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Novel Dimeric Nur77 Signaling Mechanism in Endocrine and Lymphoid Cells

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Within the nuclear receptor family, Nur77 (also known as NGFI-B) distinguishes itself by its ability to bind a target sequence (the NBRE) as a monomer and by its role in T-cell receptor (TCR)-induced apoptosis in T cells. We now report on a novel mechanism of Nur77 action that is mediated by homodimers. These dimers bind a Nur77 response element (NurRE), which has been identified as a target of CRH-induced Nur77 in the pro-opiomelanocortin (POMC) gene promoter. Both halves of the palindromic NurRE are required for responsiveness to physiological signals, like CRH in pituitary-derived AtT-20 cells. Similarly, in T-cell hybridomas, TCR activation induced NurRE but not NBRE reporters. The in vivo signaling function of Nur77 thus appears to be mediated by dimers acting on a palindromic response element of unusual spacing between its half-sites. This mechanism may represent the biologically relevant paradigm of action for this subfamily of orphan nuclear receptors.

Within the nuclear receptor family of transcription factors, Nur77 (also known as NGFI-B) is distinguished by its ability to bind a target sequence (the NBRE) as a monomer (34, 36) and by its role in T-cell receptor (TCR)-induced apoptosis in T cells (5, 21, 38). Nur77 was cloned repeatedly by numerous investigators either as a mitogen-inducible gene or as an immediate-early gene (15, 23, 25, 30). In addition, work on the role of Nur77 in T cells has clearly indicated that it fulfills a signaling function in this system. Indeed, Nur77 is induced in response to TCR activation, both at the transcriptional level and posttranscriptionally, possibly through phosphorylation (39, 41). This activation appears to occur primarily in response to calcium-dependent signals, since it is prevented by cyclosporine. Current data also suggest that Nur77 is a point of convergence of signals mediated through the protein kinase C pathway (39). The central role of Nur77 in TCR-induced signals was best illustrated by the blockade of TCR-induced apoptosis either through overexpression of a dominant negative mutant of Nur77 in vitro (21) or in vivo (5, 7) or through expression of Nur77 antisense RNA (21). These data clearly support the hypothesis that Nur77, together with related orphan nuclear receptors (7), acts as a central mediator of the signaling pathway controlling apoptosis in T cells.

Nur77 is widely expressed, in particular, throughout the brain (37, 40). Its function in the brain is not yet clear. However, Nur77 mRNA was shown to be induced in corticotropin-releasing hormone (CRH) neurons in response to stress stimuli (16). This observation suggests that Nur77 may have a signaling function in these neurons. Nur77 was also isolated as an adrenal transcription factor involved in the induction of genes encoding steroidogenic enzymes (8, 35). The CRH neurons constitute, together with the pituitary corticotroph cells and the adrenals, the hypothalamo-pituitary-adrenal (HPA) axis, which mediates the stress response. The expression of Nur77 in two components of this system, as well as the expression of orphan receptors related to Nur77 in pituitary cells (24, 26), suggested that Nur77 may play a signaling function at all three levels of the HPA axis. The corticotroph cells of the anterior pituitary produce pro-opiomelanocortin (POMC) in response to hypothalamic signals mediated through CRH and vasopressin (17). CRH-induced signals are thought to exert their effects through the cyclic AMP (cAMP) pathway, but they were also shown to involve calcium-mediated events (18). CRH activation of corticotroph cells leads to secretion of prestored adrenocorticotropin (ACTH) and to transcriptional activation of the POMC gene (17). The mechanism of this transcriptional activation is not yet clear, and various promoter targets and mechanisms have been suggested (3, 4).

Nur77 is an orphan nuclear receptor for which no ligand is known. However, Nur77 (NGFI-B) was the first nuclear receptor shown to bind DNA as a monomer. Indeed, Nur77 was shown to bind an octamer sequence, the Nur77-binding response element (NBRE), that was initially identified by genetic selection in yeast (34). The NBRE contains the hexanucleotide AGGTCA, which is typically recognized by nuclear receptors of the RAR/RXR subfamily (22), and it includes two A residues preceding this hexanucleotide; recognition of these two residues was shown to depend on NGFI-B sequences that lie outside the zinc finger domain (36). More recently, Nur77 was shown to form heterodimers with RXR and to confer 9-*cis*-retinoic acid-dependent transcription to reporters containing a DR5 regulatory element (28) and in some cases an NBRE sequence (12).

Through analysis of signals elicited by CRH in POMC-expressing cells, we now report on a natural Nur77 target sequence that is responsive to physiological stimuli, under conditions where the NBRE is unresponsive. This novel Nur response element (NurRE) mediates the physiological response of the POMC gene to CRH. The NurRE (but not the NBRE) is also responsive to TCR-induced signals in T-cell hybridomas. In contrast to NBRE binding by monomers, the NurRE binds homodimers of Nur77. Thus, the NurRE represents a biologically relevant paradigm of Nur77 signaling that

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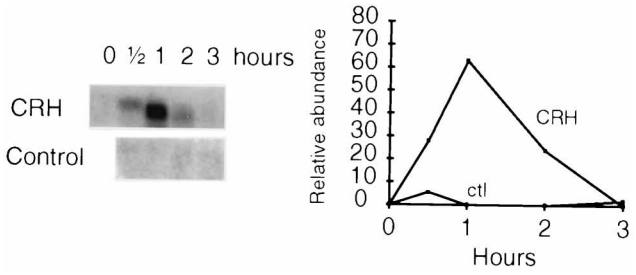

FIG. 1. CRH stimulates Nur77 mRNA in AtT-20 cells. CRH (10^{-7} M) treatment of AtT-20 cells leads to a transient induction of Nur77 mRNA as assessed by Northern blot analysis. Cellular β-actin mRNA was measured by hybridization on the same blot as control. Total AtT-20 cell RNA (20 μg) was used as previously described (11).

is physiologically responsive in both endocrine and lymphoid systems.

MATERIALS AND METHODS

Transfections. Transfections in AtT-20 cells were performed by lipofection (Lipofectin [Gibco]) with 7.5×10^5 exponentially growing AtT-20 cells in 35-mm petri dishes. The cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum stripped with dextran-coated charcoal. The cells and media were harvested 16 h after lipofection. Each sample for lipofection contained a total of 1.5 μg of DNA, including 300 ng of reporter plasmid, 300 ng of Rous sarcoma virus (RSV)-growth hormone (GH) as an internal control, 100 ng of pCMX-Nur77 expression vector, and pSP64 to make up the total amount. The POMC promoter constructs were described previously (31, 32). In particular, the NurRE and negative glucocorticoid response element (nGRE) linker-scanning replacement mutations were described previously as mutants 3 and 15 in a series of promoter mutations (31). DO 11.10 cells were electroporated in a Bio-Rad instruments at 250 V and 960 μF, with 10 μg each of reporter plasmid and expression vectors. CRH (10^{-7} M) and forskolin (10^{-7} M) were used at maximally active concentrations, and anti-CD3 (clone 145-2C11) was used at 1 μg/ml to coat the dishes. The expression plasmid for the Nur77 dominant-negative mutant was used at 3 μg/dish. Data are presented as the means ± standard errors of the mean (SEM) for three to five experiments each performed in duplicate.

Gel retardation. NBRE (5′-GATCCTCGTGCGAAAAGGTCAAGCGCTA-3′) and NurRE (5′-GATCCTAGTGATATTTACCTCCAAATGCCAGGA-3′) oligonucleotides were 3′-end labeled with Klenow polymerase and purified on polyacrylamide gels. The binding conditions and dimethyl sulfate (DMS) interference were as previously described (9, 10). Typically, about 10 ng of in vitro translated Nur77 synthesized with the Promega TNT SP6/T7 kit was used in gel retardation experiments.

RESULTS

A NurRE in the POMC promoter. Since Nur77 was previously implicated in regulation of the HPA axis (8, 16, 27), we verified that it is induced in POMC-expressing AtT-20 cells in response to CRH (Fig. 1). Since Nur77 expression is rapidly induced in response to CRH (Fig. 1), we tested whether it acts on transcription on the POMC gene. Overexpression of Nur77 was found to increase the transcription of a POMC luciferase reporter (Fig. 2A, construct 1). The only sequence of the POMC promoter that is closely related to the previously described Nur77 target, the NBRE, is found within the nGRE (9). Mutagenesis of this NBRE sequence did not prevent Nur77-dependent activation (construct 2). However, deletion of the distal region of the promoter (31) was found to abolish activity (construct 4), and further mapping of the responsive sequences with a variety of deletions and linker-scanning mutants (data not shown) led to the identification of a target sequence, the NurRE, centered around bp −395. A specific linker-scanning mutation of the NurRE abolished responsiveness to Nur77 (construct 3). To clearly define the sequences required for Nur77 response, a NurRE oligonucleotide was inserted in three copies upstream of a minimal promoter, and this response element was found to confer high responsiveness to Nur77 (construct 6). In this context, the NurRE is at least 40 times more responsive than the NBRE (construct 7) (note the scale difference).

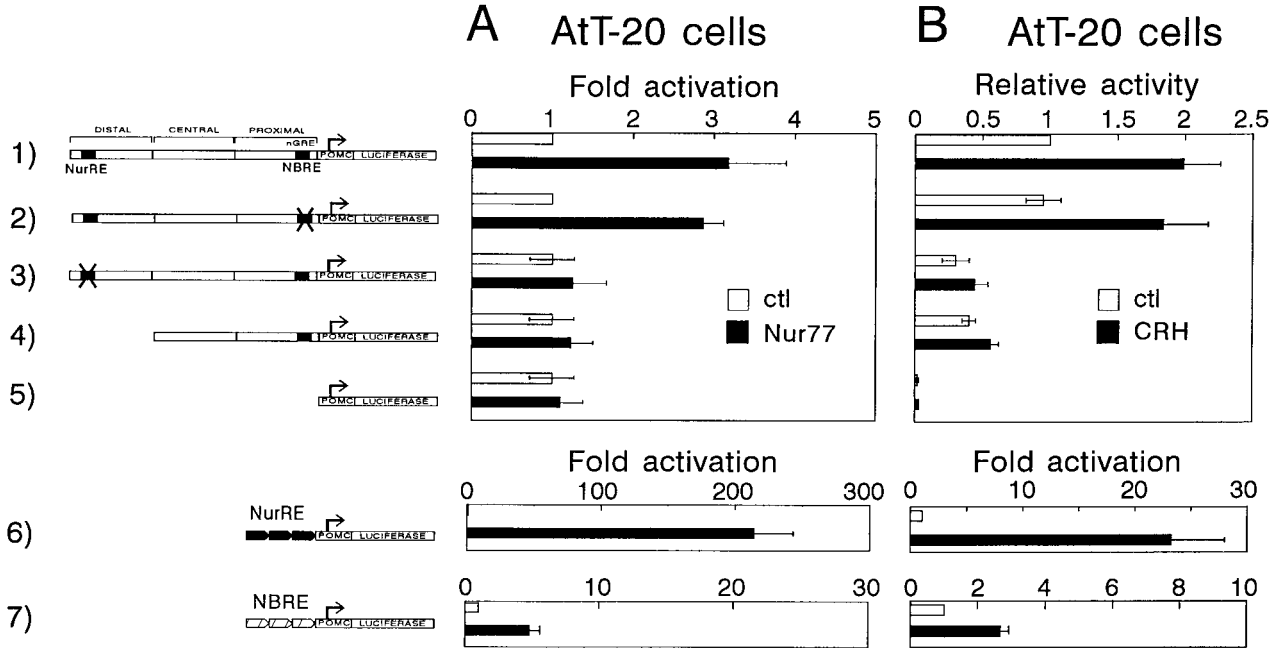

FIG. 2. The POMC gene promoter contains a Nur response element (NurRE) that confers responsiveness to Nur77 and CRH in AtT-20 cells. (A) Localization of NurRE and comparison of its activity with that of NBRE. The effect of Nur77 expression was tested on a rat POMC promoter (bp −480 to +63) fused to the luciferase reporter (construct 1), which was described previously (31). The mutations of the NBRE present within the nGRE (construct 2) and of NurRE (construct 3) contain transversions of 15 and 10 bp, respectively. Constructs 6 and 7 contain trimers (28 bp) of NurRE and NBRE inserted upstream of a minimal POMC promoter (−35 bp to +63 bp). (B) Colocalization of CRH responsiveness to the NurRE with the same constructs as in panel A for Nur77 expression.

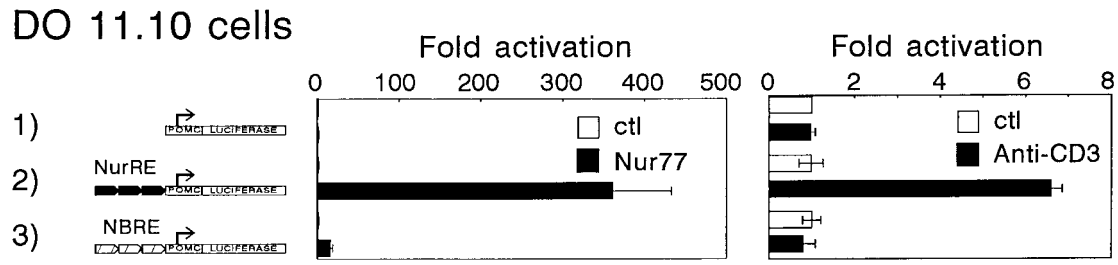

FIG. 3. TCR activation of NurRE reporter in T-cell hybridomas. The NurRE confers responsiveness to both Nur77 overexpression and treatment with anti-CD3 (leading to TCR activation by cross-linking) in the T-cell hybridoma DO 11.10. The NBRE reporter is only weakly responsive to Nur77 and unresponsive to anti-CD3.

The NurRE confers responsiveness to CRH. Since the Nur subfamily of orphan receptors has been implicated in signaling (6, 8, 16, 21, 27, 38) and since CRH induces Nur77, we tested whether the stimulatory effect of CRH on POMC transcription might be mediated through the NurRE. CRH stimulates POMC gene transcription assessed by the nuclear run-on assay by about twofold (13, 14), and its effect on POMC-luc is of the same magnitude (Fig. 2B). When promoter mutations were tested for responsiveness to CRH (Fig. 2B), it was found that NurRE confers responsiveness to CRH, since the linker-scanning mutation of this element (Fig. 2, construct 3) abolished responsiveness to the hypothalamic hormone and since oligomerization of the response element lead to a greatly enhanced response (construct 6).

TCR activation targets NurRE in T cells. In view of the importance of the Nur pathway in TCR-induced signaling, we tested the relative activity of the NurRE and NBRE in T-cell hybridomas following Nur77 expression and anti-CD3 activation of TCR signaling (Fig. 3). Whereas the NurRE reporter was induced by TCR activation, the NBRE reporter was not. Thus, the NurRE provides a paradigm for naturally occurring target sequences of the Nur orphan receptor signaling pathway.

Dominant negative mutant of Nur77 blocks the action of CRH. Since it was previously suggested that CRH may exert its effect through cAMP and protein kinase A, the response of the NurRE to forskolin was tested (Fig. 4). Interestingly, the NurRE reporter was also responsive to forskolin but less so than to CRH, suggesting that CRH may induce other pathways in addition to the cAMP pathway. To demonstrate the importance of the Nur pathway in activation of the POMC promoter in response to CRH and cAMP, we used a dominant negative mutant of Nur77 (dNurr77) that had previously been shown to block TCR-induced signals and apoptosis in T cells (38). Overexpression of dNur77 decreased basal POMC promoter activity and completely blunted CRH-induced activity (Fig. 4). In addition, dNur77 blunted the response of the NurRE reporter to CRH and forskolin. The weak activity and responsiveness of the NBRE-containing reporter were also decreased by dNur77. The complete reversal of CRH-induced POMC transcription by dNur77 suggests that this pathway is solely responsible for the transcriptional actions of CRH in AtT-20 cells.

NurRE binds dimers of Nur77. The interaction of Nur77 with NurRE was investigated directly in binding studies with in vitro-translated Nur77. Surprisingly, these binding experiments indicated that the NurRE binds homodimers of Nur77, in contrast to the monomeric interaction of this receptor with NBRE (Fig. 5A). The prevalence of dimeric complexes in gel retardation experiments suggests that dimer formation is cooperative (Fig. 5B and C). In competition experiments, NurRE and NBRE exhibited a similar specificity of binding (Fig. 5A). The interaction of Nur77 with NurRE was further defined by the DMS interference method (Fig. 6A). This analysis indicated that two Nur77 moieties interact with octamer motifs

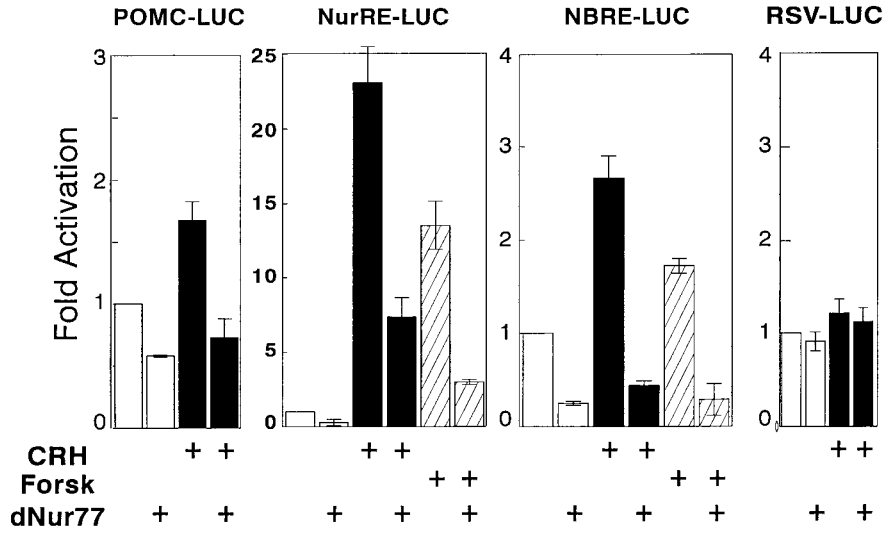

FIG. 4. Blockade of CRH and forskolin responsiveness by a dominant negative mutant of Nur77 (dNur77). The response of four reporter plasmids to CRH (10^{-7} M) and forskolin (10^{-7} M) was tested in AtT-20 cells. The reporters were POMC-luc, NurRE-luc, NBRE-luc, and RSV-luc as a negative control. The dominant negative mutant of Nur77 was described previously (38) and shown to block TCR-induced apoptosis in T cells.

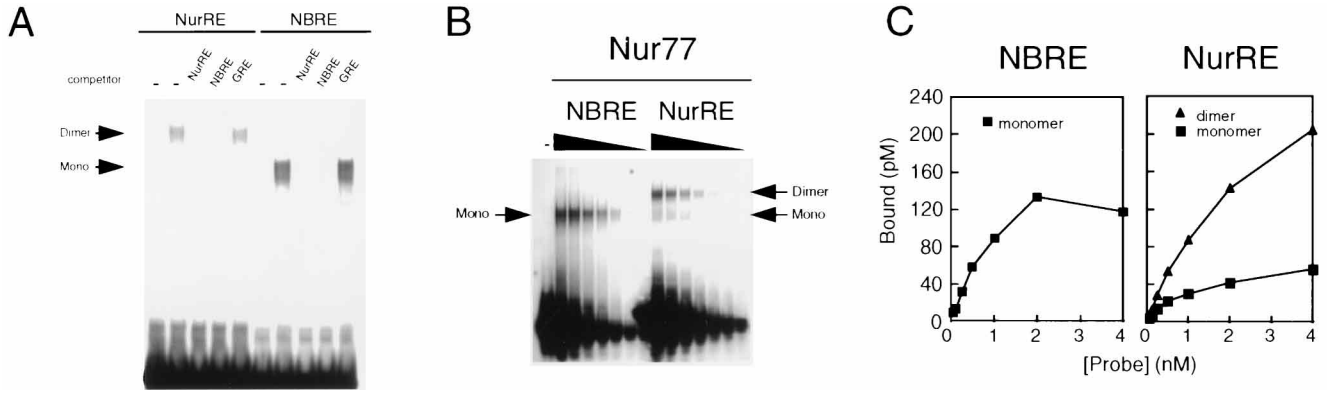

FIG. 5. Binding of Nur77 to NurRE. (A) Binding of in vitro-translated Nur77 to NurRE and NBRE (9–11). The position of monomeric (mono) and dimeric (dimer) complexes is indicated by arrows. Competitor oligonucleotides were used at 100-fold molar excess. (B) Binding curve of Nur77 in the presence of increasing concentrations of NurRE and NBRE. (C) Quantitation of binding experiments shown in panel B. Each band was quantitated with a PhosphorImager.

that are found in an inverse orientation and separated by 6 bp. Each motif is loosely related to the NBRE, AAAGGTCA (Fig. 6B), with each having two mismatches. The linker-scanning mutation used to localize the NurRE (Fig. 2A, construct 3) was targeted to the upstream motif, as indicated in Fig. 6B. However, this upstream motif is insufficient on its own to confer NurRE activity (see below).

Both halves of NurRE are required. The importance of each motif for binding of Nur77 homodimers was confirmed in gel retardation experiments; indeed, mutation of either motif (mutants M1 and M3, Fig. 6B) prevented the formation of homodimer complexes, whereas mutation of intervening sequences (mutant M2) did not (Fig. 6C). The binding of Nur77 monomers to the M1 and M3 mutants is consistent with the observation that the NurRE half-sites are similar to NBRE. The NurRE sequence is somewhat unusual in that its two inverted half-sites or NBRE-related motifs are separated by 6 or 10 bp depending on whether one considers the octamer sequence recognized by Nur77 (36) or the hexamer motif used to classify other nuclear receptor target sites (2, 22); thus, in the usual nomenclature (22), the NurRE is an ER-10 element. Previous work has shown that DNA recognition by Nur77 (NGFI-B) is extended by two A residues upstream of the canonical hexanucleotide AGGTCA and that this interaction involves amino acid residues outside of the zinc finger domain (36). These two A residues are present in each half-site of the NurRE (Fig. 6B) suggesting that this mode of DNA recognition is used, as it is for Nur77 monomer interaction with NBRE. Mutagenesis of the first A in one motif sufficed to abolish NurRE activity in response to Nur77 overexpression, as did the deletion of an octamer motif (Fig. 7). The activity of those NurRE mutants is the same as that of the NBRE reporter: it is not clear whether this activity is due to the action of Nur77 monomers or to weakly binding dimers. However, in the context of the POMC promoter, a single NBRE sequence was unresponsive to CRH or Nur77 overexpression, while a single NurRE appeared sufficient for responsiveness (Fig. 2B and C).

DISCUSSION

The identification of a natural target sequence for Nur77, the NurRE, which is much more potent than the NBRE, sug-

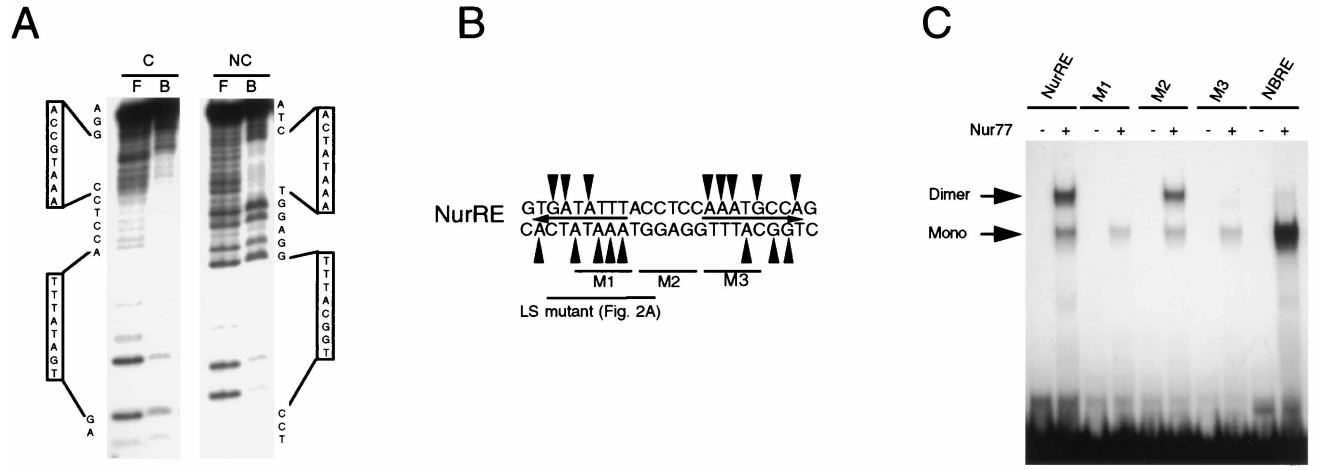

FIG. 6. Localization of NurRE. (A) DMS interference. End-labeled coding (lanes C) and noncoding (lanes NC) strands of the NurRE were used for DMS interference of Nur77 binding. DMS methylation partially revealed A residues in addition to guanosine. Residues that interfere with binding are boxed on either side of the gels. (B) The residues that interfere with binding are indicated by arrowheads on the NurRE sequence. Arrows between the strands indicate the position of the NurRE half-sites which are related to the consensus AAAGGTCA. The position of transversion mutations (M1, M2, and M3) used in binding experiments shown in panel C is indicated below the sequence. In addition, nucleotides mutated in the linker-scanning (LS) mutant used in Fig. 2 (construct 3) are indicated by a line. (C) Binding of Nur77 to wild-type and mutant NurRE. The position of each mutation is indicated below the sequence in panel B.

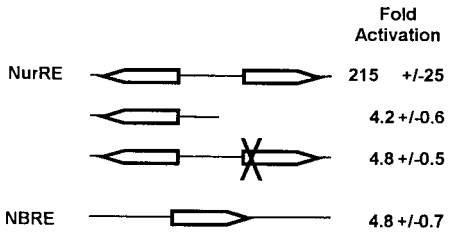

FIG. 7. Relative activity of NurRE and mutants compared to that of NBRE. The reference reporter is construct 6 of Fig. 2. The mutant NurRE has the first A of the consensus AAAGGTCA and the preceding residue replaced by transversions.

gests that the action of Nur77 as dimers may represent the physiologically relevant paradigm of action for Nur77 and for this subfamily of orphan nuclear receptors. While this difference in potency can be assessed by overexpression of Nur77 (Fig. 2A and 3), its significance is best revealed in experiments that do not involve overexpression of transcription factors but rely entirely on activation of intracellular signals through the action of physiological hormones, like CRH in AtT-20 cells (Fig. 2B) and TCR activation by anti-CD3 in T-cell hybridomas (Fig. 3). In this latter context, the NBRE reporter is inactive by comparison to the NurRE reporter, highlighting their large difference in mediating signals at the genomic level. The requirement for dimer interactions at NurRE was highlighted by mutagenesis of the NurRE (Fig. 6 and 7), where it was shown that alteration or deletion of one half-site of the palindromic NurRE results in an activity similar to that of the NBRE. The NurRE is an ER-10 inverted repeat containing two NBRE-related sequences (Fig. 6B). We have shown that this regulatory element preferentially binds dimers of in vitro-produced Nur77 (Fig. 5), and the overexpression experiments suggested that Nur77 homodimers were activating the NurRE reporters. However, it is possible that the NurRE will bind heterodimers of the Nur subfamily of nuclear receptors. Indeed, this subfamily has two other members, Nurr1 and NOR1, in addition to Nur77 (20, 26). It appears that these other Nur77-related nuclear receptors might be regulated in parallel with Nur77 in different systems (7, 26).

The identification of the NurRE as a target for binding of Nur77 dimers raises the question of the biological relevance of the NBRE, since this target sequence was originally identified in yeast (34). Later, NBREs were identified by homology in putative Nur77 target genes, in particular, in genes encoding adrenal steroidogenic enzymes (35), but formal proof that these sequences confer biological responses other than in transfection experiments is lacking. Despite its importance in TCR-induced apoptosis (5, 21, 38), there are as yet no known downstream genes of the Nur77 pathway in T cells. The identification of a potent naturally occurring NurRE should facilitate the search for Nur77 target genes which lie downstream of Nur77 in the signaling cascade leading to T-cell apoptosis.

The POMC promoter has two potential targets for Nur77: the NurRE and the NBRE, which is contained within the nGRE (9). The latter binds Nur77 monomers and exhibits a similar activity to NBRE in *trans*-activation experiments (data not shown). Although this putative Nur target site was not found to contribute responsiveness to CRH in our experiments (Fig. 2A and B), it may play a role under some physiological conditions or in other POMC-expressing cells. This latter possibility is not unlikely since the activity of the upstream NurRE is dependent on corticotroph-specific recognition of flanking promoter elements (32). Indeed, the tissue-restricted helix-loop-helix factor NeuroD/BETA2 (29a) and the *bicoid*-related factor Ptx1, which are important determinants of corticotroph-specific POMC transcription, bind just downstream of the NurRE (19). Prior work (32) clearly suggested that the NurRE would not be active in the absence of these two factors. Thus, modulation of NeuroD1 or Ptx1 activity in POMC-expressing corticotroph cells might alter the responsiveness of the POMC gene to Nur77 and consequently to signals like CRH that control Nur77 expression. Under conditions where upstream corticotroph-specific promoter regulatory elements are not active, the NBRE/nGRE might become an active target of Nur77 (24), particularly if other transcription factor(s) acting in the proximity of the NBRE/nGRE enhanced its activity.

The convergence of CRH and cAMP signals at the NurRE in the POMC gene may seem surprising. However, the POMC promoter does not contain a cAMP response element (CRE) and forskolin does not fully mimic the effect of CRH on NurRE reporters (Fig. 4), suggesting that the effects of forskolin may either be indirect or substitute for only part of the signals elicited by CRH. CRH was also shown to elevate intracellular Ca^{2+} levels (18), and Ca^{2+}-dependent signals have been implicated in POMC expression (33), as they have been for Nur activation in T cells (39, 41). Thus, it may be that signals dependent on both cAMP and Ca^{2+} converge on Nur77 to modulate POMC transcription. It was previously suggested that c-*fos* might in part mediate the effect of CRH (3); however, that work was performed with serum-starved cells, and deletion of the c-*fos* target AP1 site of the POMC promoter did not prevent CRH stimulation of POMC transcription (3; our unpublished observations). Thus, CRH activation of Nur77 might represent the primary pathway of CRH signaling in corticotroph cells; under some conditions, CRH-induced c-*fos* might also contribute to those signals (1, 3).

In conclusion, the Nur77 signaling pathway appears to be an important positive regulator of the HPA axis, since Nur77 and related factors (20, 26) mediate activation of the axis at all three levels, hypothalamus (CRH), pituitary (POMC), and adrenals (steroidogenic enzyme-coding genes). Since we show in the accompanying paper that the positive action of Nur77 is antagonized by glucocorticoids (29), the Nur77 signaling pathway may be the point of convergence for different regulatory signals in both endocrine and lymphoid systems.

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