The Factor Binding to the Glucocorticoid Modulatory Element of the Tyrosine Aminotransferase Gene Is a Novel and Ubiquitous Heteromeric Complex*

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Glucocorticoid induction of the tyrosine aminotransferase gene deviates from that of many glucocorticoid-responsive genes by having a lower EC₅₀ and displaying more agonist activity with a given antiglucocorticoid. A cis-acting element, located 3646 base pairs upstream of the start of tyrosine aminotransferase gene transcription, has been found to be sufficient to reproduce these variations with heterologous genes and promoters (Oshima, H., and Simons, S. S., J. r. (1992) Mol. Endocrinol. 6, 416-428). This element has been called a glucocorticoid modulatory element, or GME. Others have called this sequence a cyclic AMP-responsive element (CRE) due to the binding of the cyclic AMP response element binding protein (CREB). We now report the partial purification and characterization of two new proteins (GMEB1 and -2) of 88 and 67 kDa that bind to the GME/CRE as a heteromeric complex. This purification was followed by the formation of a previously characterized, biologically relevant band in gel shift assays. By several biochemical criteria, the GMEBs differed from many of the previously described CREB/CREM/ATF family members. Partial peptide sequencing revealed that the sequences of these two proteins have not yet been described. Size exclusion chromatography and molecular weight measurements of the gel-shifted band demonstrated that the GMEBs bound to the GME as a macromolecular complex of about 550 kDa that could be dissociated by deoxycholate. Similar experiments showed that CREB bound to the GME as heteromeric complexes of about 310 and 360 kDa. As determined from gel shift assays, GMEB1 and -2 are not restricted to rat liver cells but appear to be ubiquitous. Thus, these novel GMEBs may participate in a similar modulation of other glucocorticoid-inducible genes in a variety of cells.

For many years, the accepted model of steroid hormone action predicted that the responses of all regulated genes were a property of the steroid used. Thus, a gene is induced, or repressed, by agonists, and the action of agonists is prevented by antisteroids. Furthermore, the concentration of steroid required for half-maximal induction by an agonist and the amount of agonist activity possessed by a given antisteroid should be constant for each steroid and independent of the gene examined (reviewed in Refs. 1 and 2).

Recently, this model has had to be modified as exceptions were defined. Thus, jun-fos heterodimers (AP-1), and AP-1 inducers such as phorbol esters block steroid induction (3, 4) in what can be a cell-specific manner (5, 6), while jun-jun homodimers augment glucocorticoid induction (4). Cyclic AMP, via protein kinase A, can often (but not always (7-9)) cause greater induction by agonists (10, 11) and increased percentages of agonist activity for antisteroids (11, 12). Heat shock, or chemical shock, afforded a synergistic increase in glucocorticoid inducibility (13), while the immunosuppressive agent FK506 augmented the activity of subsaturating concentrations of glucocorticoids (14). Finally, dopamine can cause ligand-independent gene activation of some receptors (15). None of the above agents effected any shift in the dose-response curve for agonists except for FK506, which was postulated to increase the nuclear binding of activated complexes (14).

Other observations that did not appear to fit with the conventional model of steroid hormone action originated from studies on glucocorticoid induction of the tyrosine aminotransferase (TAT) gene in rat hepatoma tissue culture (HTC) cells, which had become a paradigm for steroid-inducible genes. We found that the dose-response curve for dexamethasone induction of TAT gene expression in the related Fu5–5 rat hepatoma cell line was left shifted compared to that in HTC cells (16). Similarly, TAT enzyme activity was induced at lower cAMP concentrations in Fu5–5 cells than in HTC cells (7). Furthermore, all antiglucocorticoids examined displayed a higher percentage of agonist activity for TAT gene expression in Fu5–5 than in HTC cells (16-18). This left shift in the TAT dose-response curve, and increased agonist activity with anti-steroids, was not a general response of all glucocorticoid-inducible genes in Fu5–5 cells (19) and occurred at the level of correctly initiated transcripts (7, 19). Surprisingly, the magnitudes both of the left shift in the dose-response curve and of the increased amount of agonist activity were not constant but varied slowly over time (17, 20) in a manner that was eventually found to be related to the density of the cells in culture (21). Therefore, it appeared that some event downstream of steroid binding to the glucocorticoid receptor selectively modulated the properties of TAT gene induction by glucocorticoid agonists and antagonists.

We previously proposed that this modulation of TAT gene induction in rat hepatoma cells occurred via the binding of a

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trans-acting factor to a cis-acting element of the TAT gene (1). Stable (22) and transient (23) transfection assays succeeded in identifying such a cis-acting element about −3646 bp of the rat TAT gene, that conveyed all of the glucocorticoid induction properties of the endogenous TAT gene to heterologous genes and promoters. This cis-acting element was called a glucocorticoid modulatory element (GME) and was found to bind a trans-acting factor(s) (23). The mechanism of action of the GME, unlike that of the commonly discussed transcription factor binding sites, does not involve synergism with the glucocorticoid response element, or GRE (24). This suggests that the GME-bound factor(s) (GMEB) might be a novel protein.

The binding site of the GMEB has also been identified as a cyclic AMP-responsive element (CRE) (25–27), but several lines of evidence indicate that two different sets of proteins are responsible for GME and CRE activity. First, the biological activities mediated by GMEB and the CRE binding protein (CREB) are quite dissimilar (Refs. 21–23).

Cells—The following double-stranded GME oligonucleotide, 5′-tgcatactgctga caggccgctagtg-3′; 3′-gaagaccgc gagctgccgtcatagc-5′ (capitalized letters correspond to the rat TAT sequence from −3654 to −3634 bp from the start of transcription; lower case letters are for added nucleotides to make Sure CALL cohesive ends), was used for gel shift assays, after filling in the single-stranded DNA with Klenow enzyme and labeling with [32P]dCTP at room temperature. Gel shift experiments were performed as described (23) with some modifications. In brief, nuclear extracts (3 μg) or cytosol preparations (7 μg) were incubated with 20,000 cpm of the 32P-end-labeled probe (0.6 fmol) in a total volume of 10 μl for 15 min at 0°C with sheared, non-denatured herring sperm DNA (0.15 μg) as a nonspecific competitor. After 60-min preincubation at 4°C in 5% non-denaturing polyacrylamide gel at 150 V in 0.4× TBE, the dried gels were autoradiographed for 12–24 h at room temperature with Kodak X-Omat XAR-5 film or were exposed to the phosphorimaging screen for the Molecular Dynamics ImageQuant system (Molecular Dynamics) for 16–72 h at room temperature. For supershift experiments, the antibodies (0.8 μl) were preincubated with the nuclear extracts or cytosol for 60 min at 0°C before adding spermine, herring sperm DNA, and [32P]-labeled GME. The upper strand sequences of blunt-ended, double strand oligonucleotides used for competition experiments were as follows: GME: 5′-CTTCTCGGTGTCAGCGCGGATCTAG-3′; M1, 5′-TGCTGAGCTCAGGCACGATTAGT-3′; M2, 5′-CTTCTGTATGAGCCCGCATGAT-3′; M3, 5′-CTTCTGCGTCATGCGTTGAT-3′; M4, 5′-CTTCTGGCTGCAGCGGATCTAG-3′; AP-1, 5′-TTCTCGGTGCTCATACAGGCTAG-3′; M5, 5′-AGACGTTTCGAGCTGAGGACAGTAGT-3′ (Refs. 23 and 24 and references therein).

Methylation interference assay—The sequence GTTTCAGGGATC of the reporter plasmid was methylated by M.HhaI, so that addition of the enzyme (100 U) to an undigested plasmid 

Materials and Methods

Chemicals—The following chemicals were obtained from the indicated sources: [32P]dCTP and [32P]dATP (3000 Ci/mmol), DuPont NEN; deoxycytidine, dimethyl sulfate, and Nonidet P-40, Sigma; p-aminoethylbenzenesulfonfonyl fluoride for 1 h at 0°C. Protein A-G agarose (Pierce) (2 μl of a 50% slurry in 25 μl Tris (pH 8.0), 0.5 mM EDTA, 1 mM bovine serum albumin) was added and incubated for 1-16 h at 0°C. The mixture was then centrifuged at 14,000 × g for 2 min at 0°C, and the supernatant (8.45 μl) was used for gel shift assays.

Fractionation Procedures for GMEB—Nuclear extracts (50 μl) in the absence or presence of 1 or 0.2 μg guanine hydrochloride were loaded onto a Microcon 100 (molecular cut-off, 100 kDa) (Amicon) and centrifuged at 2500 × g for 20–25 min until 50% of the initial volume had passed through the membrane. The retentates and pass-through fractions containing guanine hydrochloride were dialyzed against buffer 20T at 4°C for 16 h and stored at −70°C.

For size exclusion chromatography, 200 μl of nuclear extract or cytosol was separated on 6% or 8% SDS-PAGE gels (1.5 mm thick) (32) and the desired M, ranges, based on the migration of pre-stained molecular weight markers (Life Technologies, Inc., Bio-Rad) in adjacent lanes, were cut out of the gel and eluted electroeluted in 1 × SDS running buffer (25 mM Tris, 0.19 mM glycerol, 0.1% SDS) at 125 V in a model 1750 electroeluter (ISCO, Lincoln, NE) for 60 min at room temperature. After elution, the protein was precipitated with 4 volumes of acetone (−70°C), and the pellets were washed with 50% acetone, 20% buffer 20T (4°C) as described (33). The pellets were redissolved in buffer 20T and reprecipitated with acetone as before. After the second precipitation, the recovered samples were denatured and renatured in 60 μl of buffer 20T containing 6 μg guanine hydrochloride and dialyzing against buffer 20T at 4°C for 16 h. After dialysis, the renatured samples were concentrated to about 20 μl with a Microcon 10 concentrator (12,000 rpm for 15 min at 4°C) (Amicon) and stored at −70°C.

Methylation interference assay—The sequence of the double-stranded dinucleotide used was as follows (capitalized letters correspond to the rat TAT sequence from −3654 to −3634 bp from the start of transcription): 5′-tgcatactgctga caggccgctagtg-3′; 3′-ggaGaccgc gagctgccgtcatagc-5′. The probe was filled-in by Klenow fragment with [32P]dCTP or [32P]dATP for labeling specifically the upper or lower strand, respectively. The labeled probes were par-
GMEB Is a Novel Heteromeric Complex

**RESULTS**

**GMEB Is Present and Active in Non-hepatic Cells**—The GMEB-DNA complex observed in gel shift assays migrated only slightly slower than several other complexes (Fig. 1A). As expected, cytosolic solutions prepared by lysis of cells with hypotonic buffer or by the freeze-thaw techniques used to obtain crude glucocorticoid receptors (31) contained very few DNA binding species. However, each cytosolic solution still appeared to include the same GME binding species that cause the nuclear translocation of most of the cytosolic glucocorticoid receptors 

| Cells   | Agonist activity with 4 nM dexamethasone | Agonist activity with 1 μM dexamethasone-21-mesylate |
|---------|------------------------------------------|------------------------------------------------------|
|         | GREtkCAT | GMEGREtkCAT | M2GREtkCAT | GREtkCAT | GMEGREtkCAT | M2GREtkCAT |
| Fu5–5 (clone 27) | 45 | 74 | 44 | 32 | 73 | 33 |
| Fu5–5 (uncloned) | 23 | 71 | ND | 46 | 78 | ND |
| HTC (clone 28) | 35 | 54 | 38 | 26 | 47 | 28 |
| HeLa | 52 | 85 | 59 | 5 | 29 | 3 |
| L | 41 | 87 | 42 | 43 | 75 | 45 |
| PC12 | ND | ND | ND | 19 | 17 | ND |

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plasmic glucocorticoid receptors (37), there was no appreciable decrease in the intensity of the gel-shifted band with $^{32}\text{P}$ GME (data not shown). Similarly, cytosolic preparations of receptors treated with saturating concentrations of dexamethasone and then heated to activate the complexes to the DNA binding form did not display an increased amount of the GMEB complex in gel shift assays (data not shown). Therefore, we conclude that this GMEB-containing band does not involve any interaction of the glucocorticoid receptor with the GME.

GMEB bound to several polycationic columns. GMEB was eluted from heparin-agarose at 0.3–0.8 M NaCl, from DEAE-Sepharose CL-6B by 0.1–0.2 M NaCl, and from Mono Q columns with 0.2 M NaCl. Thus, GMEB would be predicted to contain at least one surface containing a net abundance of anionic charges, as would be expected for a factor involved in the modulation of transcription. GMEB also bound to phenyl-Sepharose and a Mono S polyanionic column, but the recovery was very low (data not shown).

Several results indicate that GMEB and CREB are different proteins. First, heating the nuclear extracts to 65 °C (but not 37 °C) for 10 min eliminated the formation of the GMEB-bound complex (data not shown). In contrast, CREB is stable under these conditions (data not shown, Ref. 29). Second, an antiserum against CREB antibody supershifted some of the lower two bands in gel shift assays but none of the more slowly migrating GMEB-containing bands (data not shown). This confirms the earlier report that only the lower two bands contain CREB (26). Third, a 0.5-h treatment with 10 μM of forskolin, which increases the cellular cAMP (and activates CREB), had no effect on the amount of GMEB in the gel shift assay (data not shown) or on the percent agonist activity seen for 1 μM dexamethasone 21-mesylate with the GREtkCAT reporter (30 versus 34%) and only slightly increased the percent agonist for dexamethasone 21-mesylate with the GMEGREtkCAT reporter (67 versus 57%). Fourth, a $^{32}$P oligonucleotide containing the consensus CRE (38) of the somatostatin gene (SOM/CRE) (25, 26) differs from the GME in 9 out of 19 nucleotides (see below; identical nucleotides are underlined, and lower case letters indicate flanking DNA in the reporter plasmids) and did not afford the slower migrating, GMEB-containing band in gel shift assays (GME, 5′-tcgaCTTCTGCGTCAGCGCCATgta-3; SOM/CRE, 5′-gatcCTCTCTGACGTCAGCCAAGGAgatc-3). Also, non-labeled SOM/CRE oligonucleotides only weakly competed for the formation of this band with the $^{32}$P GME oligonucleotide (data not shown).

Two factors that are closely related to CREB, which heterodimerize with CREB, and can replace CREB in vivo are CREM and ATF-1 (39). However, neither a broad spectrum antibody (anti-CREM) nor a variety of mono-specific antibodies could supershift the GMEB-containing band in gel shift assays. Similarly, immunodepletion of the nuclear extracts with these antibodies did not prevent the formation of the GMEB-containing band (data not shown). These results argue that the GMEB is not ATF-1, -2, or -3, CREB-1 or -2, CBP, or CREM. Similar experiments with anti-jun and anti-fos antibodies ruled out jun and/or fos as being the GMEB (data not shown), even though AP-1 is active with a highly homologous DNA sequence (40).

GMEB Is a Multimeric Protein—Methylation interference experiments revealed that all of the guanines in a 10-bp region of the GME are important for binding in the gel shift assay (Fig. 2). An even larger region of 26 bp is protected from DNase I digestion (data not shown). Under most circumstances, more than one protein would be required to cover such a large stretch of DNA.
When compared to molecular weight markers in the gel shift assay (41), the size of the GMEB was calculated to be ~550 kDa; the sizes of the CREB-containing bands were about 310 and 360 kDa (data not shown). A similar very large size of 600 kDa for the GMEB was observed by gel shift assays of the peak binding activity after fractionation by size exclusion chromatography on Superose 6 HR (Fig. 3, A and B) and Sepharose S-300. To determine whether this 550–600-kDa species was a monomeric or oligomeric protein, we made use of the report that deoxycholate dissociates protein-protein complexes, but not protein-DNA complexes, in a manner that can be reversed with GMEB to occur.

Raising the guanidinium hydrochloride concentration to 2.7 M-through, which would contain species ~100 kDa (Fig. 3D). Raising the guanidinium hydrochloride concentration to 2.7 M still did not allow any GMEB to appear in the flow-through of the Microcon 100 filter, as evidenced by the formation of the appropriate gel shift bands (data not shown). Collectively, these data suggest that GMEB and the ~42-kDa CREB either lived together or were processed for sequencing and autoradiographed as described under “Materials and Methods.” The DNA sequence of the top and bottom strands is shown at the left and right, respectively, of the autoradiograph. B, bound probe; F, free probe; arrows mark those guanosine residues that must remain unmethylated for complex formation with GMEB to occur.

The flow-through from this column was loaded onto a column containing tandem repeats (>10) of the biologically inactive GME mutant oligonucleotide M2 (23) to remove nonspecific binding proteins. The flow-through was then renatured prior to being probed with 32P GME oligonucleotides.

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We have called the element at –3.6 kilobases of the rat TAT gene a glucocorticoid modulatory element, or GME, because it modulates the induction properties of both subsaturating concentrations of agonists and saturating concentrations of antagonists (23). The binding of a protein(s) to the GME was observed that was directly related to the biological activity of the GME oligonucleotide (23, 24), which was different from synergism (24). We now report that the GMEB appears to be a heterooligomer of two previously unsequenced proteins, GMEB1 and GMEB2, of apparent molecular masses of 88 and 67 kDa, respectively. However, conclusive identification must await the cloning of both proteins and a demonstration of biological activity with the cloned proteins in cells lacking GMEBs.

Several properties of the GMEBs emerged during their purification that pertain to the mechanism of GME action. First, although the DNA sequence to which the GMEBs bind is very similar to that for the CREB/CREM/ATF and the Jun/Fos/AP-1 superfamilies, and CREB even binds to a non-consensus CRE at the same position as the GME at –3646 bp of the TAT gene (25–27), there is little similarity between the GMEBs and these other proteins. The GMEBs are not related to CREB by the criteria of size, biological activity (23, 24, 38), antibody reactivity, methylation interference patterns for protein binding (Fig. 2A versus Fig. 7 of Ref. 28), or amino acid sequence (Table III). Some AP-1 sites contain the CGTC of the GME, and AP-1 may bind to the GME/CRE, as it has recently been reported that 12-O-tetradecanoylphorbol-13-acetate both inhibited glucocorticoid (and cAMP) induction of TAT and caused a decreased protein occupancy of the CRE at –3646 bp (45). However, there was no similarity between the peptide sequences of the GMEBs and AP-1, an anti-AP-1 antibody did not cause a supershift of GMEB-GME complexes, and 12-O-tetradecanoylphorbol-13-acetate alone did not elicit any response from GME-containing constructs (data not shown). Thus, there is little physical or biological similarity between the GMEBs and the other factors binding to the same DNA sequence. This, then, is an additional example of different proteins that bind to the same DNA region (46, 47).

Second, we do not know if GMEB and CREB can both bind to the GME/CRE at the same time. However, it seems that CREB is unable to block GMEB action. The low levels of the protein kinase A regulatory subunit (Tse-1) in liver cells are thought to result in high amounts of active CREB that would bind to the GME/CRE (48). Nevertheless, reporter constructs containing either a single GME (GMEGREtkCAT) or other elements that are needed for CRE activity, such as multiple tandem repeats of the GME or the GME plus the downstream BIII sequence (25), show full GME activity in Fu5–5 rat hepatoma cells (23).
GMEB Is a Novel Heteromeric Complex

Cytosol from 130 g of HTC cells were sequentially purified by Mono Q DNA affinity column chromatography as described in the text (see "Materials and Methods" for details). The total protein at various stages was quantitated by the Bio-Rad protein assay with bovine serum albumin as a standard or by comparison with the silver staining with known amounts of molecular weight markers for the DNA affinity column fractions. The GMEB specific activity was estimated from the least amount of sample to give a signal for the GMEB-containing band after overnight exposure of the gel shift assay gels to Kodak XAR film at room temperature. ND, not determined.

Table II Purification of GMEBs

| Protein                      | Total activity | Specific activity | Activity recovery | Purification |
|------------------------------|----------------|-------------------|-------------------|--------------|
|                              | mg             | units × 1000      | units/μg         | %            | - fold     |
| Cytosol                      | 1816           | 10358             | 5.70              | 100          | 1          |
| Mono Q                       | 396.6          | 8596.5            | 22.2              | 83.0         | 3.89       |
| M2 DNA affinity column       | 360            | ND                | ND                | ND           | ND         |
| M3 DNA affinity column       |                |                   |                   |              |            |
| 1                            | 0.59           | 2950              | 5000              | 28.5         | 877        |
| 2                            | 0.12           | 2400              | 20000             | 23.1         | 3506       |
| 3                            | 0.065          | 2000              | 30769             | 19.3         | 5395       |

Furthermore, conditions that elevate protein kinase A activity, such as forskolin treatment, did not inhibit GME activity. Thus, while CREB binds to the same DNA sequence as GMEB, CREB does not appear to competitively inhibit GME binding in intact cells.

Third, GME activity is not limited to rat liver cells (Table I), and the GMEBs are not tissue-specific proteins (Fig. 1A). Furthermore, the fact that the GME was active with synthetic GREs and a variety of promoters, including a minimum thymidine kinase promoter (23), suggests that no tissue-specific DNA binding factors are required for GME activity.

Fourth, the GMEBs are clearly of nuclear origin but can be readily extracted from nuclei under conditions where other factors, such as CREB, stay in the nucleus. This is reminiscent of several other nuclear proteins (49), including the progesterone- and estrogen receptors, which are predominantly nuclear but appear in most cytosolic preparations. The cytosolic appearance of the GMEBs could be indicative of a dynamic equilibrium between the two cellular compartments, as established for the progesterone receptors (49–51), or may simply reflect a repartitioning of the GMEBs in the lysis buffer.

Fifth, the mass and stability of the GMEB complex are notable. The 550–600-kDa size of the protein complex seen in both gel shift assays and size exclusion chromatography (Fig. 3, A and B) argue against a nonspecific aggregate. Involvement of the 265-kDa protein CBP that binds phosphorylated CREB (52) was discounted by the observed sizes of GMEB1 and -2 and their lack of immunoreactivity with anti-CBP antibody. The most purified preparation of GMEB appeared to contain about equal amounts of GMEB1 and -2 (Fig. 5A), which would require three or four molecules of each protein in the final complex to achieve a molecular mass of 550–600 kDa. Such a massive complex is probably not too large to be extracted intact from HTC cell nuclei because identically prepared nuclei were found to be permeable to molecules as large as the 240-kDa protein complex of phycoerythrin (53). However, the GMEB complex must be quite stable to retain specific binding to the GME after extraction from the nuclei (Fig. 1), even in the presence of up to 2.7 M guanidinium hydrochloride and after various degrees of purification (Table II). Despite the stability of the GMEB complex with regard to dissociation, the rate of reassociation of the separated components was relatively slow (Fig. 4).

Finally, from the yield of purified GMEB1 and -2 in Table III, it can be calculated that there are about 40,000 molecules of each GMEB per HTC cell. This is similar to the approximately 80,000 molecules of glucocorticoid receptor that are present in an HTC cell (16). Considering the fact that most glucocorticoid-responsive genes contain two GREs, each of which binds a dimer of the receptor, the ratio of GME-bound GMEB complexes to GRE-bound receptors is about 1:2. Given the facts that the GMEBs are not limited to rat liver cells and that GME-like modulation has been observed with several other...
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The purified GMEBs from the third M3 DNA affinity column were fractionated on an 8% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to GMEB1 (88 kDa = protein 1 of Fig. 5A) and GMEB2 (67 kDa = protein 2 of Fig. 5A) were cut out and sequenced by the Keck Foundation (Yale University) as described under “Materials and Methods.” The single letter amino acid sequence of three tryptic fragments from each protein that were purified and sequenced is given, along with the yield (in parentheses) of the sequenced peptide from the original protein sample.

Table III

| Peptide                   | Sequence         | Yield (%) |
|---------------------------|------------------|-----------|
| GMEB1:                    |                  |           |
| Peptide 1–59              | PVPQQQT56VQYPQP  | 5%        |
| Peptide 1–61              | FVHLAGK         | 13%       |
| Peptide 1–109             | QVEHGEELQYTLADLER | 5%       |
| GMEB2:                    |                  |           |
| Peptide 2–73              | VPDPLLQR        | 78%       |
| Peptide 2–85              | IMDSGELDFYQNDK  | 23%       |
| Peptide 2–145             | AGLDEVICFQOEETMK | 17%       |

In summary, we have found that a heteromeric complex of two potentially new proteins binds to a cis-acting element of the TAT gene. These two proteins, GMEB1 and GMEB2, are associated with changes in the transcriptional activity of anti-glucocorticoids and low concentrations of glucocorticoids. These phenomena have not been previously described for steroid receptors and thus are of considerable mechanistic interest. It remains to be seen whether the GMEBs interact with glucocorticoid receptors and the transcriptional machinery in the manner that we have proposed (2, 23). The cloning of GMEB1 and GMEB2 and the production of specific antibodies will be of major assistance in understanding the mechanistic details of this interesting system.

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REFERENCES

1. Simons, S. S., Jr., Mercier, L., Miller, N. R., Miller, P. A., Oshima, H., Sistare, F. D., Thompson, E. B., Wasner, G., and Yen, P. M. (1989) Cancer Res. 49, 21900-21904.

Fig. 5. Purification and reconstitution of GMEB activity. A, silver-stained SDS-PAGE gel of material during various stages of purification. Unstained molecular weight markers, lanes 1 and 8; about 100 ng each of HTC cytosol, lane 2; mono Q column purified cytosol, lane 3; M2 DNA affinity column flow through, lane 4; and the GME binding fractions from the first (lane 5), second (lane 6), and third (lane 7) M3 DNA affinity column. B, protein bands 1 and 2 from the SDS gel of material during various stages of purification and reconstitution of GMEB activity in gel shift assays. The three major proteins in the eluant from the third M3 DNA affinity column (proteins 1–3 in A) were individually cut out from a silver-stained gel such as in A, electroeluted, denatured, and renatured as described under “Materials and Methods.” Aliquots (0.4 μl out of 17 μl) from the renatured sample were analyzed in the gel shift assay. Lanes 1 and 9, purified GMEB from the third M3 DNA affinity column; lanes 2–7, indicated individual proteins or mixtures of proteins that were mixed, precipitated, denatured, and renatured. C, specificity of binding activity of purified and reconstituted GMEBs. Protein bands 1 and 2 from the SDS gel of A were isolated and reconstituted as in B and then analyzed in the standard gel shift assay without (lane 2) or with a 100-fold molar excess of non-labeled specifically (GME) or nonspecifically (M2) binding oligonucleotides. The complex formed with GMEB from the third M3 affinity column, but not fractionated on SDS gels, is shown in lane 1.

glucocorticoid regulated genes (2), it will be interesting to pursue the possible role of a GME and its heteromeric binding complex in the transcription of genes other than TAT.

While CREB has been identified as a component that also binds to the DNA sequence of the GME/CRE (26), it is not known if it is the only protein in the complex. Several lines of evidence argue that the CREB-containing complexes bound to the GME are also multimeric. Most obvious is the size of the CREB-containing complexes, which were ~310–360 kDa in gel shift assays and 400 kDa on size exclusion columns (Fig. 3A and data not shown). Deoxycholate blocked the formation of the CREB-containing bands in gel shift assays, just as was observed for GMEB (Fig. 3B). Given the fact that CREB is relatively small (~42 kDa), it would seem that the CREB complexes must contain either multiple copies of CREB or other proteins. CREB will bind to the GME-containing oligonucleotide in Southwestern blots (see “Results”) and will afford gel-shifted bands with a CRE-containing oligonucleotide (26) so that homooligomeric complexes of CREB may form. However, the gel-shifted band that the sonostatin CRE formed with purified CREB exhibited a faster migration than that with crude nuclear extracts (26). Thus, the CREB-containing complex from nuclear extracts probably is not the same as that formed just from CREB and would contain some other protein(s). Further experiments are required to determine whether the suspected additional proteins are CBP (52), other members of the CREB/CREM/ATF superfamily that can homodimerize with CREB (39), or even GMEB1 or -2.

In summary, we have found that a heteromeric complex of two potentially new proteins binds to a cis-acting element of the TAT gene. These two proteins, GMEB1 and GMEB2, are associated with changes in the transcriptional activity of anti-glucocorticoids and low concentrations of glucocorticoids. These phenomena have not been previously described for steroid receptors and thus are of considerable mechanistic interest. It remains to be seen whether the GMEBs interact with glucocorticoid receptors and the transcriptional machinery in the manner that we have proposed (2, 23). The cloning of GMEB1 and GMEB2 and the production of specific antibodies will be of major assistance in understanding the mechanistic details of this interesting system.

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REFERENCES

1. Simons, S. S., Jr., Mercier, L., Miller, N. R., Miller, P. A., Oshima, H., Sistare, F. D., Thompson, E. B., Wasner, G., and Yen, P. M. (1989) Cancer Res. 49, 21900-21904.
GMEB Is a Novel Heteromeric Complex

21901

2244–2252

2. Simons, S. S., Jr., Oshima, H., and Szapary, D. (1992) Mol. Endocrinol. 6, 955–1002

3. Jorat, C., Rahmsdorf, H. J., Park, K.-K., Cato, A. C. B., Gebel, S., Ponta, H., and Herrlich, P. (1990) Cell 62, 1189–1204

4. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272

5. Cho, H., and Katzenellenbogen, B. S. (1993) Endocrinology 133, 1230–1238

6. Maroder, M., Farina, A. R., Vacca, A., Felli, M. P., Meco, D., Screpanti, I., Frati, L., and Guilina, A. (1993) Mol. Endocrinol. 7, 570–584

7. Wasner, G., and Simons, S. S., Jr. (1987) Mol. Endocrinol. 1, 109–120

8. Kazmi, S. M. I., Visconti, V., Plante, R. K., Ishaque, A., and Lau, C. (1993) J. Biol. Chem. 268, 6073–6076

9. Nordeen, S. K., Bona, B. J., and Moyer, M. L. (1993) J. Biol. Chem. 268, 112, 601–609

10. Rangarajan, P. N., Umesono, K., and Evans, R. M. (1992) Mol. Endocrinol. 6, 1451–1457

11. Sartorius, C. A., Tung, L., Takimoto, G. S., and Horwitz, K. B. (1993) J. Biol. Chem. 268, 9262–9266

12. Berk, C. A., Weigel, N. L., Moyer, M. L., Nordeen, S. K., and Edwards, D. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4441–4445

13. Sanchez, E. R., Hu, J.-L., Zhong, S., Shen, P., Greene, M. J., and Housley, P. R. (1994) Mol. Endocrinol. 8, 408–421

14. Nig, Y.-M., and Sanchez, E. R. (1993) J. Biol. Chem. 268, 6073–6076

15. Power, R. F., Mani, S. K., Codina, J., Connelly, O. M., O’Malley, B. W. (1991) Science 254, 1636–1639

16. Mercier, L., Thompson, E. B., and Simons, S. S., Jr. (1983) Endocrinology 112, 603–609

17. Mercier, L., Miller, P. A., and Simons, S. S., Jr. (1986) J. Steroid Biochem. 25, 11–20

18. Simons, S. S., Jr., and Yen, P. M. (1987) in Steroid and Sterol Hormone Action (Spelsberg, T. C., and Kumar, R., eds) pp. 251–268, M. Nijhoff, Boston

19. Wasner, G., Oshima, H., Thompson, E. B., and Simons, S. S., Jr. (1988) Mol. Endocrinol. 2, 1109–1127

20. Simons, S. S., Jr., Miller, P. A., Wasner, G., Miller, N. R., and Mercier, L. (1988) J. Steroid Biochem. 31, 1–7

21. Oshima, H., and Simons, S. S., Jr. (1992) Endocrinology 130, 2106–2112

22. Szapary, D., Oshima, H., and Simons, S. S., Jr. (1992) Endocrinology 130, 3492–3502

23. Oshima, H., and Simons, S. S., Jr. (1993) Mol. Endocrinol. 6, 416–428

24. Oshima, H., and Simons, S. S., Jr. (1993) J. Biol. Chem. 268, 26858–26865

25. Boshart, M., Weh, F., Schmid, A., Fournier, R. E. K., and Schutz, G. (1990) Cell 61, 905–916

26. Hong, S. K., Weh, F., Schmid, W., DeVack, C., Kouwenz-Leutz, E., Luckow, B., Boshart, M., and Schutz, G. (1992) EMBO J. 11, 3337–3346

27. Nitsch, D., Boshart, M., and Schutz, G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5479–5483

28. Weih, F., Stewart, A. F., Boshart, M., Nitsch, D., and Schutz, G. (1990) Genes & Dev. 4, 1437–1449

29. Shepard, A. R., Zhang, W., and Eberhardt, N. L. (1994) J. Biol. Chem. 269, 1804–1814

30. Ray, A., Sassone-Corsi, P., and Sehgal, P. B. (1989) Mol. Cell. Biol. 9, 5537–5547

31. Reichman, P. N., Yen, P. M., and Evans, R. M. (1992) Mol. Endocrinol. 7, 4441–4445

32. Whelan, S. M., and Sanchez, E. R. (1993) J. Biol. Chem. 268, 9669–9675

33. Cooney, A. J., Tsai, S. Y., and Tsai, M.-J. (1993) In Transcription Factors: A Practical Approach (Latchman, D. S., ed) p. 49, IRL Press, New York

34. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

35. Maxam, A. M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 560–564

36. Kadonaga, J. T. (1991) Methods Enzymol. 208, 10–23

37. Miller, P. A., and Simons, S. S., Jr. (1988) Endocrinology 122, 2990–2998

38. Lalli, E., and Sassone-Corsi, P. (1994) J. Biol. Chem. 269, 17359–17362

39. Hummler, E., Cole, T. J., Blendy, J. A., Ganss, R., Aguzzi, A., Schmid, W., Beer, F., and Schutz, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5647–5651

40. Gaub, M.-P., Bellard, M., Scheur, I., Chambon, P., and Sassone-Corsi, P. (1990) Cell 63, 1267–1276

41. Heinrichs, A. A., J., Bortell, R., Rahman, S., Stein, L. J., Alnemri, E. S., Litwack, G., Lian, J. B., and Stein, G. S. (1993) Biochemistry 32, 11436–11444

42. Baeuerle, P. A., and Baltimore, D. (1988) Cell 53, 211–217

43. Kupper, S. R., Marschke, K. B., Wilson, E. M., and French, F. S. (1993) J. Biol. Chem. 268, 17151–17257

44. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410

45. Reik, A., Stewart, A. F., and Schutz, G. (1994) Mol. Endocrinol. 8, 490–497

46. Steltz, T. A. (1990) Q. Rev. Biophys. 23, 205–280

47. Harrison, S. C. (1991) Nature 353, 715–719

48. Jones, K. W., Shaper, M. H., Chevre, M., and Fournier, R. E. (1991) Cell 66, 861–872

49. Guiochon-Mantel, A., Delabre, K., Lescop, P., and Milgrom, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7179–7183

50. Guiochon-Mantel, A., Lescop, P., Christin-Martre, S., Loosfelt, H., Perrot-Appelainat, M., and Milgrom, E. (1991) EMBO J. 10, 3851–3859

51. Chandran, U. R., and deFranco, D. B. (1992) Endocrinology 124, 837–844

52. Chiriva, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montmire, M. R., and Goodman, R. H. (1993) Nature 365, 855–859

53. Miyashita, Y., Miller, M., Yen, P. M., Harmon, J. M., Hanover, J. A., and Simons, S. S., Jr. (1993) J. Steroid Biochem. Mol. Biol. 46, 309–320