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Expression Level-Dependent Contribution of Glucocorticoid Receptor Domains for Functional Interaction with STAT5

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The action of the glucocorticoid receptor (GR) on β-casein gene transcription serves as a well-studied example of a case where the action of the GR is dependent on the activity of another transcription factor, STAT5. We have investigated the domain-requirement of the GR for this synergistic response in transfection experiments employing GR mutants and CV-1 or COS-7 cells. The results were influenced by the expression levels of the GR constructs. At low expression, STAT5-dependent transactivation by mutants of the GR DNA binding domain or N-terminal transactivation domain was impaired and the antiglucocorticoid RU486 exhibited a weak agonistic activity. When the N-terminal region of the GR was exchanged with the respective domain of the progesterone receptor, STAT5-dependent transactivation was reduced at low and high expression levels. Only at high expression levels did the GR exhibit the properties of a coactivator and enhanced STAT5 activity in the absence of a functional DNA binding domain and of GR binding sites in the proximal region of the β-casein gene promoter. Furthermore, at high GR expression levels RU486 was nearly as efficient as dexamethasone in activating transcription via the STAT5 dependent β-casein gene promoter. The results reconcile the controversial issue regarding the DNA binding-independent action of the GR together with STAT5 and provide evidence that the mode of action of the GR depends not only on the type of the particular promoter at which it acts but also on the concentration of the GR. GR DNA binding function appears to be mandatory for β-casein gene expression in mammary epithelial cells, since the promoter function is completely dependent on the integrity of GR binding sites in the promoter.

Modulation of gene expression by the glucocorticoid receptor (GR) involves a combination of several mechanisms such as modulation of chromatin structure (5, 27); binding to specific DNA response elements (24); interaction with sequence-specific transcription factors, coactivators, and corepressors; and ligand-dependent alterations in the balance of corepressors and coactivators bound to the receptor (20). The actual type of mechanism employed by the receptor strongly depends on the genes that are regulated and on the cellular context. There is a differential requirement for domains in the GR, depending on the prevalent mechanism utilized by the receptor. For instance, a specific subset of GR-regulated genes is affected in transgenic mice in which the wild-type GR is replaced by a mutant defective in dimerization (26). Since this mutant is strongly impaired in binding to palindromic canonical glucocorticoid response elements (GREs) (7), it can no longer regulate genes that contain functional GREs. Consistent with the observation that in most cases the binding of the GR to DNA is a prerequisite for transactivation but not for transrepression, transgenic mice expressing the dimerization mutant predominantly exhibit a defect in the expression of genes induced by glucocorticoids.

One of the exceptions where the GR can activate transcription without contacting DNA appears to be its synergistic action with STAT5 on the β-casein gene promoter (29, 30). There, GR mutants with a defective DNA binding domain (DBD) function as transcriptional activators, indicating that in this context the GR has the potential to act as a coactivator (30). A similar mechanism was suggested for the synergy between STAT3 and the GR (37). Immunoprecipitation experiments have provided evidence for direct or indirect protein-protein interactions between STAT proteins and the GR (2, 29). These data have led to the suggestion that the GR is recruited to the transcription initiation complex via STAT proteins and that this mode of interaction is of general relevance for the cross talk between STAT factors and nuclear hormone receptors. However, several reports have indicated that the synergy between STAT proteins and the GR is promoter dependent. For instance, activation of the STAT5-dependent CIS gene is not enhanced by glucocorticoids (3, 17). In addition, promoters exhibiting transcriptional synergy show reduced or completely abolished effects of the GR when binding sites for transcription factor others than STATs are deleted or mutated (3, 13, 14, 32). A problem with a more general assessment of

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the role of the GR as a coactivator in modulating gene expression is that the demonstration of its function as a coactivator has so far been made exclusively with cells overexpressing the GR. We therefore have investigated the mechanism of synergy between the GR and STAT5 under conditions where either high or low concentrations of the GR were expressed and have systematically compared the effect of mutations introduced into various domains of the GR on the transcriptional synergy with STAT5 and on the ability of the GR to transactivate in the absence of STAT5. The results obtained indicate that the coactivator function of the GR is observed only at high expression levels. In addition, overexpressed GR mutants with defective transactivation domains still retain the capacity to transactivate in conjunction with STAT5. However, at low expression levels, GR DBD or transactivation domain mutants were similarly defective in mediating transactivation in conjunction with STAT5 and without STAT5. This latter situation appears to reflect more accurately the situation in vivo, where the high expression levels obtained in transfected COS-7 cells are usually not observed.

MATERIALS AND METHODS

Plasmids. The expression vectors for the prolactin receptor, STAT5a, the C-terminally deleted form of STAT5a, and the chloramphenicol acetyltransferase (CAT) and luciferase reporter genes under the control of the rat β-casein gene promoter (sequence from −344 to −1) have been described (14, 19). The pMMTV-CAT construct was created by inserting a fragment encompassing the sequences from −1187 to +102 of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) into the *Pst*I-*Bam*HI sites of pBLCAT3 (18). The pMMTV-LUC construct was obtained by inserting a *Hin*dIII-*Bgl*II fragment from pMMTV-CAT with the MMTV-LTR into the *Hin*dIII-*Bgl*II sites of pGL3basic (Promega). The GR expression vectors employed were as follows. The first was the rat cytomegalovirus-based GR expression vector pSTC3-GR3-795 (12), which was used for the experiments described in Fig. 1. It also served as a wild-type (wt) control for the experiment in Fig. 6E with the GR mutants CS1, CS2, and CS1/CD (12). The AF-1 deletion mutant GR τ1 of the human GR and its parental wt construct have been described previously (10). It encompasses a deletion of the sequence encoding amino acids 77 to 262. The D-loop mutants GR(D4X) and A458T have been published previously (8). The GR Δ DBD construct lacks the coding region of amino acids 428 to 490. The above three mutants are derived from the parental Rous sarcoma virus-based human GR construct phGRSB (8), which was used as a wild-type control vector in the experiments with these mutants. The rat GR mutants K461A and R466A have been described previously (28). They are derived from a Rous sarcoma virus-based rat GR expression vector, which was used as a control in the experiments described in Fig. 2D and E. The constructs with the mutations R488Q, K490E, N491A, and LS7 in the second Zn^{2+} finger and their parental human wt vector are as described previously (16). The structures of the expression vectors of the human androgen receptor (11), the human progesterone receptor (15), and progesterone receptor-GR chimeric receptors (15) have been published. The high-mobility-group type 1 (HMG-1) expression vector was constructed by inserting full-length rat HMG-1 (924 bp) into the *Bam*HI site of pCDNA I/AMP (InVitrogen). The expression vectors used for normalizing the transfection efficiency were pAGLuE5 (14) and the *Renilla* luciferase expression vector pRL-SV40 (Promega). The β-casein gene promoter luciferase constructs with the mutation in the GR or STAT5 binding sites were created by excision of the *Bam*HI fragments of the respective CAT constructs (13) and cloning them into the *Bgl*II site of the luciferase expression vector pGL3 basic (Promega).

Cell culture and transfection. CV-1 and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For determining the transactivation efficiency by transient transfections, cells were split into six-well dishes at a cell density of 1×10^5 to 2×10^5 cells per well. Some of the experiments with COS-7 cells were also performed in 24-well dishes and 0.5×10^5 to 1×10^5 cells per well. The next day, transfections were carried out using the calcium phosphate coprecipitation technique as described previously (35). The total amount of DNA transfected was adjusted to the area of the culture dishes used; it was 3.3 or 0.83 μg of DNA per well for a 6-well dish or 24-well dish, respectively. To allow comparison of the relative amount of individual plasmid DNA transfected in the experiments using different culture dishes, the amount of plasmid DNA (in micrograms) indicated in the legend of each figure is consistently described for a total of 20 μg DNA transfected. At 18 h after the transfection, precipitates were washed off and replaced with fresh medium. Hormones were included at this time point when required, and extracts were prepared 24 h later.

Protein expression. GR expression was analyzed by using cell extracts prepared from HC11 cells or transfected CV-1 or COS-7 cells by homogenizing the cell pellets with 40 strokes with an A pestle in a 1-ml Dounce tissue grinder (Wheaton, Millville, N.J.) in 200 μl of 10 mM sodium phosphate (pH 7.4)–1 mM EDTA–1 mM dithiothreitol–10% glycerol–400 mM KCl supplemented with 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 1 μM pepstatin, 0.1 mM phenylmethylsulfonyl flouride, 5 μM NaF, and 0.5 μg of ocadaic acid per ml and centrifugation at 265,000 × *g* for 40 min. Samples were applied to NuPAGE 4 to 12% Bis-Tris gels (Novex), and the proteins were transferred to polyvinylidene diflouride membranes. GR-specific antibodies used for immunodetection by the enhanced chemiluminescence protocol of Amersham were the rabbit polyclonal antibodies M-20 and P-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) and the mouse monoclonal antibody 250 (referred to as #7 in reference 25).

CAT, luciferase, and *Renilla* assays. To measure the GR-dependent transactivation via the β-casein CAT reporter, transfection efficiency was normalized by determining the luciferase activity expressed by the cotransfected pAGLuE5, as described previously (14). To normalize transactivation via the luciferase reporter, the *Renilla* luciferase activity expressed by the cotransfected pRL-SV40 was measured. Details for the reporter assays were as in references 14 and 19.

RESULTS

The degree of synergy between GR and STAT5 is dependent on the GR expression level. A synergistic interaction between the GR and STAT5 can be studied in cotransfection experiments employing the β-casein gene promoter (14, 29), which contains binding sites for the GR and STAT5 (13, 14). This assay has so far been exclusively performed with COS-7 cells by cotransfecting expression vectors for the GR, STAT5, and the prolactin receptor as an activator of STAT5. Since transfected plasmids containing the simian virus 40 (SV40) origin of replication are replicated in this cell line, high expression levels are obtained, which usually greatly exceed the levels of the endogenous genes. We were therefore interested whether the parental CV-1 cells, which do not replicate plasmids and therefore express lower levels of the transfected genes than COS-7 cells, also exhibit functional synergy. As shown in Fig. 1A, this is indeed the case. At 40 ng of GR expression vector transfected, the induction levels by the glucocorticoid dexamethasone together with prolactin were enhanced in comparison to those by prolactin alone to a similar extent in CV-1 and COS-7 cells (compare the induction levels achieved at 40 ng of transfected GR construct in Fig. 1A and B). At 200 ng of transfected GR construct, the GR significantly augmented the response to prolactin even in the absence of dexamethasone in COS-7 cells but not in CV-1 cells (Fig. 1B). Such a hormone-independent activity of the GR in COS-7 cells has already been observed in early studies of its action on the MMTV LTR (6). To estimate the expression levels of the transfected rat GR receptor construct in CV-1 cells and COS-7 cells, immunoblotting experiments were performed. Since only an average of 3 to 5% of cells were transfected by our transfection procedure, the analysis was performed in the background of at least 95% of untransfected cells. It was thus necessary to overexpress the GR 20- to 30-fold to obtain the same amount of transfected GR as of the endogenous GR in untransfected cells. This was achieved by transfection of 10 μg of GR plasmid. As shown in Fig. 1C, the P-20 GR antibody, which is equally reactive with rat, mouse, and human GR, recognized similar levels of the

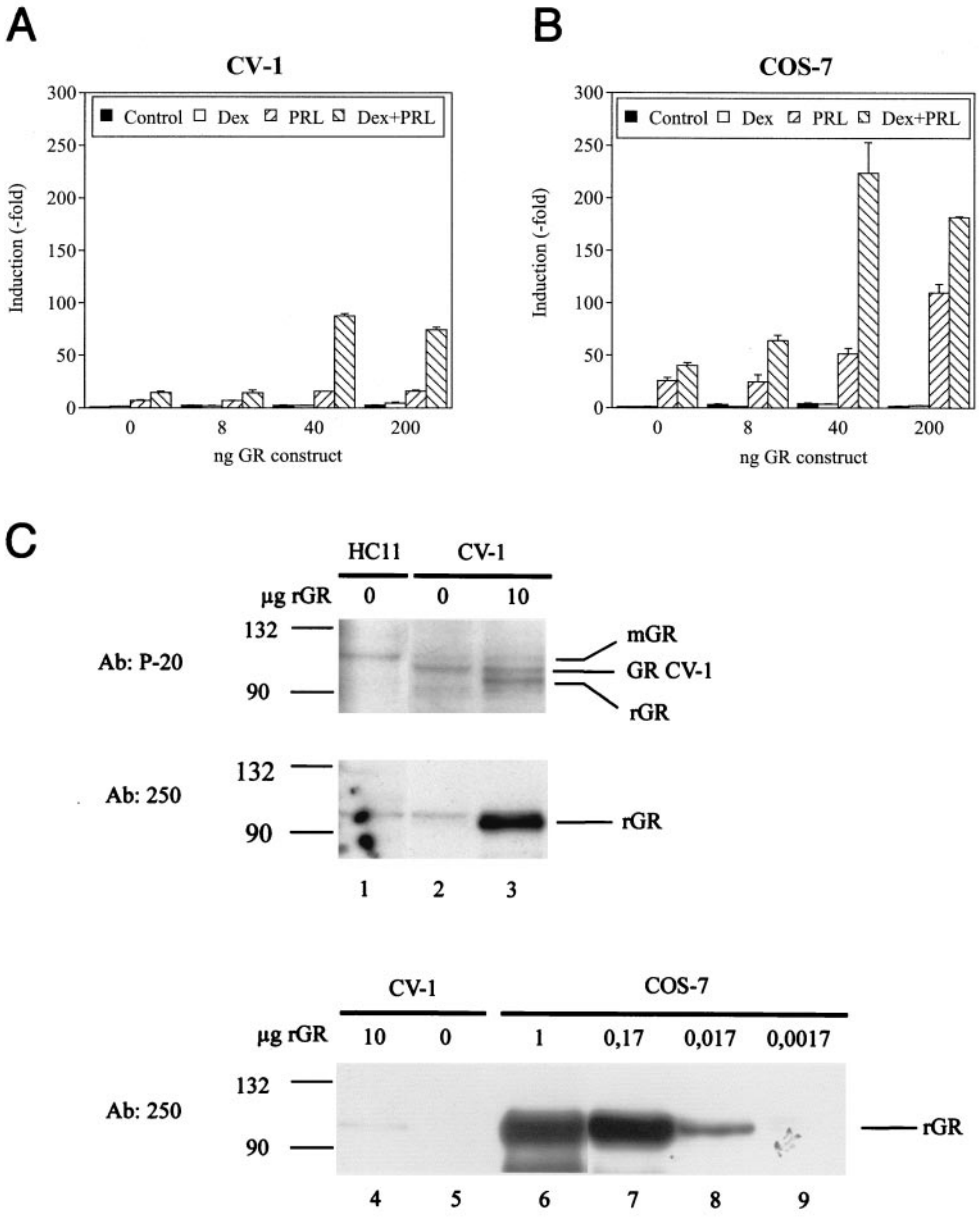

FIG. 1. Synergy between GR and STAT5 at different expression levels in CV-1 and COS-7 cells. (A and B) CV-1 (A) or COS-7 (B) cells were transiently transfected with the indicated amount of the rat GR expression vector pSTC-GR3-795, 4 μg of the mouse prolactin receptor expression vector pcDNAI-PRLR, 4 μg of the mouse STAT5a expression vector pECEStat5a, 8 μg of the β-casein gene promoter luciferase reporter pβc(−344/−1) LUC, and 0.18 μg of the SV40 *Renilla* construct pRL-SV40, and Bluescript to adjust the total DNA of 20 μg. Cells were stimulated with dexamethasone (0.1 μM) and/or prolactin (5 μg/ml) and extracts were prepared and analyzed for luciferase and *Renilla* activity as described in Materials and Methods. Transactivation activities were normalized to *Renilla* activity. Results are represented as relative induction in hormone-treated versus untreated cells not transfected with the GR construct. The means and standard errors of two separate hormone inductions are shown. Control, not hormone treated; Dex, dexamethasone treated; PRL, prolactin treated; Dex + PRL, dexamethasone and prolactin treated. (C) Expression levels of the GR were determined by immunoblotting experiments. Extracts of HC11 mammary epithelial cells (lane 1), CV-1 cells (lanes 2–5), and COS-7 cells (lanes 6 to 9) were prepared as described in Materials and Methods. In lanes 3, 4, and 6 to 9, cells were transfected with the amount of the rat GR construct per 20 μg of total DNA as indicated at the top of each lane. In lanes 2 and 5, extracts from mock-transfected cells were loaded. The positions of molecular mass markers are indicated on the left of each panel in kilodaltons. The positions of the exogenously expressed rat GR (rGR), the endogenous mouse GR (mGR), and a band corresponding to nonfunctional monkey GR protein (GR CV-1 [9]) are indicated on the right.

endogenous mouse GR in HC11 mouse mammary epithelial cells (lane 1), the endogenous, nonfunctional monkey GR in CV-1 cells (lane 2), and the transfected rat GR (lane 3). It is thus reasonable to estimate that at the much lower concentrations of the transfected GR used in the experiments in Fig. 1A, GR levels close to or below to the endogenous levels are achieved. In COS-7 cells, expression of similar levels of GR to those in CV-1 cells required only >100-fold-lower concentra-

tions of transfected DNA (Fig. 1C, compare lanes 4 and lanes 6 to 9). At 0.017 μg of GR construct transfected into COS-7 cells (lane 8), even higher expression levels were achieved than in CV-1 cells transfected with 10 μg of DNA (lanes 4). In the rest of the experiments presented here, we utilized both CV-1 and COS-7 cells for studying GR constructs with specific mutations and deletions of functional domains to assess the effect on their synergy with STAT5 over a wide range of expression levels. Since COS-7 cells and the parental CV-1 cells not only differ in the expression levels of the transfected GR constructs but also might change the GR function due to the presence and absence of large-T expression, we also compared the effect of GR in a single cell line by employing different GR concentrations for transfection.

A GR construct lacking the DBD is effective in mediating the synergy with STAT5 at high expression levels. It has been recently demonstrated that in COS-7 cells a GR lacking the DBD is able to mediate the synergy with STAT5 (30). This was taken as evidence of a lack of requirement for DNA binding of the GR when activating transcription in conjunction with STAT5. As shown in Fig. 2A, this unusual property of the GR DBD mutant is evident only in COS-7 cells at high concentrations of transfected GR. At the highest concentration employed, the transactivation by the mutant was even higher than that by the wt GR. This effect was specific for activation of the β-casein gene promoter in combination with STAT5 and was not observed with the MMTV LTR stimulated with the GR in the absence of STAT5 (compare Fig. 2A and B). Although the underlying mechanism of this effect remains unclear, it is possible that under conditions of high expression, a GR with intact DNA binding function has a negative effect on transactivation. Such a negative effect might result from competition with binding of other transcription factors required for β-casein gene transcription. Both the wt receptor and the mutant were expressed at roughly the same levels (Fig. 2F, compare lanes 1 and 2) when equal amounts of expression vectors were transfected. In CV-1 cells and at low concentrations in COS-7 cells (Fig. 2A), the DBD mutant was not able to enhance transcription of the β-casein gene promoter under conditions wherein a clear synergy with STAT5 was observed with the wt GR. The results indicate that the DBD mutant can act as a coactivator together with STAT5 at high expression levels but is defective in this property at low expression, whereas the wt GR is sufficient to promote synergy with STAT5 at both low and high expression levels.

Expression of HMG-1 enhances the effect of wt GR but not of a GR construct without DBD to promote the synergy with STAT5. The GR binding sites in the β-casein gene promoter mapped by in vitro binding studies (34) are half-palindromic suboptimal binding sites for which the GR has a lower affinity than for canonical GR binding sites (14, 34). Recently, HMG-1 and HMG-2 were shown to enhance the binding of the steroid hormone receptors to their sites on the MMTV LTR in vitro and to concomitantly increase transactivation by transiently expressed steroid hormone receptors (1). If binding of the GR to the β-casein gene promoter is required to induce transcription, one might expect that HMG proteins would also be able to exert a similar effect as on the MMTV LTR. We thus tested the potential of HMG proteins to augment the transcriptional response of the GR and STAT5 on the β-casein gene promoter. As shown in Fig. 2C, expression of HMG-1 did indeed increase transactivation over a broad concentration range of transfected GR in COS-7 cells. Transfection of HMG-2 had the same effect as transfection of HMG-1 (data not shown). The effect of HMG-1 was selective for the GR construct with an intact DBD and was not observed in conjunction with the DBD mutant (Fig. 2C, compare the last two bars). HMG did not enhance transactivation of the promoter mediated by STAT5 alone (Fig. 2C, compare the first two bars). The results are consistent with the notion that HMG acts on β-casein gene transcription by enhancing the DNA binding of the GR to DNA. On the other hand, the lack of an HMG effect on transactivation by the DBD mutant provides further evidence for a DNA binding-independent action of overexpressed GR on β-casein gene transcription and is also in accordance with a recent report that the DBD is the minimal region of steroid receptors stimulated by HMG-1 and HMG-2 (21).

Mutants of amino acids required for contact to DNA in the major groove are defective in promoting synergy with STAT5 in CV-1 cells. The study of the GR DBD by structural analysis and functional studies with mutants has made possible the definition of regions required for distinct functions such as DNA binding (22, 28), dimerization (7), and transrepression (8, 16). Since dimerization is a prerequisite for binding to palindromic GR recognition sites, dimerization mutants are in most cases also defective in DNA binding to such sites. We have analyzed a panel of mutants with mutations in distinct regions of the DBD for their capability to promote transcription of the β-casein gene promoter in conjunction with STAT5. In control experiments, the STAT5-independent transactivation activity of these mutants was tested with the MMTV LTR. For the DBD mutants employed in this study, the positions of the mutated amino acids within the structure of the two Zn^{2+} fingers are shown schematically by circles in Fig. 3.

Structural analysis of the GR DBD dimers bound to their palindromic sites in the DNA has revealed that the lysine at position 461 and the arginine at position 466 of the rat receptor are the critical residues of the DBD involved in contacting specific bases in the major groove. Mutants containing altered amino acids at these sites exhibit altered (K461A) or defective (R466A) DNA binding (28) to canonical GREs. As shown in Fig. 2D, mutants transiently expressed in CV-1 cells were strongly impaired in their transactivation via either the β-casein gene promoter or the MMTV LTR compared to the wt GR. This was not due to protein instability of the mutants as determined by Western blotting (data not shown). However, in COS-7 cells transfected with high concentrations of the mutant R466A, a significant degree of synergy with STAT5 was observed in the experiments employing the β-casein gene promoter but not with the MMTV LTR in the absence of STAT5 (Fig. 2E). Thus, under conditions where the GR is overexpressed, either DNA binding of the GR does not appear to be a prerequisite for transactivation via the β-casein gene promoter in the presence of STAT5 or the DBD mutants still exhibit binding to the noncanonical GREs present in the β-casein gene promoter.

The requirement for integrity of GR DNA binding sites in the β-casein gene promoter is relaxed at high GR expression levels. Previously, we have demonstrated the functional role of GR binding sites in the β-casein gene promoter for induction

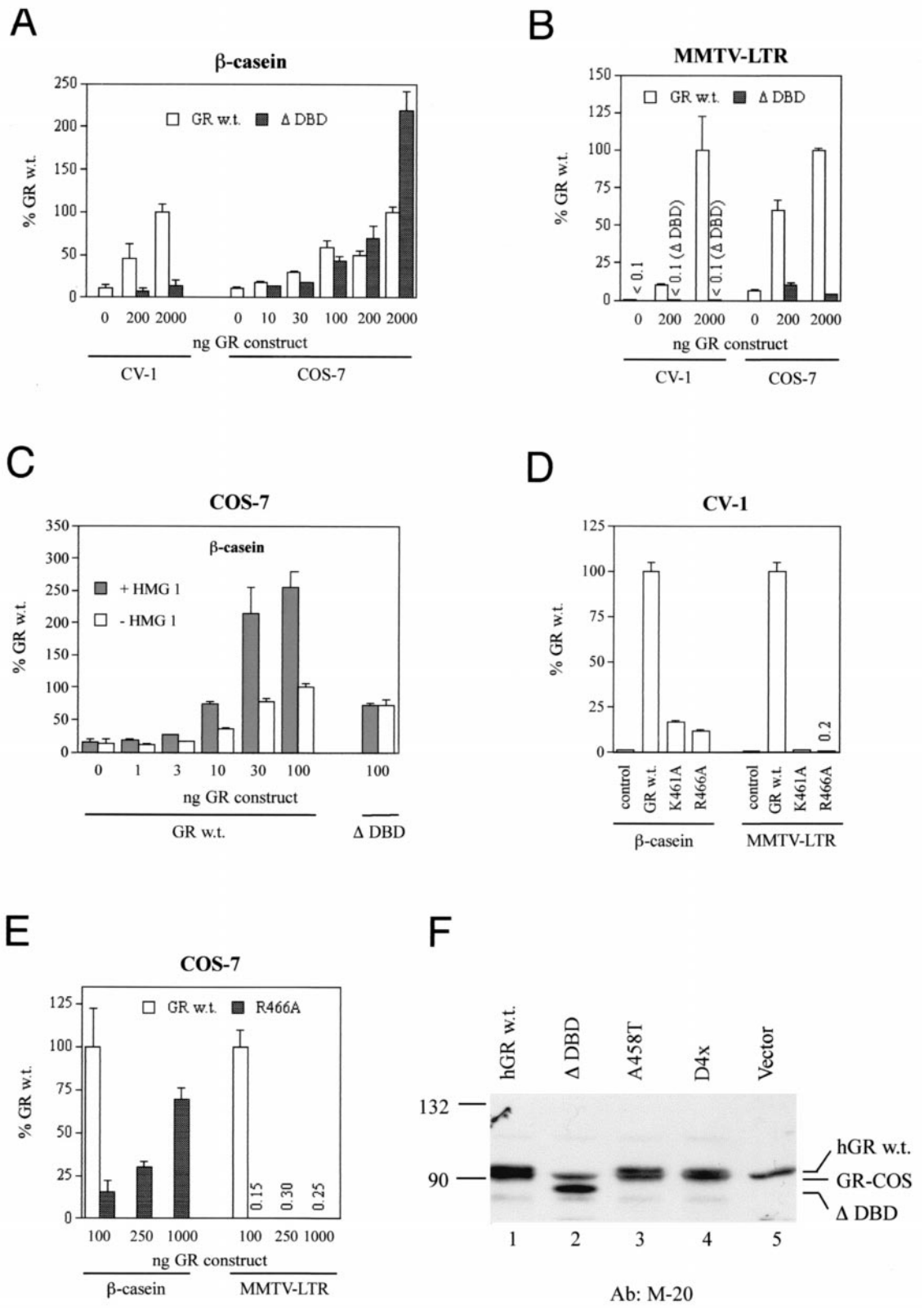

FIG. 2. Transactivation mediated by DNA binding-defective GR constructs. Cells were transfected with either wt GR (GR w.t.), a mutant GR lacking the DBD (Δ DBD), or GR mutants with mutations in amino acids required for the contact of DNA in the major groove (K461A and R466A [Fig. 3 shows the positions of mutations within the DBD]). (A to C and E) The amount of GR transfected per 20 μg total DNA is indicated at the bottom. (D). A 2-μg portion of GR construct was transfected. To assess the activity of the GR on the β-casein gene promoter (β-casein [A and C to E]), the GR constructs were cotransfected with expression vectors for the prolactin receptor, STAT5A, *Renilla*, and with the β-casein luciferase reporter as described in Fig. 1, and the cells were stimulated with both prolactin and dexamethasone. To determine the activity of the

of promoter activity (14). This was shown for both COS-7 cells expressing exogenous GR and HC11 cells expressing endogenous GR. Since, as shown in Fig. 2, a GR mutant lacking the DBD was functional at high expression levels, one would postulate that this mutant should also be able to act on a promoter lacking GR binding sites under these conditions. We therefore compared transactivation mediated by the GR wt receptor or by the GR-DBD mutant on the β-casein gene promoter (Fig. 4A) and on a promoter with mutated GR binding sites (Fig. 4B; the three proximal GR binding sites are mutated in this construct) in transfected COS-7 cells. Experiments were performed in the presence of activated STAT5 and at different concentrations of the GR constructs. As a control, the effect of GR and STAT5 on a promoter lacking the proximal STAT5 binding sites was evaluated (Fig. 4C). At 10 and 100 ng of transfected DNA, the wt GR construct was more effective than the GR DBD mutant, similar to the case already shown in Fig. 2A, whereas it remained inactive with a promoter lacking GR sites. At 1,000 ng of DNA, both the GR wt construct and the DBD mutant enhanced transcription form both promoter constructs, indicating that, indeed, at high GR expression levels the requirement for DNA binding of the GR is relaxed. Unexpectedly, the GR DBD mutant was able to transactivate the β-casein gene promoter construct with mutated GR binding sites even more efficiently than was the wt GR. This could possibly indicate a repressive function of the GR mediated by DNA binding which is lost in the DBD mutant. The construct with a mutation in the STAT5 binding site was strongly impaired in transactivation by the GR wt and the DBD mutant at all concentrations of GR constructs employed. Thus, binding of STAT5 to the β-casein gene promoter is a prerequisite for the transactivating function of the GR on this promoter, as shown previously (14). This requirement cannot be overcome by increased expression levels of the GR.

Mutants with mutations in the dimerization interface exhibit partially reduced synergy with STAT5. A point mutation (A458T) and a 4-amino-acid exchange in the D-loop (D4×) of the second Zn^{2+} finger selectively affect DNA binding and thereby impede the function of the GR as a transactivator. Transgenic mice with a targeted mutation of this domain still retain some of the GR-dependent physiological functions (26, 33). It has been speculated that these residual functions are mediated via the GR acting as a transcriptional repressor rather than a transactivator. As shown in Fig. 5A, mutants with mutations of the D loop were strongly impaired in their ability to transactivate via the MMTV LTR in transfected CV-1 and COS-7 cells compared with the wt GR (Fig. 5A and B, right-hand side). The residual activity observed with the MMTV LTR indicates that their DNA binding activity is not completely defective. Expression levels of wild-type and mutated constructs were similar, as determined by Western blotting (Fig. 2F, lanes 1, 3, and 4). When tested together with STAT5 and the β-casein gene promoter reporter construct, the A458T mutation was not significantly less effective than the wt construct in CV-1 cells and in COS-7 cells at the lowest concentration of plasmid DNA used (Fig. 5A, left side, and Fig. 5B, columns with 30 ng of GR transfected together with the β-casein gene promoter). However, in COS-7 cells at 300 or 3,000 ng of GR, the functionality of the A458T mutant was reduced for both STAT5-dependent and independent transactivation (Fig. 5B). The D4× mutant exhibited impaired transactivation in both cell lines. Thus, an intact dimerization interface is required for optimal function of the GR as a synergistic activator of STAT5.

Amino acids in the second half of the Zn^{2+} finger are required for synergy with STAT5. The amino acids 488 and 490 in the second half of Zn^{2+} finger 2 are required for transactivation and for mediation of the repression of transcription by RelA (16). As shown in Fig. 5C and D, analysis of mutants with mutations R488Q and K490E in this region provided evidence that these amino acids are also important for mediating synergy with STAT5. However, other mutants with mutations in that region, which are not defective in mediating the repression of RelA (N491A and LS7 [16]), were defective together with STAT5, indicating that the GR domain requirements for interaction between the GR and STAT5 and between the GR and RelA are different. The relative degree of functional defects of the different mutants was the same for their effects on synergy with STAT5 (Fig. 5C and D, left-hand side) and on transactivation via the MMTV LTR without STAT5 (Fig. 5C and D, right-hand side) (16). The strongest effect at all concentrations of plasmids in both CV-1 and COS-7 cells was observed with the LS7 double mutant. The K490E mutant behaved strikingly differently when expressed at low and high concentrations: at low concentrations it was the most strongly impaired mutant, whereas at the highest concentration (2,000 ng transfected into COS-7 cells) it was even more effective than the wt GR. These results are reminiscent of those observed for the mutant with the deletion of the entire DBD (Fig. 2A).

Redundant function of transactivation domains in the GR and STAT5 for activation of β-casein gene transcription. Transactivation domains have been localized in the N-terminal and C-terminal regions of the GR (20) and in the C-terminal region of STAT5 (23). We have tested the effect of deletions of the N-terminal GR transactivation domain AF-1 (also termed τ1) and the STAT5 transactivation domain on activation of β-casein gene transcription. Deletion of τ1 led to a reduction of transactivation by 70 to 86% in CV-1 cells (Fig. 6A, left-hand side). By contrast, in COS-7 cells the deleted GR resulted in transactivation efficiencies that were essentially the same as

GR in the absence of STAT5 (MMTV-LTR [B, D, and E]), the GR constructs were cotransfected with 8 μg of the MMTV-LTR LUC reporter, 0.18 μg of the SV40 *Renilla* construct pRL-SV40, and Bluescript to adjust the total DNA to 20 μg, and the cells were stimulated with dexamethasone. (C) The effect of HMG 1 expression was determined by the addition of 2.4 μg of CMV-HMG 1 expression vector (+HMG 1) or 2.4 μg of Bluescript (-HMG 1) to the transfection mixture. Either CV-1 or COS-7 cells were transfected, as indicated in panels A to E. Luciferase activity was normalized to *Renilla* activity. Results are expressed as the percentage activity of luciferase activity of wt GR transfected at the highest concentration (% GR w.t.) and are shown as the mean and standard error of three to five independent transfections. (F) Expression analysis of transfected GR constructs was performed with COS-7 cells transfected with 10 μg of the indicated GR construct and analyzed for GR expression with the M-20 antibody. The positions of the bands corresponding to the human GR w.t. (hGR w.t.), the endogenous nonfunctional GR protein (GR-COS), and the DBD mutant (Δ DBD) are indicated on the right.

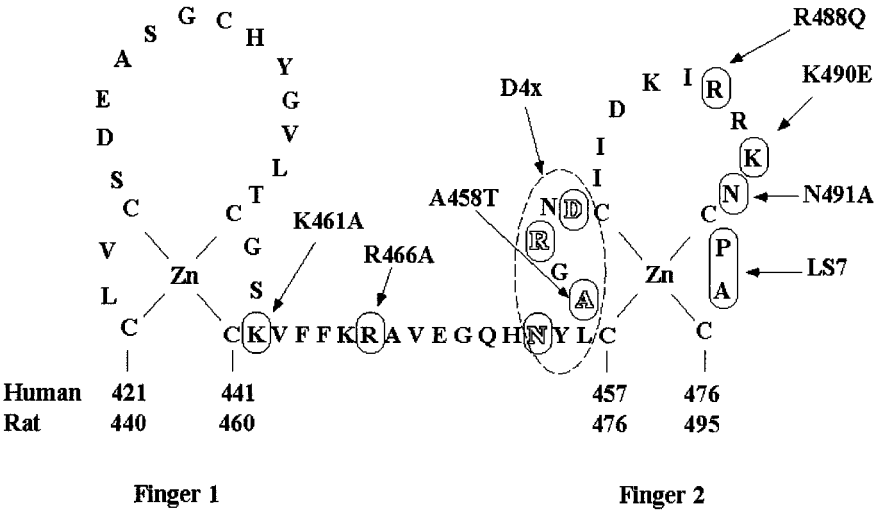

FIG. 3. Schematic outline of the DBD mutants employed. Amino acids mutated in the constructs employed in the experiments in Fig. 2 and 4 are highlighted by circles. Arrows point from the name of the respective mutants to the mutations. The amino acids mutated in the D4× mutant of the second Zn^{2+} finger are outlined. In each finger the positions of the first and fourth cysteine residues of the human and rat GR-DBD are given below the schema.

observed with the wt GR (Fig. 6B, left-hand side), indicating that under the conditions of overexpression, the transactivation mediated by the C terminus of the GR is sufficient for maximum response. By contrast, in the absence of STAT5 and with the MMTV LTR, the τ1 mutant was similarly defective at high and low expression levels (Fig. 6A and B, right-hand side). A possible explanation for the lack of requirement of the τ1 domain at high expression levels in conjunction with STAT5 is a redundant function of STAT5 and GR transactivation domains under these conditions. This hypothesis was tested by investigating the transactivation by a STAT5 deletion mutant lacking the C-terminal transactivation domain (STAT5A Δτ; Fig. 6B, middle). In combination with wt GR, the STAT5 Δτ mutant was as effective as wt STAT5. However, when STAT5 Δτ was combined with GR Δτ1, an 83% reduction of transactivation was observed. These results support the notion that the presence of the transactivation domains of either STAT5 or GR τ1 are sufficient and that only deletion of both domains results in severe impairment of transactivation.

In the same set of experiments we evaluated the effect of RU486, a glucocorticoid and progesterone receptor antagonist, on transcription mediated by STAT5 and GR. In CV-1 cells, RU486 was a partial agonist with 32% activity in comparison to dexamethasone (Fig. 6C), whereas in COS-7 cells overexpressing the GR, RU486 and dexamethasone were equally efficient (Fig. 6D). A possible explanation for this unusually strong agonistic effect of RU486 in combination with activated STAT5 is that at high GR expression levels, the ligand-dependent AF-2-mediated transactivation is not required and the ligand-independent transactivation domain τ1, together with STAT5 τ, is sufficient for maximum response. Accordingly, the RU486 liganded receptor was not fully active when the GR τ1 was absent. Under these conditions, the full agonism by RU486 was strongly reduced (18% of the effect of dexamethasone [Fig. 6D, compare the last two columns]).

We next investigated whether the observation of apparently similar effects of RU486 and dexamethasone in COS-7 cells overexpressing the GR is simply due to the fact that the overexpressed GR functions in a ligand-independent fashion. For that purpose, three different GR mutants CS1, CS2, and CS1/CD, which all have selective defects in binding of dexamethasone but not of RU486 (12), were investigated. As shown in Fig. 6E, all of these mutants still retained the capability to transactivate via the β-casein gene promoter together with STAT5 when RU486 was used but were inactive with dexamethasone. Experiments performed in the absence of either dexamethasone or RU486 (Fig. 6E, no ligand) exhibited low transactivation, indicating that ligand-independent activation of the expressed GR constructs did not account for the observed effects. Thus, we have to postulate that occupation of the ligand binding site of the GR is essential for transactivation. However, at high GR expression levels, it is irrelevant whether the receptor is liganded by agonists or partial agonists such as RU486. This finding was specific for GR in combination with STAT5, since in control experiments performed with the MMTV LTR and without STAT5, the agonistic effect of RU486 was much lower than that of dexamethasone (6%) (Fig. 6E, right-hand side).

The N-terminal region of the GR is required for efficient synergy with STAT5. The GR belongs to a subgroup of steroid hormone receptors together with the progesterone receptor (PR), the androgen receptor (AR), and the mineralocorticoid receptor, which bind to the same consensus core sequence in the DNA and have a high degree of sequence similarity in the DBD. However, several studies have reported that despite these similarities, members of this subgroup are able to discriminate between different response elements in vivo. This has been attributed to differential recognition of sequences in the DNA adjacent to the core recognition motif by the receptors (24) and/or to distinct functions in chromatin remodeling (5). We have compared the ability of the GR, PR, and AR to transactivate together with STAT5 and the β-casein gene pro-

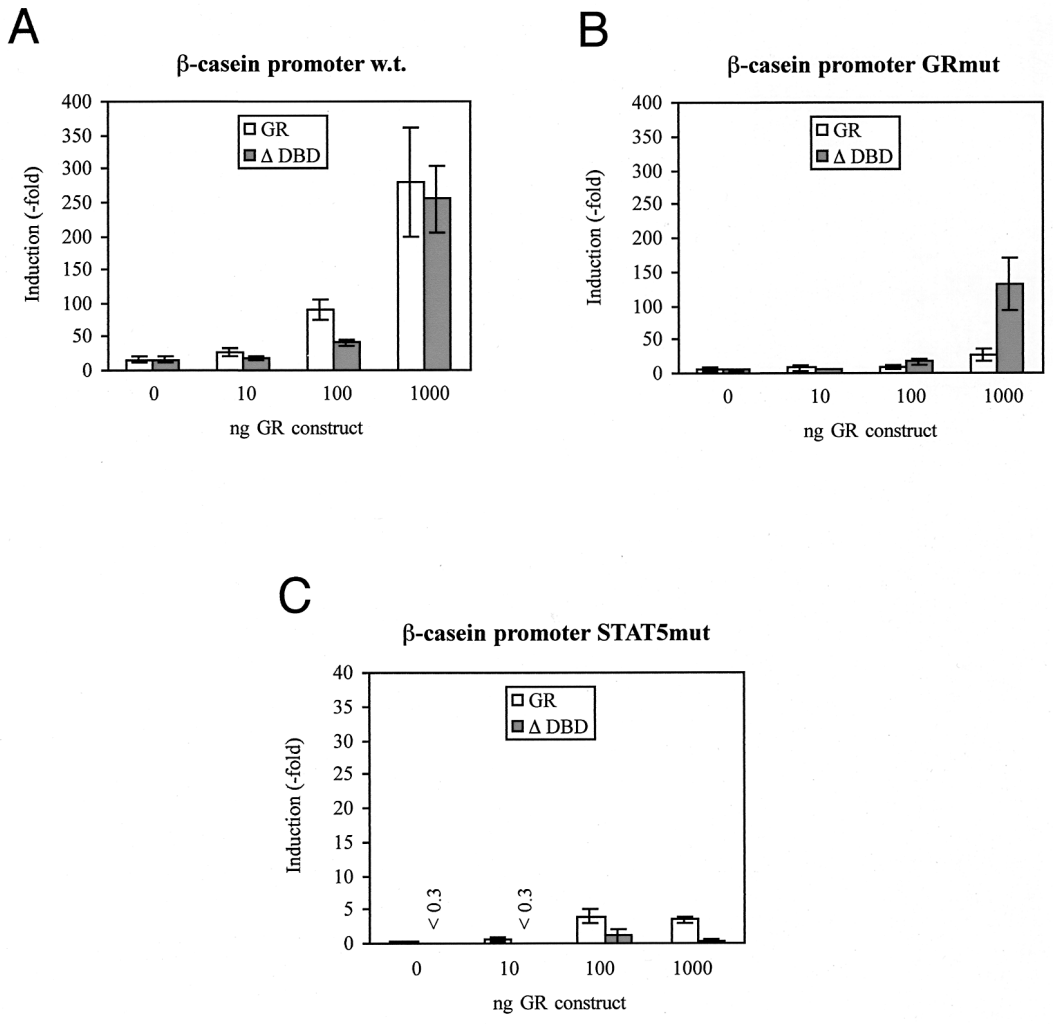

FIG. 4. Effect of β-casein gene promoter mutations on transactivation by the Δ DBD mutant. COS-7 cells were transfected with the indicated amount of GR construct. Either the GR wt construct or the Δ DBD mutant was employed, together with PRL-R, STAT5A, SV40 *Renilla* constructs, and the β-casein gene promoter construct at the same concentrations as in the experiment in Fig. 1B. (A) The unmutated −344/−1 β-casein gene promoter construct was employed. (B) The mutant of the proximal GR half sites GRc, GRd, and GRe (14) was used. (C) The mutant of the proximal STAT5 site was used. The scale of the *y* axis is reduced 10-fold to visualize the low activation levels. The results are shown as mean and standard error of four independent experiments.

moter as a template. As a control, the efficiency of transactivation in the absence of STAT5 was investigated with the MMTV LTR. Transfection experiments employing either CV-1 or COS-7 cells revealed that among these steroid receptors, only the GR was able to substantially synergize with STAT5 (Fig. 7). We further tested whether the N-terminal or C-terminal half of the GR is responsible for the more efficient function as a transactivator in comparison to the PR. Two chimeric receptors consisting of either the N-terminal half of the PR and the C terminus of the GR together with the DBD (PRN/GRC) or the converse combination (GRN/PRC) were used. Whereas these constructs exhibited almost the same efficiency to transactivate via the MMTV LTR, they exhibited distinct behaviors in combination with STAT5. The GRN/PRC chimera was 5-fold more efficient in CV-1 cells and 10-fold more efficient in COS-7 cells than was the PRN/GRC construct. Thus, the N terminus of the GR appears to contain critical regions for transactivation in combination with STAT5, which are not present in the same region of the PR. On the other hand, the C-terminal region of the PR and GR can be exchanged without strongly affecting the synergy with STAT5.

DISCUSSION

In the present study we have analyzed the mode of interaction between GR and STAT5 in promoting their synergistic effects on the β-casein gene promoter as a paradigm for the cross talk between steroid hormone receptors and members of the family of signal transducers and activators of transcription. Our results have elucidated the following: (i) specific requirements for the GR and STAT5 that are essential under high and low expression levels of the GR, (ii) redundant functional domains of the GR that are not necessary for transactivation at high expression levels, (iii) a region of the GR important for synergy that cannot adequately be replaced by the PR, and (iv)

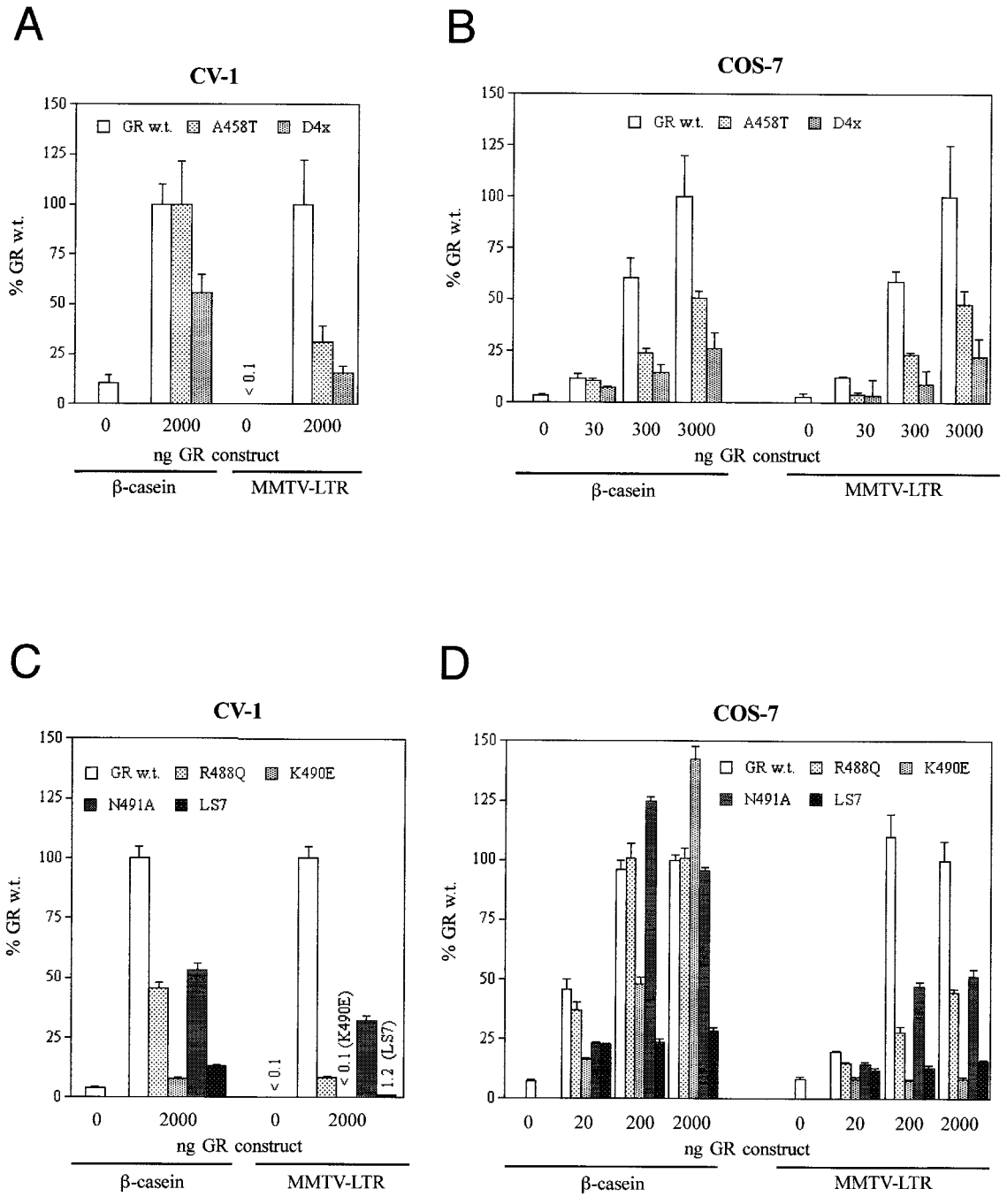

FIG. 5. Effect of the mutants with mutations of the second Zn^{2+} finger. The amounts of parental GR w.t constructs, D-loop mutants (A458T, D4× [A and B]) and mutants with mutations in the distal half of the finger (R488Q, K490E, N491A, LS7 [C and D]) per 20 μg of transfected DNA are shown. Either CV-1 cells (A and C) or COS-7 cells (B and C) were used. The conditions of transfections were as in Fig. 2, with the exception of the experiments in panel B. There, 8 μg of the β-casein CAT reporter and 0.4 μg of SV40 luciferase reporter (β-casein; left-hand side) or 8 μg of the MMTV-CAT reporter and 0.14 μg of the SV40 luciferase construct (MMTV-LTR; right-hand side) was used as the reporter construct. Hormone inductions were as in Fig. 2. Results are shown as the percentage of normalized luciferase activity (A, C, and D) or CAT activity (B), measured in cells transfected with the highest concentration of GR w.t. employed. The mean and standard error of three to four independent transfections are shown in each panel.

the potential of the glucocorticoid antagonist RU486 to act as an agonist together with STAT5.

Since the results obtained were in many cases strongly dependent on the expression levels of the GR, the experiments were performed with either CV-1 or COS-7 cells transiently transfected with different concentrations of GR constructs, thereby allowing expression over a wide range. A synopsis of the domain requirements of the GR and STAT5 at different expression levels of the GR is shown in Fig. 8. At low GR concentrations, interactions between GR and STAT5 depend

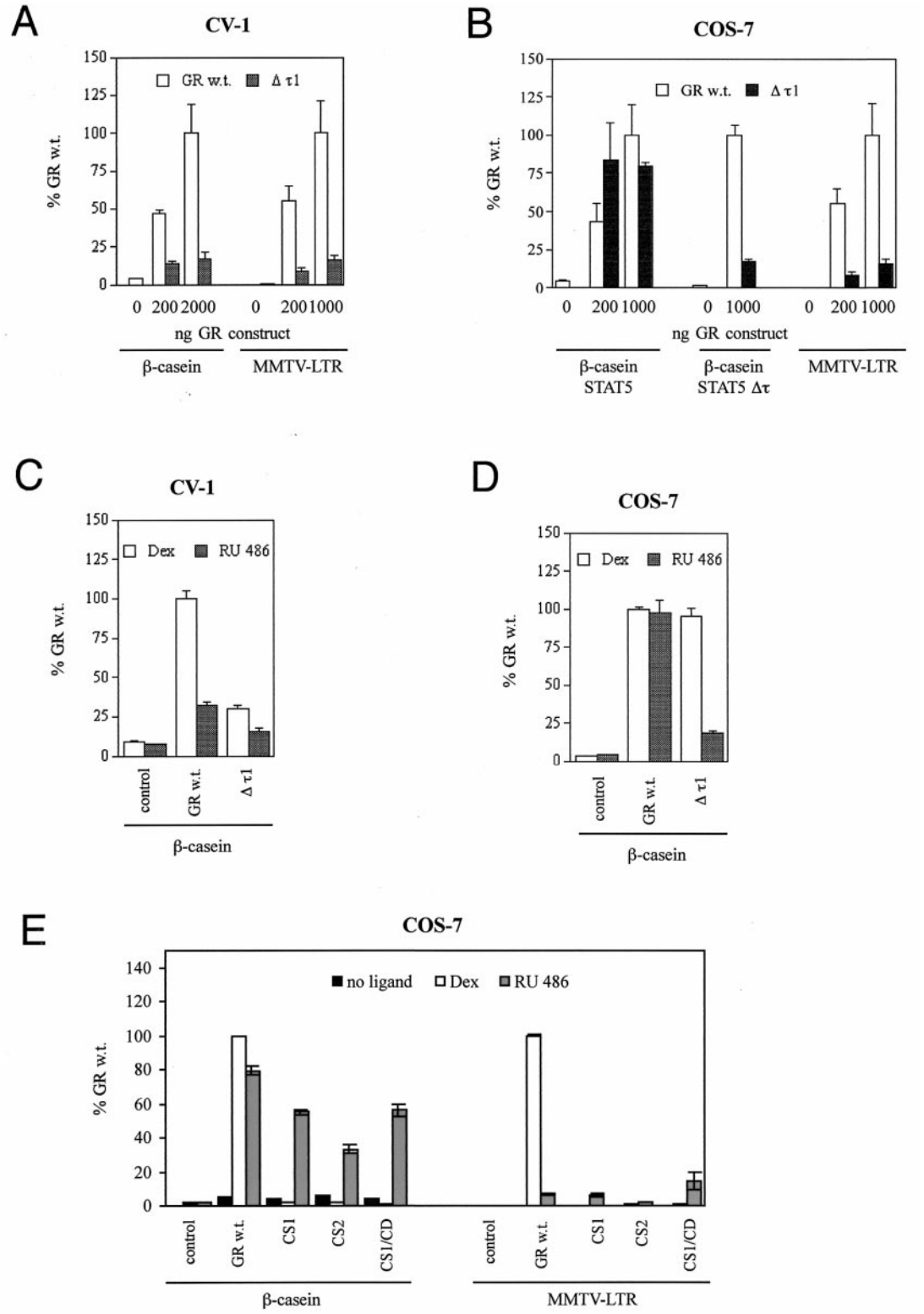

FIG. 6. Effects of mutations of the transactivation domain τ1 and of RU486. The parental wt GR construct (GR w.t.), a GR mutant with deleted AF-1 domain (Δ τ1), or mutants with mutations in the ligand binding domain inhibiting the binding of dexamethasone but not RU486 (CS1, CS2, and CS1/CD) were used as indicated at the bottom of each panel. The amount of GR construct transfected per 20 μg of total DNA is indicated at the bottom of panels A and B. It was 2 μg in panels C and D, and 1 μg in panel E. In the middle part of panel B, a STAT5A expression vector with a deletion of the carboxy-terminal transactivation domain (STAT5A Δτ) was used instead of the STAT5 wt construct. Either CV-1 or COS-7 cells were transfected, as indicated at the top of each panel. Transactivation activities in the presence (β-casein) or absence (MMTV-LTR) of STAT5 were determined as in Fig. 2 for CV-1 cells or as in Fig. 4B for COS-7 cells. (C to E) The hormone inductions were performed with 5 μg of prolactin per ml alone (no ligand), 5 μg of prolactin per ml and 0.1 μM dexamethasone (Dex), or 5 μg of prolactin per ml and 0.1 μM RU486 (R486) for the β-casein gene promoter. For the MMTV-LTR, prolactin was omitted in the inductions. Results are shown as the percentage of normalized activity measured in cells transfected with the highest concentration of wt GR employed. The means and standard errors of two to four independent transfections are shown.

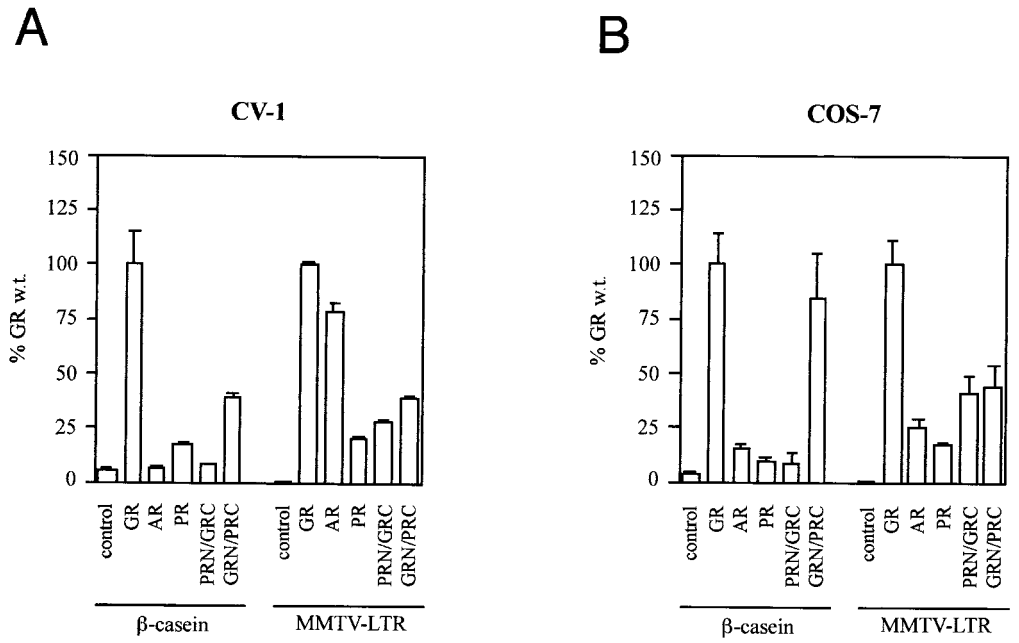

FIG. 7. Effect of chimeric progesterone-glucocorticoid receptors. CV-1 cells (A) or COS-7 cells (B) were transfected with 1 μg each of the GR, the AR, PR, PRN/GRC, or GRN/PRC (see Fig. 8 for details of structure). Transactivation activities in the presence (β-casein) or absence (MMTV-LTR) of STAT5 were determined as in Fig. 2 for CV-1 cells or as in Fig. 4B for COS-7 cells. Stimulation was with the appropriate hormones (0.1 μM dexamethasone for GR and PRN/GRC constructs, 0.1 μM R1881 for AR, and 0.1 μM R5020 for PR and GRN/PRC; 5 μg of prolactin per ml was included for the β-casein gene promoter). Results are shown as the percentage of normalized activity measured in cells transfected with the highest concentration of wt GR employed. The mean and standard error of two to four independent transfections are shown.

on the DBD domain of the GR (compare the first and second combinations in Fig. 8A). As shown in previous studies (13, 14) and schematically in Fig. 8B, the synergy is also dependent on STAT5 binding sites in the the β-casein gene promoter and on GR bound to GR half sites. At high GR concentrations, in addition to its interaction with GR half sites, the GR is able to interact with STAT5 bound to the β-casein gene promoter without utilizing its DBD. In this configuration, maximal transactivation is possible when either the transactivation domain AF-1 of the GR or the transactivation domain of STAT5 is lacking but not when both domains are lacking (Fig. 8A, third to fifth combinations). The important role of the N-terminal region of the GR in mediating the synergy with STAT5 at high and low expression levels is indicated in the last two combinations in Fig. 8A.

In accordance with a role of GR DNA binding in mediating the synergy with STAT5, mutations affecting the DNA binding function of the GR were impaired in their synergy with STAT5 when expressed in CV-1 cells. The only exception was the A458T mutant, suggesting that this particular mutant is not impaired in binding to the noncanonical GR binding sites of the β-casein gene promoter. Additional evidence for the role of GR DNA binding was provided by enhancement of GR transactivation via coexpression of HMG proteins. HMG was previously demonstrated to enhance the binding of the GR to DNA in vitro and to increase the transactivation of the GR via the MMTV LTR (1).

The particular role of GR binding to the β-casein gene promoter in providing the synergy with STAT5 might explain the different combined effects of glucocorticoid hormones and activators of STAT5 on the expression of CIS, oncostatin M, and β-casein genes: even though all three of these gene promoters contain STAT5 binding sites, only the β-casein gene was responsive to the synergistic effects of glucocorticoids (3). A similar promoter dependence was described for the interaction between the GR and STAT3. Again, a synergystic cross talk between these two transcription factors was promoter dependent and was not observed in all genes containing STAT3 binding sites (32).

At high levels of expression, GR was able to act as a transactivator even without a DBD, implying that under these conditions the GR can act as a true coactivator (Fig. 8B). Such a coactivator function of the GR in synergy with the STAT factors STAT5 and STAT3 has been postulated as a general mechanism of action based on studies performed with COS-7 cells overexpressing the GR (29, 30, 37). Our results suggest that this coactivator function does not describe the full complexity with which the GR interacts with STATs but represents one aspect of its action, which becomes predominant at high expression levels. In fact, with a pure coactivator model it would be difficult to explain the observations of the differential effect of the GR on STAT target genes. An additional property acquired by the overexpressed GR in conjuction with STAT5 shown in this study was to efficiently promote transactivation in the absence of the AF-1 domain and when liganded to the antagonist RU486 (Fig. 6). One possible explanation for this unusual behavior is that the STAT5 carboxy-terminal domain can substitute for GR AF-1 domain-mediated functions, e.g., by recruiting the same set of coactivators or by contacting the same protein surfaces in the transcription initiation complex.

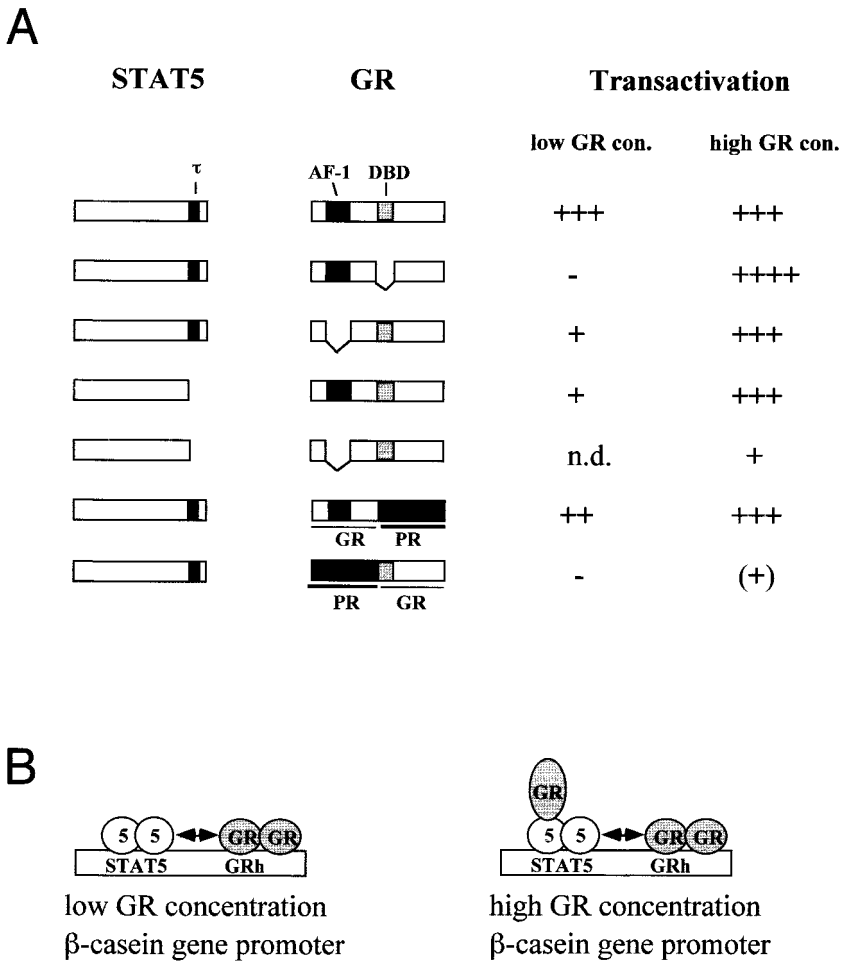

FIG. 8. Overview on the functional interactions between GR and STAT5 mediated by the β-casein gene promoter. (A) Domain dependence of synergy between GR and STAT5 at high and low GR concentrations. On the left, a schematic representation of wt STAT5 and GR constructs and constructs with deletions of the C-terminal transactivation domain of STAT5 (τ), the N-terminal transactivation domain of the GR (AF-1 or τ1), and the GR DBD (DBD) are shown. In the last two rows, the chimeric constructs GRN/PRC and PRN/GRC, used in the experiments in Fig. 7, are depicted. On the right, the relative degree of synergy between the different combinations is shown for conditions where the GR is expressed at low concentrations in CV-1 cells (bw GR con.) or high concentrations in COS-7 cells (high GR con.). Data obtained in the experiments in Fig. 2, 6, and 7 are summarized in this representation. n.d., not determined. (B) Model for the different modes of GR interactions at low and high GR concentrations. Only one each of the two functional STAT5 binding sites (STAT5) and of the several GR half sites (GRh) are shown.

This hypothesis is supported by the observation that whereas a STAT5 protein with a deletion of its transactivation domain promotes the synergy with the wt GR, it is defective in synergizing with the GR AF-1 mutant. Thus, the presence of either the GR AF-1 domain or the STAT5 C-terminal domain was sufficient. The GR still required binding of a ligand, even at high expression levels, as is evident from the failure of ligand binding-defective mutants to promote synergy with STAT5 (Fig. 6E).

It was not possible to replace the GR by the PR or AR without substantially decreasing the induction of β-casein gene transcription. Similar results were presented in a recent report (31). Results with chimeric GR/PR constructs imply that sequences within the N-terminal half of the GR outside of the DBD are important for the GR-specific effects. Further experiments will reveal whether these sequences involve regions required for the protein-protein interactions with STAT5 that have been demonstrated in coimmunoprecipitation experiments (2, 29).

Our study highlights a particular aspect of the synergism between prolactin and glucocorticoid hormones in activating β-casein gene transcription, namely, the direct, expression level-dependent type of interaction between the GR and STAT5. However, it should be emphasized that other modes of cross talk in the action of these two hormones in promoting β-casein gene expression must be considered. These include indirect effects of glucocorticoids mediated by induction or repression of inducers or repressors (4) and modulation of the rate of STAT5 dephosphorylation (36). At present it is difficult to assess the relative importance of indirect effects in comparison to the direct effects of the GR in mediating the activation of β-casein gene transcription.

A critical question posed by this study is whether the unusual property of the GR in acting as a coactivator in conjunction

with STAT5 at conditions of GR overexpression is of relevance for the in vivo situation. Previous studies have firmly established that the DNA binding function of the GR is not required for transcriptional repression of several genes. This was most convincingly demonstrated in a study with transgenic mice expressing a GR mutant with impaired DNA binding function (26). Whereas a subset of genes known to be repressed by the GR was normally regulated, expression of a set of genes induced by the GR was impaired. The mutant GR employed in the above study was a dimerization-defective GR (mutant A458T). Mice with this receptor mutant apparently lactate normally and are not altered in activation of milk protein gene expression (N. Hynes, K. Horsch, and G. Schütz, personal communication), suggesting that GR DNA binding might not be required in vivo for β-casein gene expression. However, as shown in Fig. 5A and discussed above, the GR dimerization mutant expressed by the transgene (A458T) was also not significantly impaired in its synergy with STAT5 at low expression levels, implying that it can actually bind to atypical GR binding sites in the β-casein gene promoter. This issue should be pursued further in binding studies. Our recent studies with mutants carrying mutations in the β-casein gene promoter performed with mouse mammary epithelial cells expressing endogenous levels of the GR provided evidence that in vivo and at physiological GR levels, the transactivation of the GR together with STAT5 requires binding of the GR to DNA (14). It is possible that this synergy between GR and STAT5 is additionally enhanced by GR molecules, which act as true coactivators. However, it should be emphasized that a pure coactivator function of the GR has so far been observed only in cells expressing artificially high levels.

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