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Glucocorticoids and Protein Kinase A Coordinately Modulate Transcription Factor Recruitment at a Glucocorticoid-Responsive Unit

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The rat tyrosine aminotransferase gene is a model system to study transcriptional regulation by glucocorticoid hormones. We analyzed transcription factor binding to the tyrosine aminotransferase gene glucocorticoid-responsive unit (GRU) at kb −2.5, using in vivo footprinting studies with both dimethyl sulfate and DNase I. At this GRU, glucocorticoid activation triggers a disruption of the nucleosomal structure. We show here that various regulatory pathways affect transcription factor binding to this GRU. The binding differs in two closely related glucocorticoid-responsive hepatoma cell lines. In line H4II, glucocorticoid induction promotes the recruitment of hepatocyte nuclear factor 3 (HNF3), presumably through the nucleosomal disruption. However, the footprint of the glucocorticoid receptor (GR) is not visible, even though a regular but transient interaction of the GR is necessary to maintain HNF3 binding. In contrast, in line FTO2B, HNF3 binds to the GRU in the absence of glucocorticoids and nucleosomal disruption, showing that a "closed" chromatin conformation does not repress the binding of certain transcription factors in a uniform manner. In FTO2B cells, the footprint of the GR is detectable, but this requires the activation of protein kinase A. In addition, protein kinase A stimulation also improves the recruitment of HNF3 independently of glucocorticoids and enhances the glucocorticoid response mediated by this GRU in an HNF3-dependent manner. In conclusion, the differences in the behavior of this regulatory sequence in the two cell lines show that various regulatory pathways are integrated at this GRU through modulation of interrelated events: transcription factor binding to DNA and nucleosomal disruption.

Cells react to their environment by transducing external signals into intracellular responses. Some mechanisms of hormonal signal transduction have been elucidated. Steroid hormones, such as glucocorticoids, bind directly to intracellular receptors and influence gene activity by stimulating the DNA-binding and transcriptional activation potencies of their cognate receptor (for a review, see reference 4). In contrast, peptide hormones, such as glucagon, bind to receptors located at the cell surface and activate transcription of target genes through second messengers such as cyclic AMP (cAMP). Increased intracellular cAMP levels lead to the activation of the cAMP-dependent protein kinase A (PKA), which phosphorylates a variety of nuclear transcription factors (see references 24 and 26 for reviews). Even though the molecular targets of these two hormones are different, they can act in a concerted way during some crucial regulatory responses, as in the establishment at birth of the correct pattern of gene expression in the liver (20). To study the interplay between these two regulatory pathways, we use as a model system the rat tyrosine aminotransferase (TAT) (EC 2.6.1.5) gene.

The TAT gene is expressed specifically in the parenchymal cells of the liver, where its transcription is stimulated by glucocorticoids and glucagon (for a review, see reference 19). Full glucocorticoid induction of the rat TAT gene is achieved through the cooperative interaction of two remote glucocorticoid-responsive units (GRUs) located at kb −2.5 (−2.5 GRU) and kb −5.5 (17, 22), whereas the glucagon-cAMP response is mediated by a cAMP-responsive unit located at kb −3.6 (9). Each unit consists of the assembly of binding sites for widely expressed hormone-responsive transcription factors as well as liver-enriched factors (9, 14, 18, 32, 38). Both hormones stimulate the recruitment of transcription factors at these regulatory units. Glucagon triggers the phosphorylation of the cAMP response element-binding protein (CREB), and this event stimulates its interaction with the cAMP-responsive unit at kb −3.6 by increasing its affinity for its DNA target site (31, 43). Glucocorticoids allow the interaction of the glucocorticoid receptor (GR) with the −2.5 GRU, and this interaction promotes a rearrangement of the local chromatin structure that affects two specifically phased nucleosomes (10, 35). Work performed with two related hepatoma cell lines has led to conflicting results concerning the identities of the factors stably recruited at this GRU following glucocorticoid stimulation. In some studies, glucocorticoid-dependent changes of reactivity toward dimethyl sulfate (DMS) have been interpreted as revealing the permanent interaction of the GR with DNA (6, 35). These changes have not been observed in other studies, revealing that the GR is not stably bound to DNA in these cases (14, 37). In these same studies, analyses performed with DNase I have revealed a glucocorticoid-dependent interaction of another transcription factor, hepatocyte nuclear factor 3 (HNF3) (formerly called HNF5 [37]). Since HNF3 interacts in a glucocorticoid-independent manner with the other TAT GRU at kb −5.5, where glucocorticoids are not required for the establishment of an open nucleosomal structure, we have proposed that glucocorticoids allow the recruitment of HNF3 at the −2.5 GRU by promoting a disruption of the nucleosomal structure (37). A similar mechanism is believed to be responsible for the glucocorticoid-dependent recruitment

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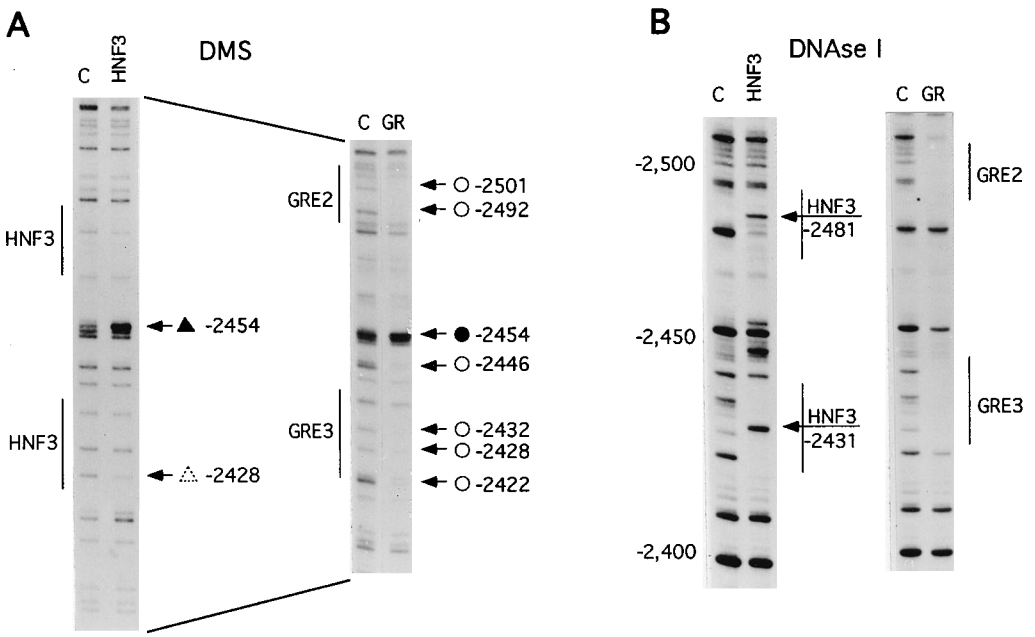

FIG. 1. Comparative analysis of the in vitro footprints of HNF3 and the GR obtained with DMS and DNase I. Results for the lower strand of the −2.5 GRU are shown. Before treatment with the footprinting agent, the probe was incubated without protein (lanes C [control]), with HNF3 purified from rat liver nuclear extract by affinity chromatography (38), or with purified recombinant GR DNA-binding domain (27). The same relative amounts of factor and DNA were used with each reagent. (A) DMS footprinting. The protected guanosines are indicated by the open symbols, and the hyperreactive guanosines are indicated by the closed symbols. The dotted symbol corresponds to protection at a lower level. (B) DNase I footprinting. The corresponding footprints are indicated by vertical bars, and the arrows indicate the hyperreactive bands specific for HNF3 interaction. The numbers indicate positions with respect to the transcription start site (in nucleotides).

of nuclear factor 1 with the mouse mammary tumor virus promoter (2, 33).

For the TAT gene, cross-talk between either the cAMP or the glucocorticoid pathway and other signal transduction pathways has been observed. Both insulin and protein kinase C negatively regulate the activity of each type of hormone-responsive unit at the TAT gene (16, 36). However, the cAMP and glucocorticoid pathways were believed to act independently through distinct regulatory units. In this report, we show that there is an additional level of pathway cross-talk: PKA can modulate the activity of the −2.5 GRU through the HNF3-binding sites. The cross-talk between the two pathways affects the interactions of both the GR and HNF3 with DNA.

MATERIALS AND METHODS

In vitro footprinting, in vivo footprinting, and genomic sequencing. In vitro footprinting with DNase I or DMS was performed as described elsewhere (14). HNF3 was prepared as described previously (38).

In vivo footprinting was performed as follows. Before treatment with either DMS or DNase I, hepatoma cells were cultured for 60 min in serum-free medium and then treated with hormones as follows: dexamethasone (10^{-7} M) alone for 60 min, forskolin (10 μM) alone for 120 min, or forskolin (10μM) for 60 min and then both forskolin (10 μM) and dexamethasone (10^{-7} M) for 60 min. When indicated (see Fig. 5), dexamethasone was replaced by corticosterone. Non-treated cells were incubated with 0.1% ethanol as a control. DMS treatment of the cells and DNA preparation were performed as described elsewhere (35). Chemical sequencing reactions and piperidine cleavage were as described previously (28). DNase I treatment of permeabilized hepatoma cells was as described previously (37), except that DNA preparation was performed as for DMS-treated samples. For genomic sequencing, we used the ligation-mediated PCR procedure as described previously (14, 37). For each footprinting experiment presented, the complete experiment was performed two or three times, with no important changes in the resulting pattern. To assess the extents of the changes in reactivity, the relevant bands were quantified with a Phosphorimager (Molecular Dynamics).

Plasmid constructions, cell culture, and transfections. All plasmid constructions were as described elsewhere (14, 38).

FTO2B and WT-8 cells were cultured as described previously (23), except that the medium was also supplemented with penicillin, streptomycin, and amphotericin B (Fungizone). H4II cells were cultured as described previously (17). Electroporation was performed with a Gene Pulser and a Capacitance Extender from Bio-Rad. Cells were trypsinized, washed in cold phosphate-buffered saline (PBS), and resuspended in PBS at a concentration of 2×10^7 cells per ml. A 400-μl portion of the suspension was mixed with 20 μg of plasmid DNA. After 5 min at room temperature, cells were pulsed at 960 μF and 250 V for FTO2B and WT-8 cells or 220 V for H4II cells. After a 10-min incubation at 37°C for FTO2B and WT-8 cells or at room temperature for H4II cells, the suspension was diluted in medium and cultured. Prior to extract preparation, cells were treated for 24 h with either 10 μM forskolin, 10^{-7} M dexamethasone, or 0.1% ethanol. Cell extract preparation and TAT and chloramphenicol acetyltransferase assays were done as described previously (17).

RESULTS

In vitro characterization of the signatures of the interaction of HNF3 and GR with DNA as analyzed with DMS and DNase I. Both HNF3 and the GR interact in vitro with the −2.5 GRU of the rat TAT gene at neighboring and overlapping sites (18). To clearly establish the identities of the factors interacting in vivo, we have performed a comparative analysis of their in vitro footprints obtained with either DMS or DNase I. The GR gives clear footprints with DMS, whereas HNF3 only slightly modifies the reactivity of the guanines toward this reagent (Fig. 1A). Therefore, DMS allows essentially only the detection of the GR interaction. In contrast, when DNase I is used, both factors give clear in vitro footprints (Fig. 1B). DNase I is particularly suitable for the detection and identification of the HNF3 interaction. Indeed, this interaction leads to the appearance of one novel specific hypersensitive site on each DNA strand at each HNF3-binding site (Fig. 1) (18, 37, 38). Since this band is not present on naked DNA, it allows the detection of partial occupancy by HNF3 (37). This characteristic band is a signature of HNF3 binding, since it is not obtained with the GR (Fig. 1) or with any of the other factors that can be detected in vitro (14, 18).

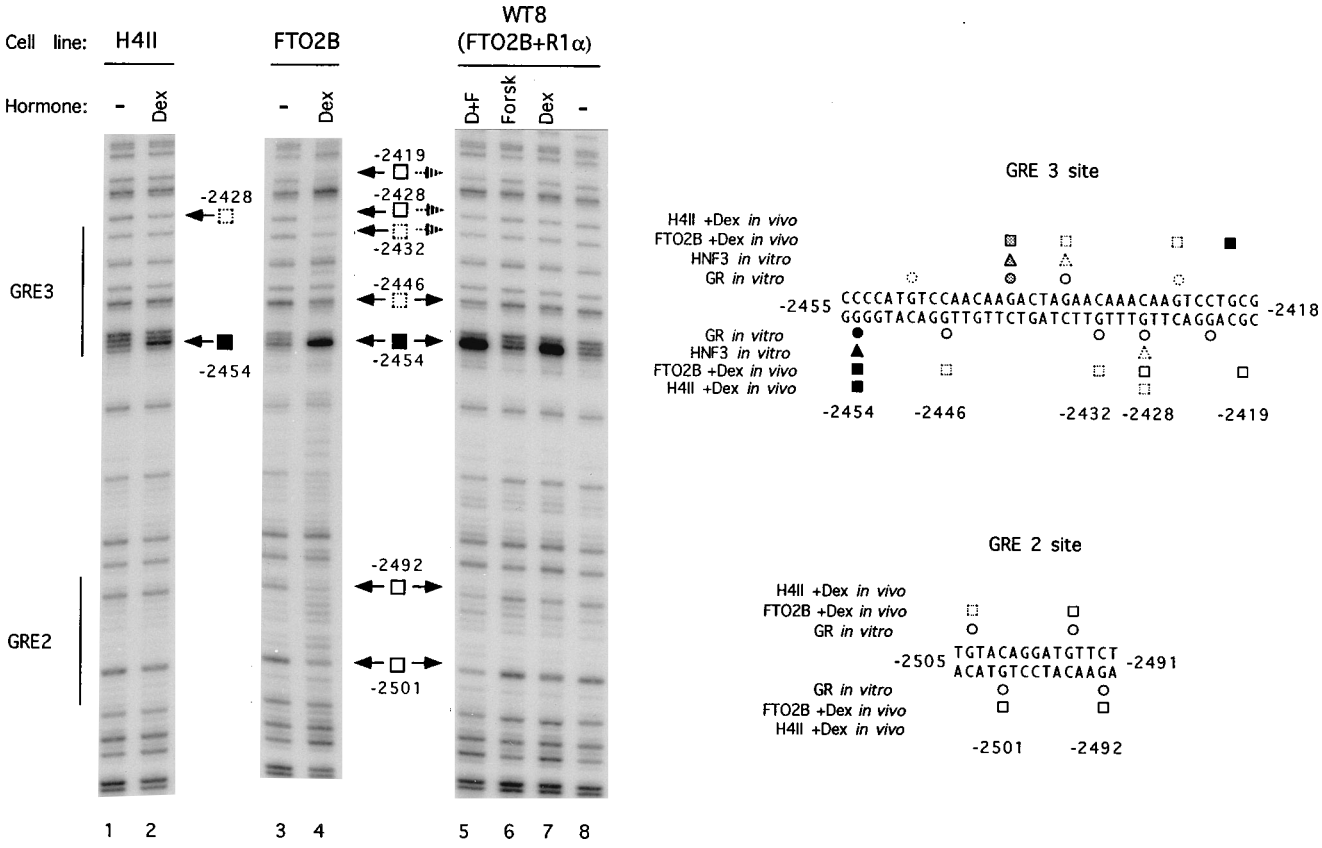

FIG. 2. In vivo footprinting analysis of the −2.5 GRU with DMS. Results for the lower strand are shown. Hepatoma cells were treated with hormones as described in Materials and Methods (Dex, dexamethasone; Forsk, forskolin; D+F, both dexamethasone and forskolin). Hormone-dependent changes of reactivity are indicated by either open squares (corresponding to protection), closed squares (corresponding to enhancement), or dotted symbols (corresponding to protection at a lower level). The hatched arrows indicate changes in reactivity that are not as marked in WT-8 cells treated with both dexamethasone and forskolin as in FTO2B cells treated with dexamethasone. The right side of the figure summarizes the changes in reactivity at the GRE2 and GRE3 sites. The changes in reactivity induced in vitro upon GR or HNF3 binding are indicated by circles and triangles, respectively. The hormone-dependent changes observed in FTO2B and H4II cells are indicated by squares. Open symbols, protection; dotted symbols, protection at a lower level; closed symbols, enhancement; shaded symbols, enhancement at a lower level. The numbers indicate positions with respect to the transcription start site (in nucleotides).

Cell line-specific variability of the in vivo footprints of glucocorticoid-activated GR. In vivo footprinting analyses of the −2.5 GRU of the TAT gene have not led consistently to the detection of GR binding to DNA upon glucocorticoid treatment (6, 14, 35, 37). These analyses were performed with two related but distinct rat hepatoma cell lines named H4II and FTO2B. To clearly establish the basis of these discrepancies, we have performed a comparative DMS footprinting analysis of these two cell lines. This analysis, and those that follow, have been reproduced two or three times, each time on both strands. Figure 2 shows that only minor glucocorticoid-dependent changes in reactivity towards DMS can be seen in H4II cells (compare lanes 1 and 2), whereas several changes in reactivity are observed in FTO2B cells (compare lanes 3 and 4). Two GR-binding sites show changed reactivity in this cell line: a high-affinity site that closely resembles a canonical palindrome with a 3-bp spacing, namely the GRE2 site, and a low-affinity site, not closely related to the canonical palindrome, namely the GRE3 site. The GRE3 site is also a high-affinity HNF3-binding site (18, 38), and both factors bind in a mutually exclusive way with this site (37). In FTO2B cells, the changes in reactivity observed at the GRE2 site correspond exactly to those obtained in vitro with the GR (Fig. 2); from a quantitative analysis it can be estimated that ~50% of the site is occupied by the GR. The interpretation of the changes in reactivity observed at the GRE3 site is more ambiguous. The changes may result from a partial and mixed occupancy of the sites by either the GR or HNF3 (Fig. 2). At the 3′ border of the GRE3 site, there are two changes in reactivity (positions −2419 and −2420) that cannot be accounted for by either of these factors on the basis of their in vitro DMS footprints. In H4II cells, the clearer glucocorticoid-induced change in reactivity is a hyperreactivity at position −2454. This hyperreactivity is obtained in vitro with both HNF3 and the GR, and it is the more distinct change resulting from HNF3 interaction (Fig. 1A). It is compatible with the glucocorticoid-dependent interaction of HNF3 (see below) but not with that of GR, since most of the protections conferred by GR in vitro are not visible in vivo upon glucocorticoid stimulation. In conclusion, the patterns of transcription factor interaction in the two cell lines are different, and the interaction of GR with the GRU is visible only in FTO2B cells.

PKA activity is responsible for the in vivo visibility of the interaction of GR with the −2.5 GRU. Even though the GR interaction is not visible in H4II cells, the magnitudes of glucocorticoid induction of TAT gene transcription are similar in FTO2B and H4II cells (about 20-fold in both cell lines [data not shown]). Furthermore, GR stimulation leads to similar rearrangements of chromatin structure at the −2.5 GRU in the two cell lines (10, 35). However the basal TAT level is higher

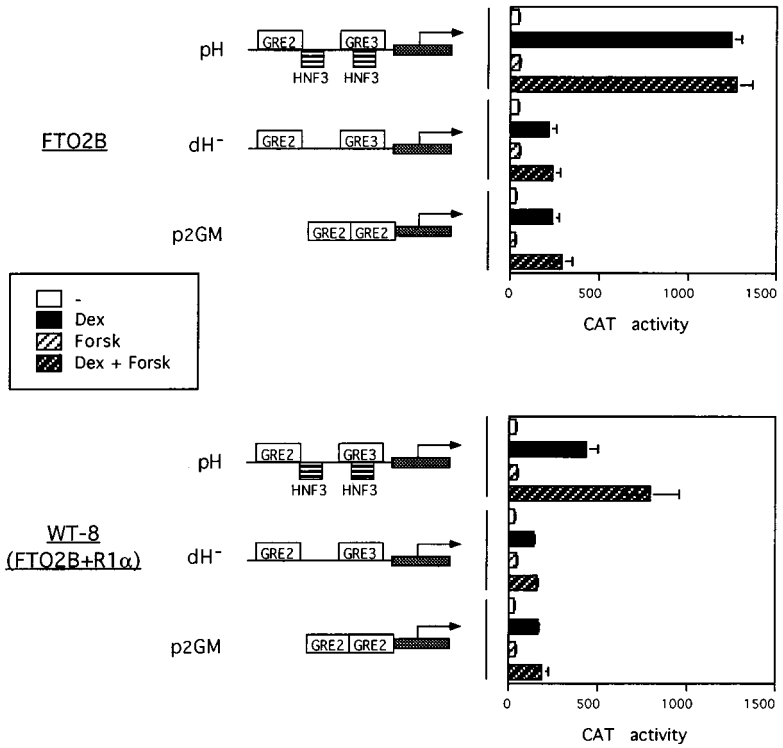

FIG. 3. Transient-expression analysis of the role of PKA activity in the function of the −2.5 GRU. The reporter plasmids tested are represented on the left. The gray box with an arrow represents the minimal promoter. The GRE2-, GRE3-, and HNF3-binding sites of the −2.5 GRU are indicated by boxes. The absence of HNF3 boxes indicates mutation of the corresponding binding sites. p2GM corresponds to a duplicated GRE2-binding site where the Ets-binding site has been inactivated (14). The chloramphenicol acetyltransferase (CAT) activities represented are the means from at least three independent experiments. The bars indicate the standard errors of the mean; when these values are low, the bars are not visible. As indicated, cells were treated with dexamethasone (Dex) (10^{-7} M) and/or forskolin (Forsk) (10 μM) 24 h prior to collection.

in FTO2B cells than in H4II cells (70 versus 20 mU/mg of protein). In FTO2B cells, the basal transcription level has been linked to the enhancer located around kb −3.6 (9). In these cells, the constitutive activity of this enhancer is due to a constitutive PKA activity that results from the imbalance of the levels of the catalytic and repressive regulatory subunits of the kinase (8, 9, 23). Overexpression of the regulatory subunit R1α shuts off the constitutive PKA activity and the basal activity of the enhancer (8, 23). In H4II cells, in accordance with the lower basal TAT activity, the −3.6 enhancer shows no basal activity (as assessed by transient-expression analyses [data not shown]). This suggests that the PKA pathway is not constitutively active in this cell line. Since it has been proposed that PKA can enhance the DNA-binding activity of the GR (34), we tested whether the constitutive PKA activity in FTO2B cells could contribute to the detection of the GR footprints. Therefore, we analyzed the changes in reactivity towards DMS observed following hormonal stimulation of an FTO2B-derived cell line named WT-8, whose basal PKA activity is shut off following overexpression of R1α (23). Extinction of the constitutive PKA activity causes a loss of the glucocorticoid-dependent GR footprint at both GREs (compare lanes 7 and 8 in Fig. 2). PKA can be reactivated upon treatment with forskolin, an agent that stimulates adenylate cyclase (39). The GR footprints on the GRE2 site can be recovered following activation of both the GR and PKA by a combined treatment with dexamethasone and forskolin (Fig. 2, lane 5). On the GRE3 site, the GR footprints are also significantly recovered, but the protections are not as marked as they are in FTO2B cells. Forskolin treatment has no effect on its own (Fig. 2, lane 6). Therefore, PKA activity is required in FTO2B cells in order to permit GR interaction with DNA to persist sufficiently to leave a detectable footprint.

The different behavior of the GR in H4II and FTO2B-derived cells cannot be attributed solely to differences in PKA activity. Indeed, forskolin treatment does not allow recovery of the glucocorticoid-dependent GR footprints in H4II cells, even though it is able to increase TAT activity (data not shown).

PKA stimulates the glucocorticoid response of the −2.5 GRU via HNF3. To assess the functional consequence of the modulation of transcription factor interaction by PKA, transient-expression assays with FTO2B and WT-8 cells were carried out (Fig. 3). When a reporter corresponding to the −2.5 GRU linked upstream to a minimal promoter (plasmid pH) is used, PKA activity stimulates the glucocorticoid response: overexpression of R1α decreases the glucocorticoid-induced level threefold (compare data for dexamethasone treatment in both cell lines; $P < 0.01$ in a paired t test), and forskolin treatment of WT-8 cells leads to a twofold increase in the glucocorticoid induction (compare data for dexamethasone treatment with and without forskolin in WT-8 cells; $P < 0.03$ in a paired t test). To define the target sequences for PKA action, we further dissected the GRU. We analyzed whether PKA acts directly on the GR by testing a reporter containing a duplicated GR-binding site isolated from the context of the GRU (plasmid p2GM) (Fig. 3). Forskolin addition has no effect on the level of expression of this construct in WT-8 cells. To test whether HNF3 is involved in the PKA response, we analyzed the activity of a mutated −2.5 GRU in which the HNF3-binding sites are specifically inactivated (plasmid dH⁻) (14,

38). As previously described (14, 38), inactivation of the HNF3-binding sites decreases glucocorticoid induction (compare the levels of expression of pH and dH^- in dexamethasone-induced cells [Fig. 3]). The remaining activity is no longer influenced by the cAMP-increased PKA activity in WT-8 cells (compare data for dexamethasone treatment with and without forskolin). Finally, in agreement with the absence of an effect of PKA stimulation on the interaction of the GR with DNA in H4II cells, forskolin treatment does not stimulate the glucocorticoid response mediated by the −2.5 GRU in this cell line (data not shown).

In conclusion, PKA activity can stimulate the glucocorticoid response mediated by the −2.5 GRU of the TAT gene, and this modulation requires the interaction of HNF3.

HNF3 can bind to the −2.5 GRU in the absence of the glucocorticoid-dependent rearrangement of the chromatin structure. To analyze the in vivo interaction of HNF3 with the −2.5 GRU, we used DNase I because of its high sensitivity for the detection of this interaction (Fig. 1). In the following studies, we have highlighted the changes in the reactivity that corresponds to the HNF3 signature. The data presented here correspond to one strand, but the analyses performed with the other strand confirm the modalities of the HNF3 interaction. These studies were performed with permeabilized cells under conditions that minimize the risk of stripping away the factors from their binding sites (37). However, despite these precautions, most of the GR footprint is lost with this protocol as assessed by DMS footprinting analysis of permeabilized FTO2B cells (data not shown). Therefore, in contrast to DMS, DNase I, as used here, allows the in vivo detection of HNF3 but not of the GR.

As previously described (37), in H4II cells, the interaction of HNF3 with the −2.5 GRU is strictly glucocorticoid dependent (compare lanes 1 and 2 in Fig. 4); it does not occur in the absence of the nucleosome disruption triggered by the GR (the slight band that can be seen at the GRE3 site in the absence of hormone is shifted by one base from the HNF3 band and is obtained with naked DNA [Fig. 1]). In contrast, in FTO2B cells, in the absence of glucocorticoids, HNF3 already binds to its two sites on the −2.5 GRU as assessed by the presence of the characteristic HNF3-dependent bands (Fig. 4, lane 5). In these cells, without glucocorticoids, a nucleosome apparently covers the two HNF3-binding sites (35), but this nucleosome is not sufficient to preclude HNF3 interaction in FTO2B cells. As in H4II cells, glucocorticoid induction results in an increased amount of factor interacting with DNA (compare lanes 5 and 6 in Fig. 4), but the level of HNF3 recruitment is lower in FTO2B cells (compare lanes 5 and 6 with lanes 1 and 2).

As shown for the GR (Fig. 2), but to a smaller extent, the interaction of HNF3 is affected by PKA activity in FTO2B-derived cells. Extinction of the constitutive PKA activity upon R1α overexpression (WT-8 cells) decreases the amount of HNF3 interacting both in the absence and in the presence of glucocorticoids (compare lanes 5 and 6 with lanes 7 and 8 in Fig. 4). Reactivation of PKA following forskolin treatment increases the HNF3 interaction about twofold (as quantified with a Phosphorimager), up to an occupancy level similar to that found in FTO2B cells (Fig. 4, lanes 9 and 10). This increase in the HNF3 interaction with DNA was not observed in vitro with nuclear extracts of WT-8 cells stimulated or not by forskolin (data not shown). Therefore, the interactions of both the GR and HNF3 with DNA are stabilized in FTO2B cells upon PKA stimulation. In contrast, in H4II cells, forskolin treatment does not lead to increased DNA binding of either the GR (see above) or HNF3 (Fig. 4, lanes 3 and 4).

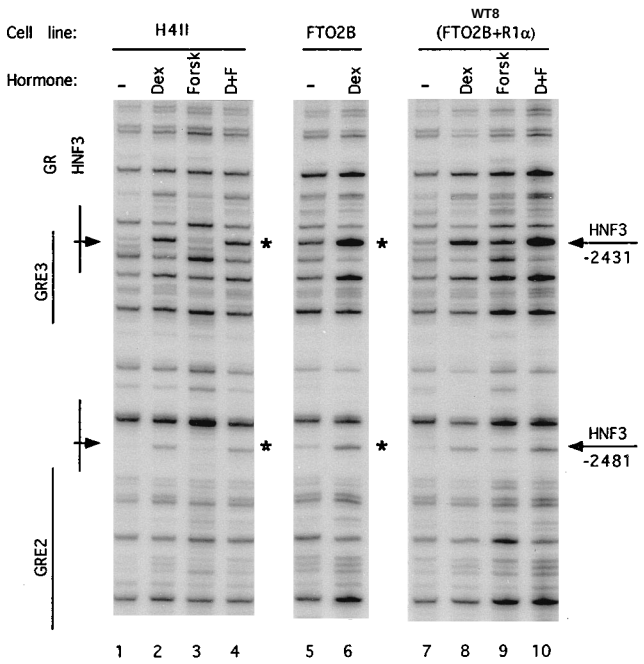

FIG. 4. In vivo footprinting analysis of the −2.5 GRU with DNase I. Results for the lower strand are shown. The data are presented as described in the legend to Fig. 2. The left side of the figure shows the locations of the GR- and HNF3-binding sites as represented by bars. The arrows and asterisks indicate the HNF3-associated hyperreactive bands.

In H4II cells, continuous activation of the GR is necessary for HNF3 interaction with the −2.5 GRU. The differences in the patterns of transcription factor interaction in H4II and FTO2B cells could be taken as an indication that transcriptional activation by the GR proceeds through different mechanisms in these cell lines. In FTO2B cells, where the GR interaction is sufficiently continuous to be detectable, the GR must be continuously activated to maintain an open chromatin structure (35). In H4II cells, since the interaction of the GR is not detectable per se, a continuously activated GR might not be necessary to maintain the system in an active state, and transcriptional activation could have proceeded through a "hit-and-run" mechanism (37). To test this hypothesis, we analyzed the requirement for the continuous presence of glucocorticoids to maintain the HNF3 interaction in this cell line. Figure 5 shows that most of the interaction of HNF3 is lost 1 h after the hormone is washed out of the cells (compare lanes 4 and 5). Thus, the GR must repetitively associate with DNA to maintain the region accessible to HNF3, although it does not remain at its sites long enough to be detected. The situation in H4II cells can be better described by a permanent dynamic interplay of transcription factors rather than by a simple hit-and-run mechanism (37).

DISCUSSION

Dynamics of transcription factor interaction during glucocorticoid stimulation. In both the H4II and FTO2B cell lines, in the absence of hormone, a phased array of nucleosomes is positioned over the −2.5 GRU of the TAT gene (10, 35). Following glucocorticoid addition, the hormone-activated GR interacts with DNA in a closed chromatin conformation and triggers a chromatin structural alteration that affects two neighboring nucleosomes (10, 35). The in vivo footprints corresponding to the same hormonal status are different in the

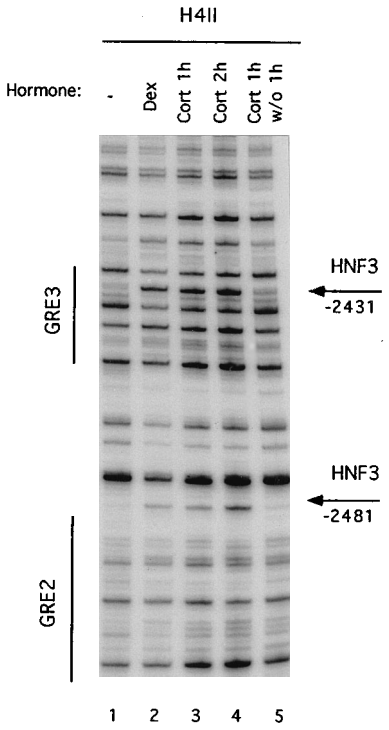

FIG. 5. The continuous presence of glucocorticoids is necessary to maintain HNF3 interaction. The lower strand of the −2.5 GRU is analyzed by in vivo footprinting with DNase I. The data are presented as described in the legend to Fig. 2. For the washout experiment, hydrocortisone was used instead of dexamethasone because the rate of deinduction is faster (35). The samples are from uninduced cells (−); from cells induced for 1 h with 10^{-7} M dexamethasone, for 1 h with 10^{-7} M hydrocortisone (Cort 1h), or for 2 h with hydrocortisone (Cort 2h); and from cells induced with hydrocortisone for 1 h followed by a washout and a 1-h incubation without hormone (Cort 1h w/o 1h).

two hepatoma cell lines. Transcription factor interaction with the −2.5 GRU is modeled in Fig. 6. The main feature of this scheme is the dynamic equilibrium between different states.

In H4II cells, in the absence of glucocorticoids, the interaction of HNF3 with the −2.5 GRU is not detected. Following GR activation and nucleosomal disruption, HNF3 binds to its two binding sites in the −2.5 GRU (Fig. 4) (37). Nucleosomal disruption is likely to be the event that allows the glucocorticoid-dependent binding of HNF3. Indeed, in H4II cells, HNF3 interacts independently of the presence of glucocorticoids with the −5.5 GRU, where there is a glucocorticoid-independent disruption of nucleosomal structure (37). At both GR-binding sites, the interaction of the GR is not detected by footprinting. Therefore, the states represented in Fig. 6, in which the GR is interacting with DNA, are transient. The most frequent state when an equilibrium is established is that in which the sites are occupied by HNF3 but not by the GR (represented on the right side of Fig. 6). The evidence for the occurrence of a transient state in which the GR is interacting with DNA is provided by the experiment in which the glucocorticoids were withdrawn. In this case, HNF3 binding is lost (Fig. 5), and the equilibrium is shifted toward the left. If the GR was activating by a simple hit-and-run mechanism and was binding only transiently at the early stage of activation, one would predict that it would not be required for the maintenance of the open conformation of the system. The requirement for the hormone for this maintenance can be most simply explained by the regular requirement for a transient interaction of the activated GR with the −2.5 GRU, even if this interaction is not sufficiently long-lived to be detected by footprinting.

In FTO2B cells, the predominant states at the equilibrium are different, but the overall sequence of states is likely to be very similar. In the absence of hormone, the closed nucleosomal structure does not appear to be different from that observed in H4II cells (10, 35). However, this closed conformation does not totally preclude HNF3 interaction (see below). Upon hormone addition, the state at the equilibrium in which the GR interacts with DNA is much more frequent than it is in H4II cells: half of the GRE2 sites in the population are occupied by the GR. Occupancy of the GRE3 site cannot be easily quantified because both HNF3 and the GR bind to this site, in a mutually exclusive manner (37). The data are compatible with a mixed occupancy by either the GR or HNF3. In FTO2B cells, hormone withdrawal also leads to a reversal of the equilibrium toward the closed conformation (35). This is in agreement with a common overall succession of stages in the two cell lines despite the different levels of occupancy of the GR-binding sites.

In conclusion, we propose that the important differences in the patterns of transcription factor interaction with DNA observed in the two cell lines reflect differences in the equilibrium constants of a common dynamic process rather than two distinct processes.

Interplay between chromatin structural features and transcription factors. Chromatin structural features play pivotal roles in the regulation of gene expression (see references 1, 7, 15, and 44 for reviews). The chromatin structure of a tissue-specific gene is generally in a tightly closed conformation in the tissue where the gene is not expressed (defined here as the locked conformation). A general loosening of the locked chromatin conformation can be observed over the entire gene when it is expressed or poised for expression (defined here as the closed conformation). A full local opening of the chromatin structure, corresponding to the disruption of one or a few nucleosomes, is generally associated with the recruitment of transcription factors with regulatory regions (defined here as the open conformation). This conformation is usually detectable as a hypersensitivity of the underlying DNA to various nucleases.

Transcription factors could be classified on the basis of their behavior toward these chromatin structural features: some factors require an open conformation to bind DNA, while others can bind to a closed conformation (1, 42). This second category can be further divided into two classes. One corresponds to the factors that promote a structural alteration upon binding. The GR falls in this first class, as it can promote the opening of the chromatin structure as shown by the appearance of a nuclease-hypersensitive site on several of its cognate regulatory regions (see references 5 and 46 for examples). The other class includes the factors that bind without apparently modifying the chromatin structure. We have previously shown that some members of the Ets family fall in this second class (14). One of the Ets-binding sites present in the −2.5 GRU lies in a region that is wrapped around a nucleosome in the absence of glucocorticoids. This site is occupied in both hepatoma cell lines studied here independently of the presence of glucocorticoids (reference 14 and data not shown). Therefore, the interaction of Ets factors with this site neither requires nor triggers nucleosomal disruption. Strikingly, this site is not occupied in fibroblasts in which the TAT gene is silent, despite the presence of the cognate DNA-binding activity (14). This indicates that these factors cannot interact with DNA in a locked chromatin configuration.

Members of the HNF3 family cannot be classified in a single

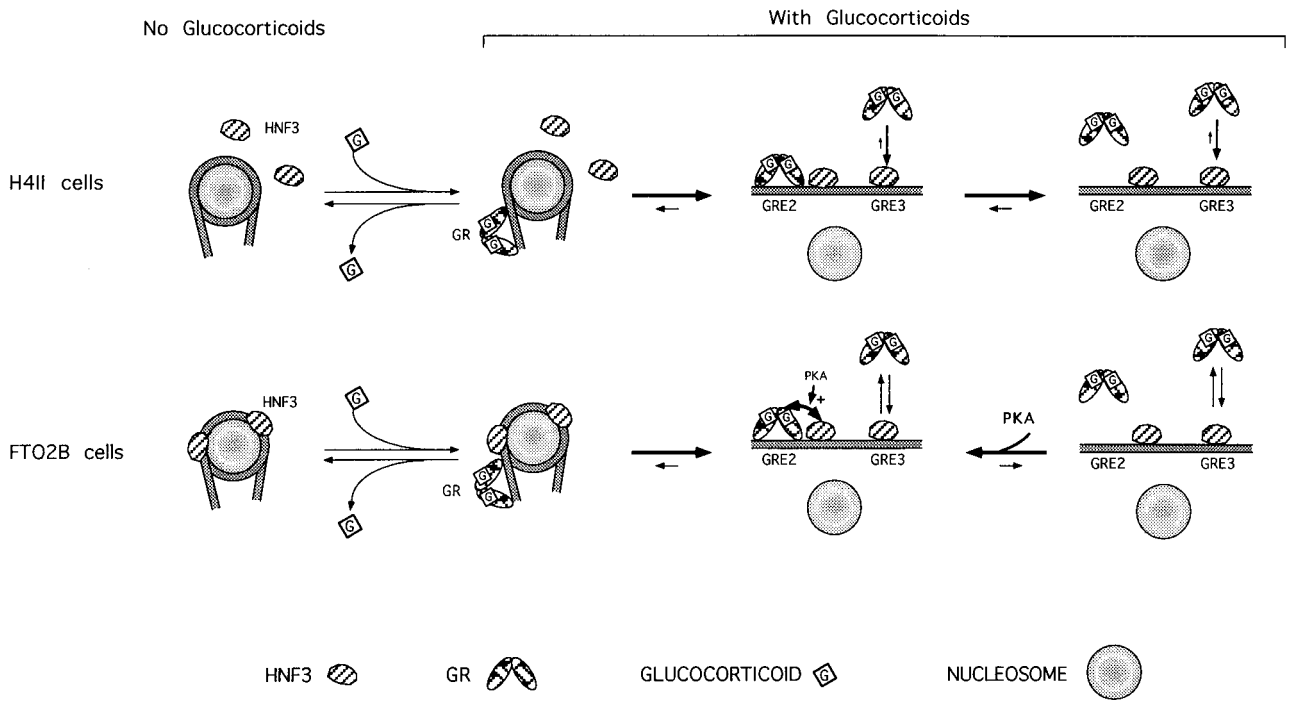

FIG. 6. Schematic representation of the dynamic equilibrium of transcription factor interaction with the −2.5 GRU. The model describing the successive stages of transcription factor interaction with the −2.5 GRU in the two cell lines is described in the text (see Discussion). An array of phased nucleosomes covers the −2.5 region (10), but a single nucleosome, that covering the HNF3-binding sites, is shown. The glucocorticoid-induced nucleosomal disruption has been represented as a nucleosome displacement for simplicity, but other types of nucleosomal alteration can be considered (1). The first state following hormone addition, in which the activated GR interacts with DNA in a closed chromatin conformation, is presumed. It is represented for clarity, even though it is too transient to be detected.

category; in H4II cells, they appear to be unable to bind to the −2.5 GRU in the closed conformation (without glucocorticoid), whereas in FTO2B cells, they can. In the FTO2B cell line, they behave like the Ets factors, as they bind to DNA without triggering a nucleosomal disruption. In liver cells, HNF3 interacts in vivo with DNA wrapped in a "nucleosome-like particle" at another regulatory sequence, the mouse albumin enhancer (29). However, it is likely that the nucleosome-like particle in the albumin enhancer does not correspond to the closed conformation seen in the absence of glucocorticoid at the −2.5 GRU, since the albumin enhancer is hypersensitive to DNase I in the liver (29).

The origin of the differences in the interactions of HNF3 with the −2.5 GRU in the two hepatoma cell lines is unknown and could result from several mechanisms. There could be differences in the nature of the HNF3 activities. Similar overall levels of different HNF3 family members are expressed in the two cell lines. HNF3α, -β, and -γ are expressed in FTO2B cells, whereas HNF3β accounts for most of the DNA-binding activity detected in H4II cells (reference 38 and data not shown). These different proteins might have different abilities to interact with nucleosomal DNA. Alternatively, differential modification, for example, phosphorylation, of HNF3 or accessory proteins could modulate this interaction. There could also be differences in the positioning of the nucleosomes. The previously published analyses of the nucleosomal phasing at the −2.5 GRU show similar positioning in the two cell lines (10, 35). However, the resolution of these analyses does not allow the conclusion that both rotational and translational phasings are identical. Finally, there could be differences either in the protein composition of the nucleosome core or among the factors that affect DNA-binding protein interaction with nucleosomes, such as histone acetylation (25), nucleoplasmin or a similar activity (11), or the SWI-SNF complex (12). Whatever the mechanisms involved, our data show that the interaction of a transcription factor with nucleosomal DNA is not an all-or-none situation. This interaction is susceptible to modulation in vivo, thus corresponding to an additional level at which gene expression can be regulated.

Cross-talk between the glucocorticoid and cAMP pathways. The glucocorticoid response is integrated with other regulatory networks: cross-talk with the PKA, protein kinase C, and insulin pathways has been described (see references 16, 30, 34, 36, and 47 for examples). However, the outcome of the interaction between the pathways can vary depending on the cell type or the gene considered. For example, both activation and repression of the GR response by protein kinase C have been reported (30, 36).

Here we show that in FTO2B-derived hepatoma cells, PKA stabilizes the binding to DNA of the glucocorticoid-activated GR (Fig. 2) and stimulates the glucocorticoid response mediated by the TAT −2.5 GRU (Fig. 3). PKA also affects GR function in other systems involving different cell types and/or regulatory elements. PKA has been reported to increase the GR level (13), but in the experiment here, no increase in the GR level was observed by Western blot analysis (data not shown). In other cases, following overexpression of PKA, increased DNA-binding activity of the GR has been observed (34). It is not clear if this general increase of GR binding to a simple GRE relates to the increased interaction of the GR with the −2.5 GRU observed here. Indeed, this effect was correlated with an increased enhancer activity of a dimer of a simple GRE that is not seen in FTO2B-derived cells. We show that in this cell line, PKA stabilizes the interaction with DNA not only of the glucocorticoid-activated GR but also of HNF3 (Fig. 2 and 4). Furthermore, we show that the PKA-dependent

stimulation of the glucocorticoid response mediated by the TAT −2.5 GRU depends on the integrity of the HNF3-binding sites (Fig. 3). Even though there are indications that transient transfections do not fully reflect chromatin-dependent effects on gene regulation (3, 40), it is tempting to consider that the forskolin effect observed by using in vivo footprinting and that observed in transient-expression assays are due to the same cause. The most parsimonious interpretation of this set of data is that in FTO2B cells, PKA stabilizes the GR interaction with DNA through HNF3. This could result from a direct interaction of the GR with HNF3 or could require additional factors. Heterogeneity in the presence of such factors or in the behavior of members of the HNF3 family could account for the absence of stabilization of the GR interaction following forskolin treatment of H4II cells.

The major transcriptional mediators of PKA are members of the CREB family (see references 24 and 26 for reviews). CREB has been shown to contribute both positively and negatively to the glucocorticoid response of target genes. In the phosphoenolpyruvate carboxykinase gene promoter, CREB binding to a CRE located in the vicinity of a GRU is important for the full glucocorticoid response (21, 45), whereas competition between CREB and the GR has been proposed to be responsible for the negative regulation of the gene for the glycoprotein hormone α subunit (41). As shown here, HNF3 is also a member of the PKA-responsive factor family that participates in the integration of this response with other regulatory networks in liver cells.

HNF3 is also involved in the negative regulation by insulin of the TAT gene glucocorticoid response (16). Since insulin also antagonizes the activity of CREB (16), both of the transcriptional mediators of PKA in the liver, CREB and HNF3, are negatively affected by this hormone. It has been shown that the effect of insulin is downstream of the phosphorylation of CREB by PKA, because this phosphorylation is not affected by insulin (16). Similarly, it is unlikely that insulin simply reverses the PKA-mediated phosphorylation event that affects GR interaction with DNA, since insulin treatment of FTO2B cells does not lead to the loss of the glucocorticoid-dependent GR footprint (data not shown).

In conclusion, we have observed a dynamic interplay of interrelated events: transcription factor interaction with DNA and chromatin structural alteration. These events can be controlled by various stimuli allowing the integration of the glucocorticoid response with other regulatory networks within a single GRU. Regulated changes in the equilibria of the interaction of factors with DNA result in varied interplay between transcription factors and chromatin structural features. In some instances, these changes are not reflected by profound changes in the extent of transcriptional activation, thus showing the redundancy of the transcriptional activation potentials of the various states and the plasticity of the regulatory mechanisms involved.

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REFERENCES

1. **Adams, C. C., and J. L. Workman.** 1993. Nucleosome displacement in transcription. Cell **72:**305–308.
2. **Archer, T. K., M. G. Cordingley, R. G. Wolford, and G. L. Hager.** 1991. Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumor virus promoter. Mol. Cell. Biol. **11:** 688–698.
3. **Archer, T. K., P. Lefebvre, R. G. Wolford, and G. L. Hager.** 1992. Transcription factor loading on the MMTV promoter: a bimodal mechanism for promoter activation. Science **255:**1573–1576.
4. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56:**335–344.
5. **Becker, P., R. Renkawitz, and G. Schütz.** 1984. Tissue-specific DNAase I hypersensitive sites in the 5′-flanking sequences of the tryptophan oxygenase and the tyrosine aminotransferase genes. EMBO J. **3:**2015–2020.
6. **Becker, P. B., B. Gloss, W. Schmid, U. Strähle, and G. Schütz.** 1986. In-vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. Nature (London) **324:**686–688.
7. **Bonifer, C., A. Hecht, H. Saueressig, D. M. Winter, and A. E. Sippel.** 1991. Dynamic chromatin: the regulatory domain organization of eucaryotic gene loci. J. Cell. Biochem. **47:**99–108.
8. **Boshart, M., F. Weih, M. Nichols, and G. Schütz.** 1991. The tissue-specific extinguisher locus TSE1 encodes a regulatory subunit of cAMP-dependent protein kinase. Cell **66:**849–859.
9. **Boshart, M., F. Weih, A. Schmidt, R. E. Fournier, and G. Schütz.** 1990. A cyclic AMP response element mediates repression of tyrosine aminotransferase gene transcription by the tissue-specific extinguisher locus Tse-1. Cell **61:**905–916.
10. **Carr, K. D., and H. Richard-Foy.** 1990. Glucocorticoids locally disrupt an array of positioned nucleosomes on the rat tyrosine aminotransferase promoter in hepatoma cells. Proc. Natl. Acad. Sci. USA **87:**9300–9304.
11. **Chen, H., B. Li, and J. L. Workman.** 1994. A histone-binding protein, nucleoplasmin, stimulates transcription factor binding to nucleosomes and factor-induced nucleosome disassembly. EMBO J. **13:**380–390.
12. **Coté, J., J. Quinn, J. L. Workman, and C. L. Peterson.** 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science **265:**53–60.
13. **Dong, Y., M. Aronsson, J.-A. Gustafsson, and S. Okret.** 1989. The mechanism of cAMP-induced glucocorticoid receptor expression. J. Biol. Chem. **264:**13679–13683.
14. **Espinás, M. L., J. Roux, J. Ghysdael, R. Pictet, and T. Grange.** 1994. Participation of Ets transcription factors in the glucocorticoid response of the rat tyrosine aminotransferase gene. Mol. Cell. Biol. **14:**4116–4125.
15. **Felsenfeld, G.** 1992. Chromatin as an essential part of the transcriptional mechanism. Nature (London) **355:**219–224.
16. **Ganss, R., F. Weih, and G. Schütz.** 1994. The cyclic adenosine 3′, 5′-monophosphate- and the glucocorticoid-dependent enhancers are targets for insulin repression of tyrosine aminotransferase gene transcription. Mol. Endocrinol. **8:**895–903.
17. **Grange, T., J. Roux, G. Rigaud, and R. Pictet.** 1989. Two remote glucocorticoid responsive units interact cooperatively to promote glucocorticoid induction of rat tyrosine aminotransferase gene expression. Nucleic Acids Res. **17:**8695–8709.
18. **Grange, T., J. Roux, G. Rigaud, and R. Pictet.** 1991. Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific factor. Nucleic Acids Res. **19:**131–139.
19. **Granner, D. K., and J. L. Hargrove.** 1983. Regulation of the synthesis of tyrosine aminotransferase: the relationship to mRNA-TAT. Mol. Cell. Biochem. **53/54:**113–128.
20. **Greengard, O.** 1969. Enzymic differentiation in mammalian liver. Injection of fetal rats with hormones causes the premature formation of enzymes. Science **163:**891–895.
21. **Imai, E., J. N. Miner, J. A. Mitchell, K. R. Yamamoto, and D. K. Granner.** 1993. Glucocorticoid receptor-cAMP response element-binding protein interaction and the response of the phosphoenolpyruvate carboxikinase gene to glucocorticoids. J. Biol. Chem. **268:**5353–5356.
22. **Jantzen, H. M., U. Strähle, B. Gloss, F. Stewart, W. Schmid, M. Boshart, R. Miksicek, and G. Schütz.** 1987. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell **49:**29–38.
23. **Jones, K. W., M. H. Shapero, M. Chevrete, and R. E. K. Fournier.** 1991. Subtractive hybridization cloning of a tissue-specific extinguisher: TSE1 encodes a regulatory subunit of protein kinase A. Cell **66:**861–872.
24. **Lalli, E., and P. Sassone-Corsi.** 1994. Signal transduction and gene regulation: the nuclear response to cAMP. J. Biol. Chem. **269:**17359–17362.
25. **Lee, D. Y., J. J. Hayes, D. Pruss, and A. P. Wolffe.** 1993. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell **72:**73–84.
26. **Lee, K. A. W.** 1991. Transcriptional regulation by cAMP. Curr. Opin. Cell Biol. **3:**953–959.
27. **Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler.** 1991. Crystallographic analysis of the interaction of the glu-

cocorticoid receptor with DNA. Nature (London) **352:**497–505.
28. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. **65:**499–560.
29. **McPherson, C. E., E.-Y. Shim, D. S. Friedman, and K. S. Zaret.** 1993. An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array. Cell **75:**387–398.
30. **Moyer, M. L., K. C. Borror, B. J. Bona, D. B. DeFranco, and S. K. Nordeen.** 1993. Modulation of cell signaling pathways can enhance or impair glucocorticoid-induced gene expression without altering the state of receptor phosphorylation. J. Biol. Chem. **268:**22933–22940.
31. **Nichols, M., F. Weih, W. Schmid, C. DeVack, K.-L. E., B. Luckow, M. Boshart, and G. Schütz.** 1992. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J. **11:**3337–3346.
32. **Nitsch, D., M. Boshart, and G. Schütz.** 1993. Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. Proc. Natl. Acad. Sci. USA **90:**5479–5483.
33. **Piña, B., U. Brüggemeier, and M. Beato.** 1990. Nucleosome positioning modulates accessibility of regulatory proteins to the mouse mammary tumor virus promoter. Cell **60:**719–731.
34. **Rangarajan, P. N., K. Umesono, and R. M. Evans.** 1992. Modulation of glucocorticoid receptor function by protein kinase A. Mol. Endocrinol. **6:** 1451–1457.
35. **Reik, A., G. Schütz, and A. F. Stewart.** 1991. Glucocorticoids are required for establishment and maintenance of an alteration in chromatin structure: induction leads to a reversible disruption of nucleosomes over an enhancer. EMBO J. **10:**2569–2576.
36. **Reik, A., F. Stewart, and G. Schütz.** 1994. Cross-talk modulation of signal transduction pathways: two mechanisms are involved in the control of tyrosine aminotransferase gene expression by phorbol esters. Mol. Endocrinol. **8:**490–497.
37. **Rigaud, G., J. Roux, R. Pictet, and T. Grange.** 1991. In vivo footprinting of the rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell **67:**977–986.
38. **Roux, J., R. Pictet, and T. Grange.** 1995. Hepatocyte nuclear factor 3 determines the amplitude of the glucocorticoid response of rat tyrosine aminotransferase gene. DNA Cell Biol. **14:**385–396.
39. **Seamon, K. B., W. Padgett, and J. W. Daly.** 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl. Acad. Sci. USA **78:**3363–3367.
40. **Smith, C. L., T. K. Archer, G. Hamlin Green, and G. L. Hager.** 1993. Newly expressed progesterone receptor cannot activate stable, replicated mouse mammary tumor virus templates but acquires transactivation potential upon continuous expression. Proc. Natl. Acad. Sci. USA **90:**11202–11206.
41. **Stauber, C., J. Altschmied, I. E. Akerblom, J. L. Marron, and P. L. Mellon.** 1992. Mutual cross-interference between glucocorticoid receptor and CREB inhibits transactivation in placental cells. New Biol. **4:**527–540.
42. **Taylor, I. C., J. L. Workman, T. J. Schuetz, and R. E. Kingston.** 1991. Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: differential function of DNA-binding domains. Genes Dev. **5:**1285–1298.
43. **Weih, F., A. F. Stewart, M. Boshart, D. Nitsch, and G. Schütz.** 1990. In vivo monitoring of a cAMP-stimulated DNA-binding activity. Genes Dev. **4:** 1437–1449.
44. **Wolffe, A. P.** 1994. The transcription of chromatin templates. Curr. Opin. Genet. Dev. **4:**245–254.
45. **Xing, L., and P. G. Quinn.** 1993. Involvement of 3′,5′-cyclic adenosine monophosphate regulatory element binding protein (CREB) in both basal and hormone-mediated expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene expression. Mol. Endocrinol. **7:**1484–1494.
46. **Zaret, K. S., and K. R. Yamamoto.** 1984. Reversible and persistent changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. Cell **38:**29–38.
47. **Zhang, H., Y.-C. Li, and A. P. Young.** 1993. Protein kinase A activation of glucocorticoid-mediated signaling in the developing retina. Proc. Natl. Acad. Sci. USA **90:**3880–3884.