAL-promoter sequences −128 to −106 and −66 to −44 [14], respectively, as well as restriction sites at each end (underlined).

Antibodies and recombinant transcription factors. Specific Jun rabbit polyclonal antibodies were generated by immunization with synthetic peptides coupled to keyhole limpet hemocyanin. The peptides used were: c-Jun, SYGAAGLAFPSQPQQ; JunB, ISYLPHAPPFAGG; and JunD, GCQLLPQHQVPAY. No cross-reactivity was observed between the different antisera [23]. The polyclonal antibody CREB was generated by immunization with bacterially expressed CREB [24]. This antibody reacts with several members of the CREB/ATF family (CREB1, 2 and ATF1, 2 and 3 have been explored and they all crossreact; personal communication Dr Mats Nilsson, Center for Nutrition and Toxicology, Karolinska Institutet). The CREB1 antibody (C-21), specific for CREB1, the CREB2 antibody (C-20), specific for CREB2/ATF4, and the ATF1 (C41-5.1), ATF2 (C19; cat#sc-187) and ATF3 (C19; cat#sc-188) antibodies, all specific for the corresponding proteins, respectively, were obtained from Santa Cruz Biotechnology Inc (CA).

Transcription factors were synthesized *in vitro* by transcription-translation of plasmids containing cJun, cFos and JunD cDNAs. Recombinantly produced CREB1 was kindly provided by Dr Johan Lund (Dept. of Medical Nutrition, Karolinska Institutet).

RESULTS

Functional identification of promoter-regions essential for basal and PMA-induced transcription in human neuroblastoma cells. Since we found previously that PMA increases GAL-mRNA in SH-SY5Y cells [14], we co-transfected the 131GAL-luc plasmids with 1.2 μg RSV-cJun and 0.6 μg RSV-cFos. Luciferase activity was increased 2.8±0.5 fold compared to 1.6±0.3 fold increase for the pXP2 plasmid (mean±SD; n=3 each). When the 131GAL-luc was co-transfected with RSV-cJun and RSV-cFos and treated with PMA (10 nM), highly significant and apparent synergistic inductions were obtained (Fig. 2A). As control for *trans*-activation of cJun and cFos following transient transfections, we used the control plasmid PK3-tk-CAT, which has three AP1 sites upstream of the tk-promoter. When the PK3-tk-CAT plasmid was co-transfected with the plasmids RSV-cJun and RSV-cFos and treated with PMA (10 nM), an apparent synergistic induction was obtained similar to that observed with the two GAL-luc plasmids (Fig. 2B).

To determine the minimal sequence required for PMA (10 nM) induced GAL-transcription, we tran-

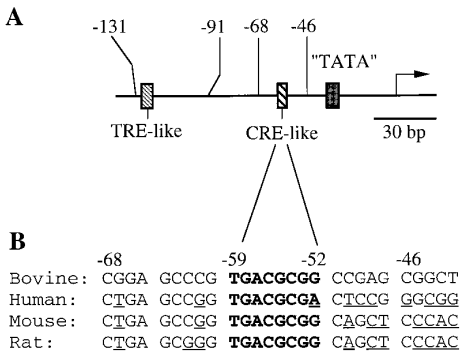

FIG. 1. Schematic drawing of the bovine GAL-promoter [14] and species comparison of the nucleotides in the GAL-gene surrounding the CRE-like sequences. (A) Representation of potential DNA:protein-interactions in the GAL-promoter. (B) Comparision of the bovine, human, mouse and rat GAL nucleotide sequences in the promoter-region to which a functional PMA-response was mapped in this study. Underlined nucleotides indicate differences from the bovine sequence and bold characters the CRE-like element.

siently transfected human SH-SY5Y cells with plasmids containing different deletions of the GAL-promoter coupled to luciferase; i.e. 131, 91, 68 and 46 bp of the promoter as well as the promoterless pXP2 plasmid. In doing so, we found that a PMA-responsive element appeared to be present between −68 and −46 bp (Table I). The basal promoter activities of the 91GAL-luc, 68GAL-luc and 46GAL-luc constructs were approximately 77, 39 and 3 % of the 131GAL-luc construct (100 %), respectively, suggesting that additional 5′-promoter sequences (−131 to −68 bp) may be needed to restore apparent maximal basal GAL-luc activity.

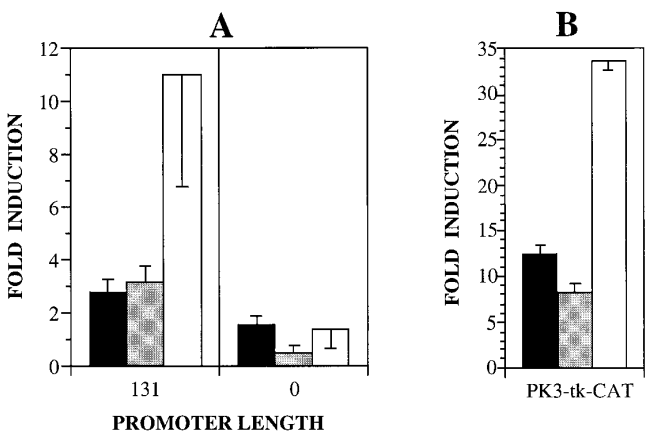

FIG. 2. AP1-response of the 131GAL-luc and the 0GAL-luc (pXP2) and PK3-tk-CAT-control plasmids in human neuroblastoma cells. (A) Luciferase- and (B) CAT-activity (fold induction corrected for transfection efficency using β-galactiosidase) obtained after co-transfection with plasmids expressing AP1-proteins (cJun/cFos; bold bars), treatment with PMA (10 nM; hatched bars) or a combination of the two procedures (open bars) for 18 hours. The bars in A represent mean±SD from three independent experiments performed in triplicates or duplicates whereas one representative experiment is shown in B.

TABLE 1

Basal and PMA Induced Luciferase Activity in Human Neuroblastoma (SK-N-SH-SY5Y) Cells Transfected with the Galanin/Luciferase Plasmids

	Basal activity (arbitrary units)	PMA (10 nM) (fold-induction)
Galanin/luciferase		
131	459 ± 90	2.1 ± 0.4
91	354 ± 192	2.3 ± 0.7
68	179 ± 91	1.6 ± 0.3
46	14 ± 14	0.7 ± 0.2
Control		
0 (promoterless parent; pXP2)	2.4 ± 1.4	0.8 ± 0.2

Note. Basal luciferase activity (arbitrary units) for the various bovine GAL-promoter plasmids corrected for transfection efficiency using β-galactosidase activity. The numbers for the various promoters indicate base pairs up-stream from the transcriptional start site. Luciferase activity (fold induction) after treatment with 10 nM PMA for 18 hours. Data represent mean±SD for 6 independent experiments performed in duplicates or triplicates using at least two batches of plasmids for each construct.

Electrical mobility shift assay identify a CRE-like-specific complex. To demonstrate the presence of PMA-inducible nuclear factors in SH-SY5Y cells we performed EMSAs. In the experiments shown in figure 3 we used a labelled CRE-like oligonucleotids (Fig 1) and nuclear extracts prepared from SH-SY5Y cells treated with DMSO (control) or PMA (10 nM). Two major DNA-binding protein-complexes were usually formed with the CRE-like probe and these complexes were further analyzed by competition-experiments. A 50-fold molar excess of unlabelled probes containing the TRE- and CRE-consensus sequences as well as the TRE- and CRE-like sequences present in the bovine GAL-promoter were used. The upper complex (1), as well as a part of the lower complex (2), could be competed with TRE- and CRE-consensus probes, the latter being more efficient. Only a slight reduction in intensity of both complexes could be observed with the TRE-like probe. However, the protein-binding in complex 1 and 2 was efficently competed with the CRE-like probe. When the unlabelled CRE-consensus probe was used (≥100-fold excess) complex 1 was completely removed while a part of complex 2 still remained (Fig. 4, lane 2-11), indicating that complex 2 represent different protein-probe-interactions that migrate very closely. In addition, as seen from figure 4 (lanes 2 to 9), one or several of the nuclear protein(s) that bind to the labelled CRE-like probe (complex 2) after preabsorption with unlabelled CRE-consensus probe is also PMA-inducible.

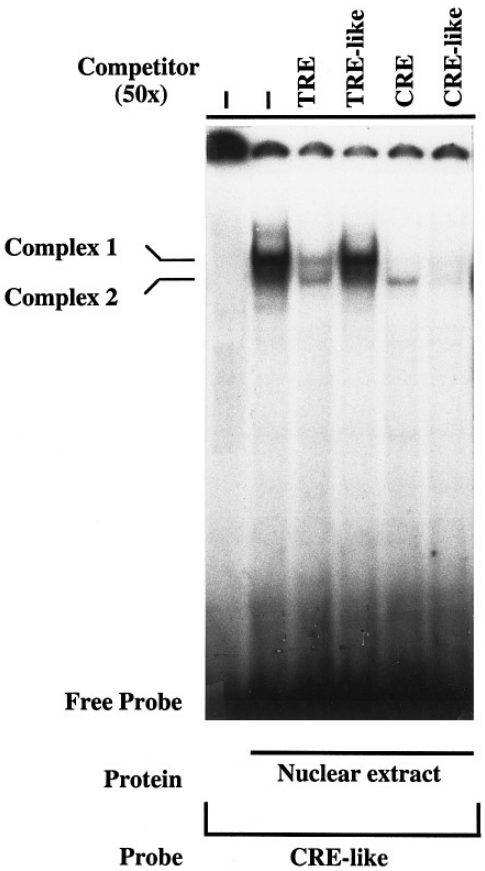

FIG. 3. Competition-electrical mobility shift assay (EMSA) of ^{32}P-labelled CRE-like binding activities in human neuroblastoma nuclear extracts after treatment with 10 nM PMA for 18 hours. Competition was performed with a 50-fold molar excess of unlabelled competitor: TRE-consensus, TRE-like, CRE-consensus and CRE-like oligonucleotides.

These protein(s) bind preferentially to the CRE-like probe since the addition of a further 100-fold of the CRE-consensus probe to 200-fold (lane 11), compared with the addition of 100-fold CRE-like probe (lane 10), did not reduce the intensity of the PMA-induced nuclear protein(s) binding in complex 2. Initial time-response studies performed with the CRE-consensus (data not shown) and CRE-like probes (Fig. 4; lane 12-15), demonstrated that the PMA-inducible (10 nM) binding appeared to change in a time-dependent fashion.

Members of the AP1- and CREB/ATF-families are part of the CRE-like protein-complexes. To identify specific proteins present in the CRE-like complexes, a panel of different antibodies (Fig. 5A and B) directed against some proteins known to interact with the CRE-consensus sequence were used in EMSA. Complex 1 was disrupted with the c-Jun and JunD antibodies and partially disrupted by the JunB directed antibody. In this context, we also observed that the CRE-like probe could bind *in vitro* translated JunD/c-Fos (Fig. 5A) as well as cJun/cFos (data not shown). The general polyclonal CREB/ATF antibody, which recognizes several members of the CREB/ATF family, disrupted the binding of all components contributing to complex 1 and the further, more specific, analysis revealed that the ATF2, ATF3, CREB2/ATF4 and CREB1 antibodies disrupted complex 1 in varying degrees (Fig. 5A and B). In this context we also observed that the CRE-like probe could bind recombinantly produced CREB1 and that the amount of the CREB/ATF antibody used could abolish the CREB1 binding (Fig. 5A).

Complex 2 was also efficently disrupted with the general CREB/ATF antibody and in fact all individual members of the CREB/ATF family (Fig. 5B) seemed to be involved in the formation of complex 2 in varying degrees. The Jun specific antibodies had only a slight, if any, effect on complex 2. Thus, the antibody-experiments suggest that the nuclear protein(s) contributing to complex 2 appear to be mainly related to the known members of the CREB/ATF-family. However, since complex 2 could not be completly disrupted with the general CREB/ATF antibody, it may be suggested that an additional protein(s) exist that can bind to the CRE-like probe and that these protein(s) probably do not belong to the CREB/ATF family.

DISCUSSION

We have previously shown, that both GAL mRNA and peptide levels in bovine adrenal chromaffin cells and in human neuroblastoma cells, are stimulated by PMA, suggesting that the PKC pathway may be involved in mediating this response [11, 14]. Moreover, we have shown that the bovine GAL-promoter contains a number of consensus binding sequences for *trans*-activating proteins that are known to be activated by PMA, i.e. AP2, EGR1 and NF-kB, as well as a half CREB sequence [14].

To start dissecting the nature of possible mediators of PMA-induced increases of GAL mRNA, we first explored whether or not the classical AP1-protein family members Jun/Fos [25] could *trans*-activate the bovine GAL-gene. Thus, co-transfection of the 131GAL-luc plasmid with the expression-plasmids for cJun/cFos resulted in approximately three-fold elevations of the basal promoter activity. To further understand the mechanism by which PMA induces GAL mRNA in SH-SY5Y-cells, we transfected a set of plasmids containing different length of the GAL-promoter into these cells. We obtained a significant induction in response to PMA with all GAL-plasmids harboring 68 bp or more of the promoter, but not with 46GAL-luc and the promoterless plasmid pXP2. Thus, a PMA-responsive-element

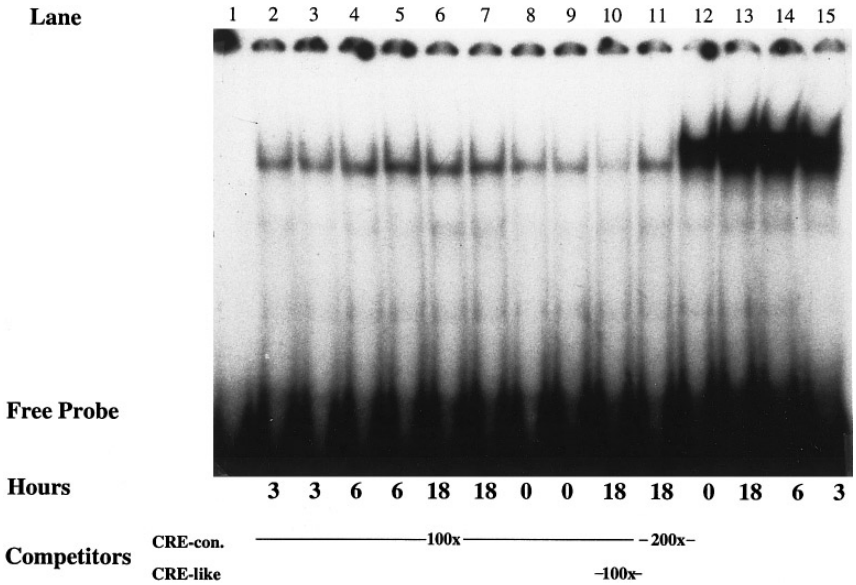

FIG. 4. Electrical mobility shift assay (EMSA) of ^{32}P-labelled CRE-like binding activities in human neuroblastoma nuclear extracts after treatment with 10 nM PMA for 0 to 18 hours (h): 0 h (lanes 8, 9 and 12), 3 h (lane 2, 3 and 15), 6 h (lanes 4, 5 and 14), 18 h (lanes 6, 7, 10, 11 and 13). Competition was performed with unlabelled CRE-consensus (100-fold molar excess) oligonucleotide in lanes 2 to 10; in lane 10 a 100-fold excess of unlabelled CRE-like oligonucleotide was also added, and in lane 11 a 200-fold excess of unlabelled CRE-consensus oligonucleotide was added.

on the bovine GAL-gene appears to reside somewhere in the promoter-region 68 to 46.

Since there were only a half CREB sequence (the CRE-like site), we explored the latter for protein-binding in EMSAs. In addition, a TRE-like site located further upstream in the promoter-region (−131 to −91; Fig. 1)) that may also be involved in the PMA-responsiveness was also explored for protein-binding in EMSAs. In doing so, we found that only the former probe containing the CRE-like sequence (TGACGCGG: −59 to −52) bound PMA-inducible proteins present in nuclear extracts prepared from SH-SY5Y cells. During the progress of this work, Anouar and coworkers [15] reported that PMA-inducible-nuclear-proteins, isolated from primary cultures of bovine adrenal chromaffin cell, could also bind to a probe containing the CRE-like site. Moreover, they found that the binding of bovine PMA-inducible proteins to their CRE-like probe was reduced by unlabelled probes containing CRE- or TRE-consensus sequences which is in agreement with our results using nuclear extracts obtained from human neuroblastoma SH-SY5Y cells. Identical (Fig. 1) CRE-like sequences are present in the mouse and rat GAL-promoters [26, 27] whereas the human GAL-promoter CRE-like sequence (TGACGCGA) is different at one nucleotide position (underlined) [28]. Similar non-palindromic TRE- and CRE-like sequences have also been found in a number of PMA and/or cAMP-inducible genes ([29, 30] and reference therein), but contrary to the consensus TRE- and CRE-elements, the protein-binding to these degenerate elements is somewhat different since most of them do not bind Jun/Jun homodimers and they bind Jun/Fos heterodimers with lower affinity [29, 30]. In view of this, we tested the ability of some proteins belonging to the AP1- and CREB/ATF-families to bind to the CRE-like element. We found that CREB1 proteins alone, or combinations of JunD/cFos or cJun/cFos proteins, did bind to the CRE-like probe whereas cJun/cJun binding could not be detected (data not shown).

In this study, an immunological characterization of the SH-SY5Y nuclear-proteins binding to the CRE-like element in the GAL-promoter was performed for the first time using antibodies against different members of the AP1- and CREB/ATF-families. We found that a general CREB/ATF polyclonal antibody inhibited the formation of almost all protein-CRE-like-interactions in complex 1 and 2, whereas specific ATF and CREB antibodies as well as different Jun antibodies inhibited in varying degrees the formation of such complexes. This suggests that the majority of the PMA-inducible proteins in human SH-SY5Y cells, which bind to the CRE-like sequence in the bovine GAL-promoter, appear to be members of the Jun- and CREB/ATF-families, forming either Jun/ATF and CREB/ATF heterodimers and possibly also CREB/CREB and ATF/ATF homodimers. However, the competitive EMSAs indicate that the CRE-like containing probe may bind additional apparent specific PMA-inducible proteins, since some protein-binding to this probe (complex 2) always

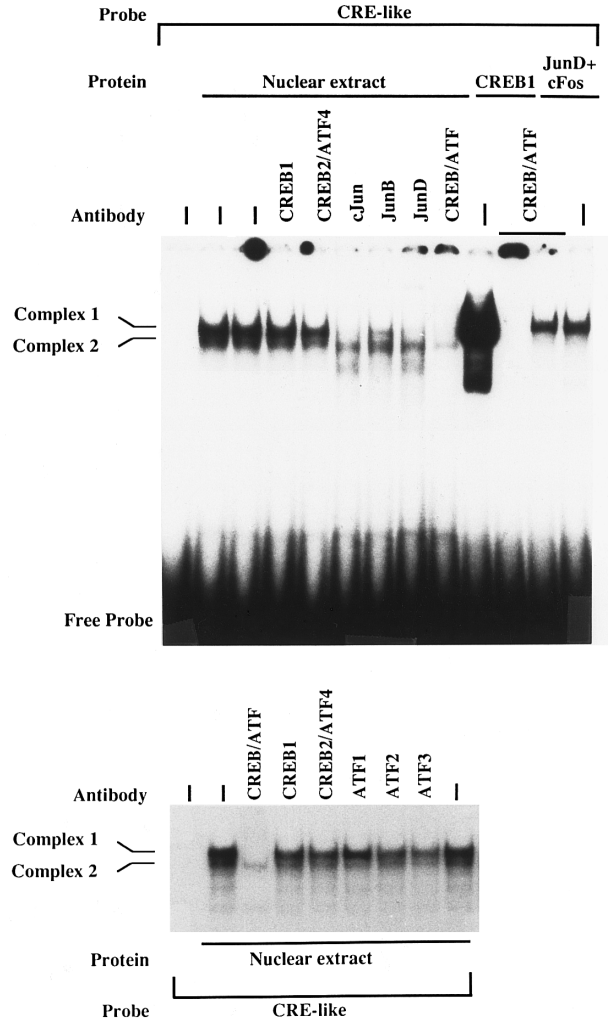

FIG. 5. Identification of protein-forming-complexes with the ^{32}P-labelled CRE-like probe. Electrical mobility shift assay was performed using human neuroblastoma nuclear extracts prepared after treatment with 10 nM PMA for 18 hours. Equal amounts of nuclear extracts (4 μg), recombinantly produced CREB1 (0.2 μg) and *in vitro*-translated JunD/cFos (2 μl) were incubated with ^{32}P-labelled CRE-like probe. Proteins were immunologically identified using antibodies specifically recognizing: (A) cJun, JunB and JunD, (B) ATF1, ATF2, ATF3, as well as (A and B) CREB1, CREB2/ATF4 and CREB/ATF. In (A) 4-10 μl of the antibodies were used and in (B) 1 μl of each antibody.

remained after preincubation with the general polyclonal CREB/ATF antibody. A similar and/or identical, protein-binding to the CRE-like probe also remained after competition with an excess of unlabelled CRE-consensus oligonucleotide. Taken together, these latter results indicate that there exist additional apparent PMA-inducible nuclear proteins in human SH-SY5Y cells that bind to the CRE-like sequence identified in the GAL-promoter; these protein(s) appear not to belong to the CREB/ATF-family or the Jun family. Functionally, cJun and GAL are co-regulated and increased in the dorsal root ganglia following neuronal damage/transection of the siatic nerve [31]. Thus, the binding of AP1 and/or PMA-inducible proteins to the identified CRE-like element as demonstrated in this study may be involved in the *trans*-activation of the GAL-gene in connection with i.e. damage to specific GAL-expressing neurons as well as in the basal forebrain in Alzheimer's patients [5].

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