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Functional Interference between the Ubiquitous and Constitutive Octamer Transcription Factor 1 (OTF-1) and the Glucocorticoid Receptor by Direct Protein-Protein Interaction Involving the Homeo Subdomain of OTF-1

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The ubiquitous and constitutive octamer transcription factor OTF-1 (Oct 1) is the target of positive regulation by the potent herpes simplex virus *trans*-activator VP16, which forms a complex with the homeodomain of OTF-1. Here we present evidence that the glucocorticoid receptor can negatively regulate OTF-1 function by a mechanism that is independent of DNA binding. In vivo-expressed glucocorticoid receptor inhibited in a hormone-dependent manner activation of a minimal promoter construct carrying a functional octamer site. Moreover, expression of the receptor in vivo resulted in hormone-dependent repression of OTF-1-dependent DNA-binding activity in nuclear extract. In vitro, the DNA-binding activity of partially purified OTF-1 was repressed following incubation with purified glucocorticoid receptor. Cross-linking and immunoprecipitation experiments indicated that the functional interference may be due to a strong association between these two proteins in solution. Finally, preliminary evidence indicates that the homeo subdomain of OTF-1 that directs formation of a complex with VP16 may also be critical for interaction with the glucocorticoid receptor. Thus, OTF-1 is a target for both positive and negative regulation by protein-protein interaction. Moreover, the functional interference between OTF-1 and the glucocorticoid receptor represents a novel regulatory mechanism in the cross-coupling of signal transduction pathways of nuclear receptors and constitutive transcription factors.

Signal transduction by glucocorticoid hormones is mediated by a specific intracellular receptor protein, which, like many other members of the steroid receptor superfamily, functions as a ligand-activated nuclear transcriptional regulator (for a review, see reference 15). The mechanism by which the receptor activates the transcriptional response of target genes to glucocorticoids is poorly understood. However, the transcriptional activation is known to be mediated by interaction of the receptor with positive control elements (glucocorticoid response elements) which are present in single or multiple copies upstream of or within target genes (for a review, see reference 3). The transactivation potential of the glucocorticoid receptor can be substantially increased when mediated via synthetic promoters containing a glucocorticoid response element placed in the immediate vicinity of the binding site of a constitutive transcription factor (54). In addition to its positive effect on gene transcription the glucocorticoid receptor is also capable of inactivating target genes. This effect appears to be carried out either by competition with upstream transcription factors (for a review, see reference 48) or with general transcription factors (such as, possibly, TFIID [60]) for overlapping DNA target sites, or, alternatively, by repression mechanisms independent of DNA binding. The latter mechanism is typified by the recently identified transcriptional interference (also termed cross coupling; for a review, see reference 53) between the glucocorticoid receptor and c-Fos/c-Jun involving a possible interaction between these proteins and the formation of abortive complexes (29, 37, 55, 70). Under certain conditions interference between the glucocorticoid receptor and Fos/Jun signal transduction pathways appears also to occur on composite glucocorticoid response elements that have the potential to bind both the receptor and the Fos/Jun complex (12).

In contrast to the inducible glucocorticoid receptor, octamer transcription factors (OTFs; also referred to as Oct factors) represent constitutive factors which have been implicated in transcriptional activation of a variety of genes through interaction with sequences that contain the octamer motif (ATGCAAAT). Among these factors, the 90-kDa OTF-1 protein is ubiquitously distributed and has been shown to be involved in, for instance, cell cycle regulation of the human histone H2b gene and the constitutive expression of small nuclear RNA genes (6, 18, 34, 39, 61 [and references therein]), whereas the ~60-kDa OTF-2 protein is expressed in a tissue-restricted pattern and appears to be involved in the lymphoid-specific expression of immunoglobulin promoters and enhancers (7, 31, 38, 50, 51). In addition, OTF-1 mediates adenovirus DNA replication (43). Interestingly, OTF-1 and -2 display identical DNA-binding specificities (45 [and references therein]) and structurally belong to the POU family of transcription factors which all are characterized by an extended region of homology including a C-terminal homeo box subdomain (for a review, see reference 26). Additional members of the OTF family of proteins have been identified during embryogenesis (for a review, see reference 52) and in the nervous system (24, 63).

Apart from being involved in DNA binding, the OTF-1 homeodomain directs formation of a complex with the herpes simplex virus transactivator VP16 resulting in strong activation of herpes simplex immediate-early promoters (33, 58, 59 [and references therein]). In this report we show that

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the hormone-activated glucocorticoid receptor can function as a negative regulator of OTF activity. DNA-binding, coimmunoprecipitation, and protein cross-linking experiments suggested that transcriptional interference is mediated by direct interaction between the hormone-activated glucocorticoid receptor and the homeodomain of OTF-1, leading to inhibition of OTF-1-dependent DNA-binding activity. In vivo, interference between glucocorticoid receptor and OTF-1 function is hormone-dependent, indicating that the surface mediating transcriptional interference may be masked in the inactive, latent receptor form.

MATERIALS AND METHODS

Cells and extract preparation. Hepa 1c1c7 cells (22) were grown in minimum essential medium as previously described (8, 68). Stably transfected and amplified CHO K1 cells expressing full-length glucocorticoid receptor were cultured as previously described (1). Nuclear extracts were prepared either essentially as described by Dignam et al. (13) or by a small-scale cell extract procedure as previously described (1).

Transient transfection experiments and RNA analysis. Parental or stable, amplified CHO cells expressing glucocorticoid receptor were transiently transfected with the lipofectin reagent (Bethesda Research Laboratories) (16) with 1 μg of reference plasmid OVEC-Ref (66) and 20 μg of the minimal promoter construct Octa(1) containing a synthetic octamer element from the immunoglobulin heavy-chain intron enhancer fused to the β-globin TATA box per 100-mm dish (38). In control experiments, the cells were transfected with the OVEC(S) (66) vector, which contains a β-globin TATA box but no upstream regulatory element. Hormone treatment of the cells was routinely carried out for 12 to 16 h at a concentration of 0.5 μM dexamethasone. Cytoplasmic RNA was isolated and analyzed by an S1 nuclease protection assay essentially as described elsewhere (47). An end-labeled single-stranded oligonucleotide extending between positions −18 bp and +75 bp relative to the transcription start site of the rabbit β-globin gene (66) was used as probe.

Protein purification and in vitro translation of transcription factors. The glucocorticoid receptor was purified from rat liver to apparent homogeneity as previously described (69). The minimal DNA-binding domain of the glucocorticoid receptor was expressed as a fusion protein with protein A in *Escherichia coli*, cleaved with α-chymotrypsin, and purified as previously described (9). The glycosylated species of OTF-1 was partially purified from Hepa 1c1c7 cells by wheat germ agglutinin affinity chromatography essentially as previously described (28, 39). Briefly, hepatoma cell nuclear extract was incubated with the affinity resin for 1 h at 0 to 4°C and at high ionic strength (~420 mM KCl) in 20 mM Tris-HCl (pH 7.9)–20% (vol/vol) glycerol–0.2 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride. The column was washed extensively with the application buffer and eluted with 400 mM *N*-acetyl glucosamine in buffer containing 100 mM KCl. This protocol generally resulted in 150- to 200-fold purification of OTF-1 yielding an up to 5% pure preparation of this protein. Importantly, 60 to 80% of the input OTF-1-dependent DNA-binding activity was recovered by this protocol, indicating that OTF-1 resides predominantly in its glycosylated form in these cells. In indicated experiments, wild-type or mutant OTF-1 or VP16 was expressed by in vitro translation in rabbit reticulocyte lysate (Promega) by using input mRNA generated by in vitro transcription of the plasmids pBSoct-1$^+$ (61), pBSΔOct-1(2)1He$_2$(3)1 (Oct-1/He$_2$2 [58]), and pGT65 (20), respectively.

DNA-binding assays. The OTF-1-dependent DNA-binding activity was monitored by an electrophoretic gel mobility shift assay as previously described (46, 51). A ^{32}P-labeled, double-stranded oligonucleotide spanning the octamer element of the histone H2b promoter (18) was used as a specific probe. To monitor the formation of VP16-induced complexes with OTF-1, we used an oligonucleotide probe containing the TAATGARAT motif of the herpes simplex virus ICP0 promoter (20). The DNA-binding activity of the adenovirus major late transcription factor USF was analyzed as previously described (21) by using as a probe a double-stranded oligonucleotide containing the USF recognition sequence of the adenovirus major late promoter. Protein-DNA complexes were resolved on 4% (30:1 cross-linked) low-ionic-strength native polyacrylamide gels.

Detection of multiprotein complexes by immunoprecipitation. Purified glucocorticoid receptor (20 μl; 7 μg of protein per ml) was incubated for 20 min at 25°C with purified OTF-1 or in vitro-translated OTF-1 in reticulocyte lysate in a total volume of 100 μl of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9)–60 mM KCl–2 mM dithiothreitol–10% (vol/vol) glycerol. Complexes were immunoprecipitated essentially as previously described (10) by using the monoclonal anti-glucocorticoid receptor antibody 7 (42) or control immunoglobulin immobilized on CNBr-activated Sepharose (Pharmacia) beads. The protein complexes were incubated with the immunoglobulin-Sepharose for 1 h at 0 to 4°C prior to centrifugation. Pelleted material was washed with 20 mM sodium phosphate (pH 7.2)–10% (vol/vol) glycerol–150 mM NaCl–1 mM EDTA prior to extraction with (i) sodium dodecyl sulfate (SDS) sample buffer and subsequent analysis by SDS-polyacrylamide gel electrophoresis or (ii) phosphate buffer containing 1 M NaCl and 0.01% (vol/vol) Nonidet P-40 and subsequent DNA-binding experiments.

Protein cross-linking experiments. ^{35}S-labeled, in vitro-translated OTF-1 was incubated in a total volume of 50 μl with purified glucocorticoid receptor in 12 mM HEPES (pH 7.9)–12% (vol/vol) glycerol–60 mM KCl–5 mM $MgCl_2$–0.6 mM EDTA prior to addition of the homobifunctional cross-linking agent dithiobis(succinimidylpropionate) (Pierce) to a final concentration of 2 mM and further incubation at room temperature for 1 h. The cross-linking reaction was then stopped by addition of ethanolamine to a final concentration of 0.1 M. Cross-linked protein complexes were immunoprecipitated by using Sepharose beads containing the monoclonal anti-glucocorticoid receptor antibody 7 or control immunoglobulin as described above. In certain experiments cross-linked material was also reduced by boiling the pelleted material in SDS-polyacrylamide gel electrophoresis sample buffer containing 15% (vol/vol) β-mercaptoethanol prior to analysis on SDS-polyacrylamide gels.

RESULTS

The hormone-activated glucocorticoid receptor interferes with endogenous OTF-1 function in vivo. Given the background that the glucocorticoid receptor can antagonize function of c-Jun and c-Fos, which both are components of the phorbol ester-activated AP-1 transcription factor (12, 29, 37, 55, 70), we were interested to test whether the glucocorticoid receptor could affect the activity of a ubiquitous factor such as OTF-1 involved in constitutive gene expression. To this end, we used a minimal β-globin promoter construct (con-

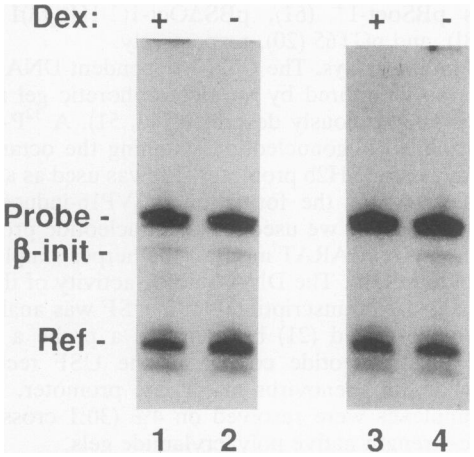

FIG. 1. Hormone-dependent repression of an octamer-dependent promoter by the glucocorticoid receptor. Parental CHO cells (lanes 1 and 2) or stably transfected pMTGR-CHO cells expressing full-length glucocorticoid receptor (lanes 3 and 4) were transiently transfected with the Octa(1) reporter plasmid and the internal standard plasmid OVEC-Ref. The cells were treated for 16 h with 0.5 μM dexamethasone (Dex) as indicated. The activity of the promoters was determined by S1 nuclease protection analysis of cytoplasmic RNA. The bands labeled β-init indicate correctly initiated β-globin transcripts derived from the Octa(1) construct, whereas the Ref band is generated by RNA initiated from the internal standard plasmid OVEC-Ref.

taining only an octamer element, a TATA box, and the simian virus 40 enhancer downstream of the β-globin transcription unit). This reporter construct is strongly activated in lymphoid cells but is also activated, albeit at a lower level, by the ubiquitous OTF-1 factor in nonlymphoid cells (30 [and references therein]). In the present experiments the octamer/TATA box promoter construct was introduced into either parental CHO K1 cells or into stably transfected and amplified CHO cells (pMT-GR [1]) expressing glucocorticoid receptor. Parental CHO K1 cells are considered receptor negative since the endogenous glucocorticoid receptor is expressed at very low levels and is hardly detectable by ligand-binding or immunochemical assays (1, 27 [and references therein]). As a negative reporter control we used the OVEC(S) promoter which contains only the β-globin TATA box and a downstream simian virus 40 enhancer but no upstream regulatory element (66). S1 nuclease protection analysis demonstrated that this TATA box promoter construct showed no detectable activity in parental or receptor-expressing CHO cells (data not shown). However, the octamer-containing promoter gave a detectable, albeit low, signal in the parental CHO cells (Fig. 1). In these cells the expression level of the octamer/TATA box promoter was unaltered in the presence of hormone (compare lanes 1 and 2). In contrast, we were surprised to observe that the β-globin transcript was strongly down-regulated by dexamethasone in the CHO cells expressing glucocorticoid receptor (compare lanes 3 and 4), implying that the hormone-activated receptor may repress the transcriptional activity of the OTF-1 protein in these cells. The simian virus 40 enhancer-driven internal reference promoter OVEC-Ref (66) produced a strong signal in both parental and receptor-expressing CHO cells (Fig. 1). This signal remained unchanged upon hormone treatment, demonstrating that, under the experimental conditions used, expression of the glucocorticoid receptor did not have a general effect on transcription.

The glucocorticoid receptor down-regulates the DNA-binding activity of OTF-1 in vivo. To further investigate whether expression of the glucocorticoid receptor affected functional properties of OTF-1, we next examined the DNA-binding activity of OTF-1 in parental CHO cells and in the pMT-GR cells overexpressing the glucocorticoid receptor. To this end we used an electrophoretic mobility shift assay employing a labeled, synthetic octamer sequence from the histone H2b promoter (18) as a specific probe. In the absence of hormone treatment a single protein-DNA complex was generated in nuclear extract from either the parental or pMT-GR CHO cells (Fig. 2A, compare lanes 2 and 4). The specificity of this complex was established by DNA competition experiments. The complex was not formed in the presence of an excess of the unlabeled octamer sequence, whereas formation of the complex was not affected by the presence of an excess of a synthetic glucocorticoid response element (Fig. 2C). Moreover, the relative mobility of the complex was very similar or identical to that generated by in vitro-translated OTF-1 mRNA (data not shown). We therefore conclude that the complex most likely represents an OTF-1-dependent DNA-binding activity. Treatment of the parental CHO cells with dexamethasone did not affect the relative levels of this DNA-binding activity (Fig. 2A, compare lanes 2 and 3).

Interestingly, however, the OTF-1-dependent complex was markedly decreased in intensity so that it was barely detectable in nuclear extract of hormone-treated pMT-GR cells (Fig. 2A, compare lanes 3 and 5), indicating that the DNA-binding activity of OTF-1 was repressed by expression of the glucocorticoid receptor. In control experiments, the OTF-1-dependent DNA-binding activity was not affected by transfection of CHO cells with the pMT expression vector alone (data not shown). To standardize the nuclear extracts from untreated and hormone-treated cells, we also monitored the DNA-binding activity of the ubiquitous and constitutive adenovirus major late transcription factor USF. This protein represented a rather abundant factor in nuclear extracts from either the parental or the glucocorticoid receptor-expressing CHO cells (Fig. 2B). The specificity of the USF-dependent complex was verified by DNA competition experiments using an excess of a synthetic USF-binding site from the adenovirus major late promoter (21) versus an unrelated sequence motif spanning a glucocorticoid response element (data not shown). Importantly, the relative levels of the USF-dependent DNA-binding activity were not altered by treatment of the various CHO cell lines with dexamethasone (Fig. 2B, compare lanes 2 and 4 with lanes 3 and 5).

The full-length glucocorticoid receptor represses the DNA-binding activity of OTF-1 in vitro. The purified glucocorticoid receptor does not bind to the octamer motifs of either the histone H2b or the immunoglobulin light-chain or heavy-chain promoters (data not shown), strongly arguing that the receptor does not antagonize OTF-1 function by direct interaction with the octamer element. The hormone-dependent inhibition of OTF-1-dependent transcriptional and DNA-binding activities in CHO cells expressing glucocorticoid receptor may therefore be due to structural interference between the receptor and the DNA-binding activity of OTF-1. To test this notion we partially purified OTF-1 from hepatoma cells by a wheat germ agglutinin affinity chromatography (28, 39). This method also resulted in a rapid and significant concentration of OTF-1. Moreover, 60 to 80% of the input DNA-binding activity of OTF-1 was recovered by this method, indicating that the majority of OTF-1 molecules

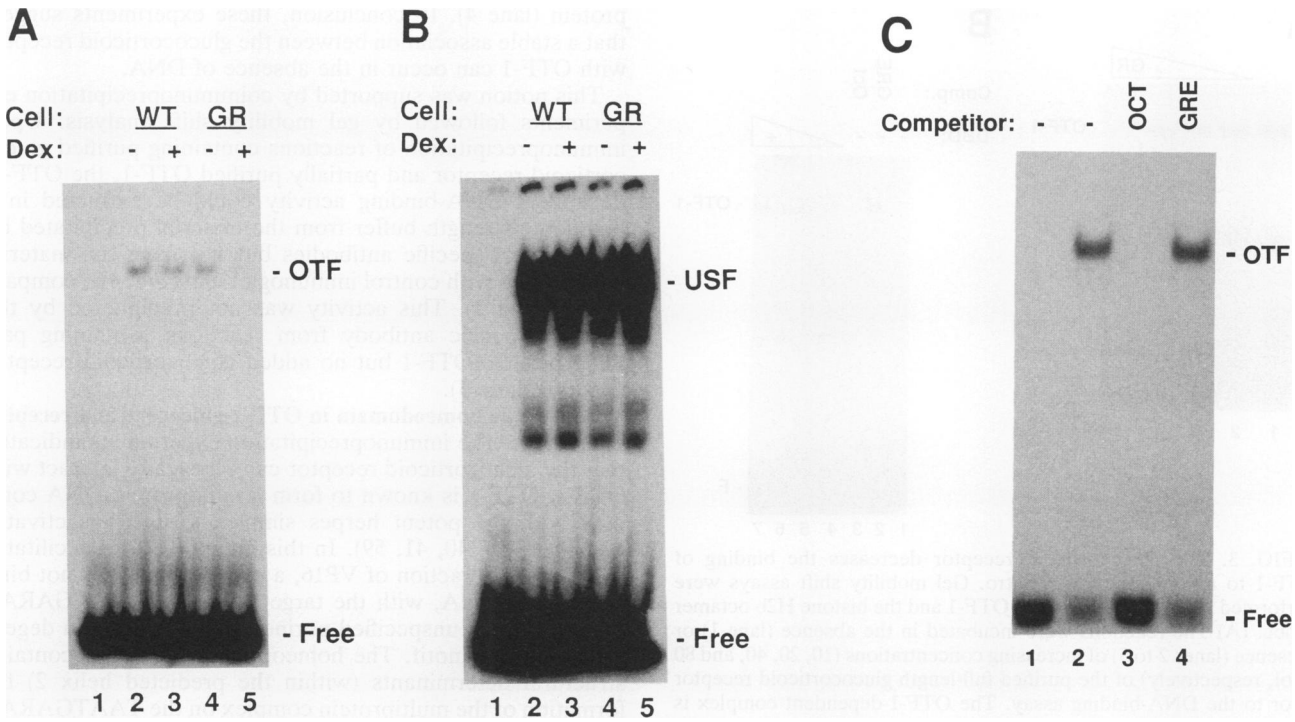

FIG. 2. Hormone-dependent inhibition of the DNA-binding of OTF-1 by the glucocorticoid receptor in vivo. Parental CHO cells (WT) or glucocorticoid receptor-expressing pMTGR CHO cells (GR) were incubated for 16 h in the absence or presence of 0.5 μM dexamethasone (Dex) as indicated, and rapid, small-scale nuclear extracts were prepared. These nuclear extracts were analyzed in gel mobility shift experiments for the DNA-binding activities of OTF-1 and the adenovirus major late transcription factor USF. (A) OTF-1-dependent DNA-binding activities were monitored by using a probe containing an octamer element from the human histone H2b promoter. OTF, the OTF-1–DNA complex. (B) The same nuclear extracts were also analyzed for DNA-binding activity of the constitutive USF factor. The probe used spans the USF-binding site of the adenovirus major late promoter. The USF-specific complex is indicated. (C) The specificity of the octamer binding reaction was assessed in DNA competition experiments. Nuclear extract from untreated parental CHO cells was incubated with the labeled histone H2b octamer probe in the absence (lane 2) or presence of either a 200-fold molar excess of the unlabeled octamer element (Oct; lane 3) or an identical excess of a synthetic glucocorticoid response element (GRE, lane 4). The mobilities of the unbound octamer and USF probes in the absence of any added protein (Free) are shown in lane 1 of the different panels.

were present in a glycosylated form. The partially purified OTF-1 preparation was incubated with a labeled probe spanning the octamer element of the histone H2b promoter in the absence or presence of increasing concentrations of glucocorticoid receptor purified to near homogeneity. These reactions were assayed for OTF-1-dependent DNA-binding activity by gel mobility shift analysis. In the presence of the glucocorticoid receptor, we observed an inhibition of the DNA-binding activity of OTF-1 (Fig. 3A). The specificity of the OTF-1-dependent protein-DNA complex was established by DNA-binding competition experiments using an excess of the unlabeled octamer site versus an excess of an unrelated sequence motif spanning a glucocorticoid response element (data not shown; Fig. 3B, compare lanes 2 and 3). The inhibitory effect of the glucocorticoid receptor on the OTF-dependent DNA-binding activity showed dose dependency, so that, at the highest dose of receptor tested (80 fmol), the DNA-binding activity of OTF-1 was virtually extinguished (Fig. 3A, compare lanes 2 to 5).

Failure of the minimal DNA-binding domain of the glucocorticoid receptor to repress the DNA-binding activity of OTF-1. We next examined if the DNA-binding activity of OTF-1 was affected by exposure to a truncated glucocorticoid receptor derivative spanning the minimal DNA-binding domain (the zinc finger motif [23, and references therein]) of the glucocorticoid receptor. A nearly homogenous (at least 95% pure) preparation of the bacterially expressed DNA-binding domain of the receptor (9) was used in these experiments. The expressed protein binds DNA with the same specificity as the full-length receptor (9, 19). In contrast to the full-length protein, however, it did not appear to significantly affect the DNA-binding activity of OTF-1 in coincubation experiments (Fig. 3B). Very low, if any, levels of inhibition of OTF-1-dependent DNA-binding activity were detected following exposure of partially purified OTF-1 to very high concentrations (8 pmol) of the purified glucorticoid receptor DNA-binding domain in vitro (Fig. 3B, compare lanes 4 and 7). On a molar basis these concentrations of the DNA-binding domain by far exceed those of the full-length glucocorticoid receptor that produced repression of OTF-1 activity. These data suggest that structures other than the DNA-binding domain may be important for the inhibitory effect of the receptor on OTF-1 activity. Alternatively, posttranslational modification of the DNA-binding domain of the receptor may be required for interference with OTF-1. For instance, both the glucorticoid receptor and OTF-1 are known to be posttranslationally modified by phosphorylation. The glucocorticoid receptor appears to be hyperphosphorylated in hormone-treated cells (25), whereas OTF-1 is differentially phosphorylated during the cell cycle (49).

DNA-independent protein-protein interaction between the glucocorticoid receptor and OTF-1. A simple model explaining the observed in vivo and in vitro inhibition of OTF-1 activity by the glucocorticoid receptor may be functional

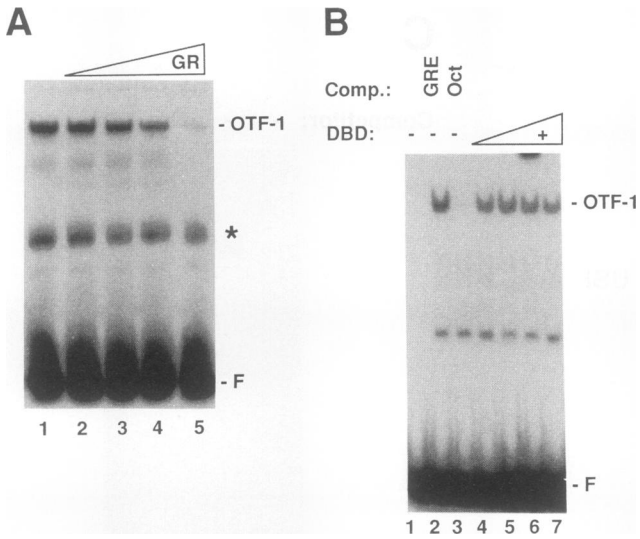

FIG. 3. The glucocorticoid receptor decreases the binding of OTF-1 to an octamer site in vitro. Gel mobility shift assays were performed with partially purified OTF-1 and the histone H2b octamer probe. (A) The reactions were incubated in the absence (lane 1) or presence (lanes 2 to 5) of increasing concentrations (10, 20, 40, and 80 fmol, respectively) of the purified full-length glucocorticoid receptor prior to the DNA-binding assay. The OTF-1-dependent complex is indicated. The band indicated by a star represents a nonspecific protein-DNA complex. (B) The DNA-binding reactions were assembled in the absence (lanes 2 and 3) or presence (lanes 4 to 7) of increasing concentrations (1, 2, 4, and 8 pmol, respectively) of the purified, bacterially expressed DNA-binding domain (DBD) of the glucocorticoid receptor. The specificity of the OTF-1-dependent complex was analyzed by DNA competition experiments using either a 200-fold molar excess of an unlabeled octamer element (Oct; lane 2) or an identical excess of a synthetic glucocorticoid response element (GRE, lane 3). Lane 1 shows the mobility of the unbound ^{32}P-labeled histone H2b octamer probe (F) in the absence of any added protein.

interference due to a direct physical interaction between these proteins. In initial experiments, we could not detect any ternary glucocorticoid receptor–OTF-1–DNA complex by gel mobility shift analysis (data not shown) with probes containing both a glucocorticoid response element and an octamer motif (separated by 2 and 15 bp, respectively). To investigate a possible interaction between the glucocorticoid receptor and OTF-1 in the absence of any DNA target sequences, radiolabeled OTF-1 was synthesized by in vitro translation of OTF-1 mRNA in reticulocyte lysates and incubated in the absence or presence of purified full-length glucocorticoid receptor. Subsequently, these reactions were immunoprecipitated either with an anti-glucocorticoid receptor monoclonal antibody or with control immunoglobulin coupled to cyanogen bromide-activated Sepharose (10), and the immunoreactive material was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The anti-glucocorticoid receptor antibody did not precipitate labeled OTF-1 in the absence of the glucocorticoid receptor, nor did it precipitate any bands in control reactions containing reticulocyte lysate primed with irrelevant mRNA (Fig. 4A, lanes 1 and 2). In the presence of the receptor, however, the anti-receptor antibody coprecipitated the labeled OTF-1 band on SDS-polyacrylamide gels (lane 3), indicating protein complex formation. In parallel reactions containing both the glucocorticoid receptor and in vitro-translated OTF-1, control immunoglobulin did not precipitate the labeled OTF-1 protein (lane 4). In conclusion, these experiments suggest that a stable association between the glucocorticoid receptor with OTF-1 can occur in the absence of DNA.

This notion was supported by coimmunoprecipitation experiments followed by gel mobility shift analysis. Upon immunoprecipitation of reactions containing purified glucocorticoid receptor and partially purified OTF-1, the OTF-1-dependent DNA-binding activity could be extracted in a high-ionic-strength buffer from the material precipitated by the receptor-specific antibodies but not from the material precipitated with control immunoglobulin (Fig. 4B, compare lanes 1 and 2). This activity was not precipitated by the receptor-specific antibody from reactions containing partially purified OTF-1 but no added glucocorticoid receptor (Fig. 4B, lane 3).

Role of the homeodomain in OTF-1-glucocorticoid receptor interaction. The immunoprecipitation experiments indicated that the glucocorticoid receptor can physically interact with OTF-1. OTF-1 is known to form a multiprotein-DNA complex with the potent herpes simplex virus transactivator VP16 (20, 32, 40, 41, 59). In this manner OTF-1 facilitates functional interaction of VP16, a protein that does not bind strongly to DNA, with the target sequence TAATGARAT (where R is an unspecified purine), which harbors a degenerate octamer motif. The homeodomain of OTF-1 contains structural determinants (within the predicted helix 2) for formation of the multiprotein complex on the TAATGARAT sequence (32, 58, 59). These determinants are not conserved in the OTF-2 homeodomain, and OTF-2 forms only a very weak complex with VP16 (20, 33, 59). Thus, substitution of the predicted helix 2 of OTF-1 with the corresponding sequence from OTF-2 yields a chimeric protein, Oct-1/He2-2, that fails to interact with VP16 (59). Interestingly, incubation of the labeled, in vitro-translated chimeric Oct-1/He2-2 protein with purified glucocorticoid receptor also failed to produce a complex that was precipitated by the monoclonal anti-glucocorticoid receptor antibody (Fig. 4A, compare lanes 3 and 5). In line with this observation, the octamer-binding activity of the in vitro-translated Oct-1/He2-2 chimeric protein was not repressed by exposure to concentrations of glucocorticoid receptor that repress wild-type OTF-1 (Fig. 4C). We interpret these results to indicate that the OTF-1 homeodomain plays an important role in mediating the interaction, direct or indirect, between the glucocorticoid receptor and OTF-1.

The glucocorticoid receptor interferes with the interaction between the OTF-1-VP16 complex and the TAATGARAT target sequence motif. In direct DNA-binding experiments we monitored the formation of a VP16-induced complex with OTF-1 by gel mobility shift analysis with a labeled TAATGARAT probe derived from the herpes simplex virus ICP0 promoter. Although VP16 can interact directly with OTF-1 (58), complex formation is facilitated by a host cell factor(s) (20, 33 [and references therein]). We therefore used in vitro-translated VP16 in reticulocyte lysate as a protein source. OTF-1 binds to the TAATGARAT sequence in the absence of VP16 (20) (Fig. 5, lane 2), whereas no protein-DNA complex was generated with the TAATGARAT probe by exposure to either in vitro-translated VP16 (20) (Fig. 5, lane 3) or purified glucocorticoid receptor (Fig. 5, lane 4). The specificity of the OTF-1-dependent protein-DNA complex was verified by DNA competition experiments (data not shown). The VP16-induced multiprotein complex was detected upon coincubation of the TAATGARAT element with both in vitro-translated VP16 and OTF-1 (lane 5). Although VP16-induced complex formation appears to stabilize DNA-

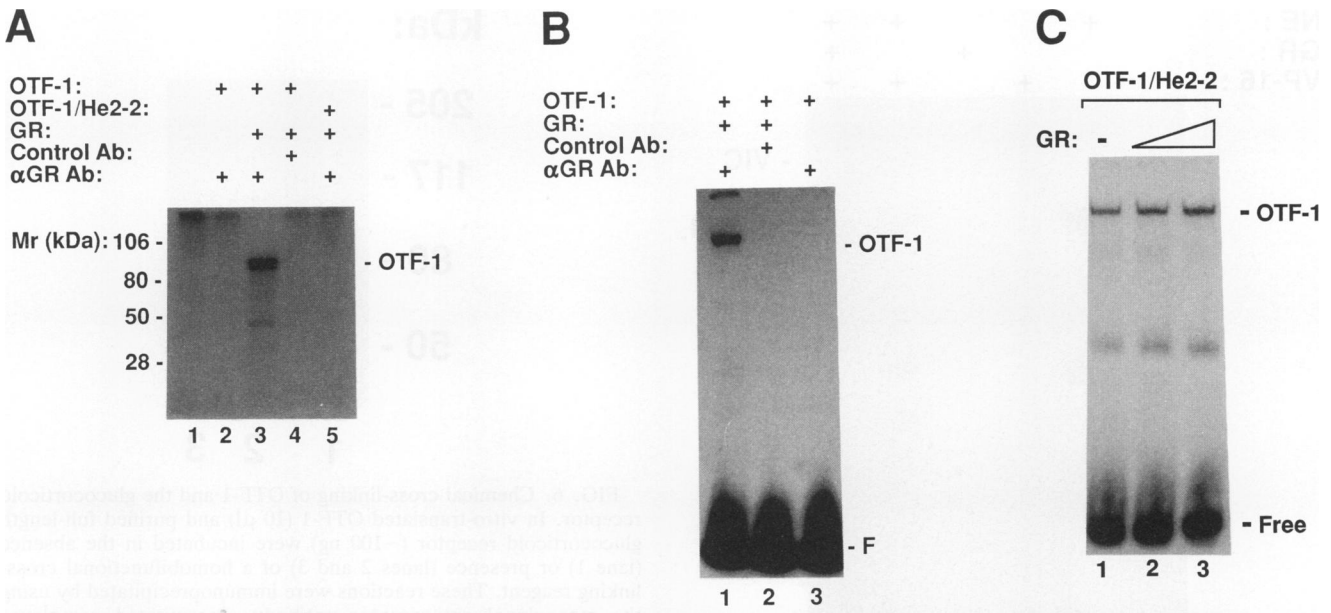

FIG. 4. Immunoprecipitation of OTF-1–glucocorticoid receptor complexes. (A) Wild-type OTF-1 or the OTF-1/He2-2 fusion protein were expressed by in vitro translation in rabbit reticulocyte lysate. The in vitro-translated proteins (10 μl) were incubated in the absence or presence of ~100 ng of purified glucocorticoid receptor (GR), a receptor-specific monoclonal antibody (αGR Ab) or control immunoglobulin (Control Ab). These reactions were immunoprecipitated, and resulting complexes were analyzed by autoradiography following separation on an SDS–10% polyacrylamide gel. The mobilities of protein standards are indicated. (B) The immunoprecipitation reactions were also performed with partially purified OTF-1 (~2 μg) which was incubated in the absence or presence of purified glucocorticoid receptor (~100 ng). Glucocorticoid receptor-associated factors were extracted from the precipitated material in a high-salt buffer and characterized by gel mobility shift analysis with a labeled histone H2b octamer probe. The OTF-1-dependent protein-DNA complex is indicated, whereas F indicates the mobility of the free (unbound) probe. (C) The OTF-1/He2-2 fusion protein was expressed by in vitro translation in rabbit reticulocyte lysate and incubated in the absence (lane 1) or presence (lanes 2 and 3) of increasing concentrations (50 and 100 fmol, respectively) of the purified full-length glucocorticoid receptor prior to the DNA-binding assay. The reactions were analyzed by the gel mobility shift assay as described above. The mobility of the expressed OTF-1 fusion protein is indicated.

binding (33, 58 [and references therein]), we also observed a marked decrease in intensity of the band generated by the VP16-induced complex with OTF-1 in the presence of the glucocorticoid receptor (Fig. 5, compare lanes 5 and 6). Thus, the glucocorticoid receptor appears to also negatively regulate the DNA-binding activity of the VP-16-induced multiprotein complex toward the TAATGARAT sequence. Alternatively, it is possible that the glucocorticoid receptor directly interferes with the interaction between OTF-1 and VP16 and that VP16 and the glucocorticoid receptor depend on a similar structural motifs for interaction with OTF-1. However, this issue remains to be addressed in an experimental system that does not depend on DNA binding.

Evidence for direct interaction between the glucocorticoid receptor and OTF-1. To investigate if the glucocorticoid receptor could interact directly with OTF-1, we performed reversible protein cross-linking experiments. Purified full-length glucocorticoid receptor and was incubated with radiolabeled OTF-1 generated by in vitro translation of OTF-1 mRNA in reticulocyte lysates in the absence or presence of the homobifunctional and reversible cross-linking reagent dithiobis(succinimidylproprionate). The reactions were immunoprecipitated by the monoclonal anti-glucocorticoid receptor antibody. In the absence of the cross-linking reagent only the OTF-1-dependent band was visualized by SDS-polyacrylamide gel electrophoresis of the precipitated material (Fig. 6, lane 1). In addition to this band a larger complex of around 180 kDa was precipitated in the presence of the cross-linking reagent (lane 2). If these immune complexes were exposed to a reducing reagent (that cleaves the cross-linker), only the bona fide OTF-1-dependent band was detected (lane 3). The molecular mass of the large cross-linked complex is consistent with that of a heteromeric complex of OTF-1 and the glucocorticoid receptor. Given the background that OTF-1 appears to be monomeric in solution (2, 35, 46) and that the 180-kDa complex (which theoretically could harbor a homodimeric form of OTF-1) was not detected after cross-linking of the labeled OTF-1 protein in the absence of the glucocorticoid receptor (data not shown), these data argue that the glucocorticoid receptor may directly interact with OTF-1.

DISCUSSION

The glucocorticoid receptor forms a nonproductive complex with the ubiquitous and constitutive transcription factor OTF-1. The present experiments indicate that the OTF-1 factor may be the target for repression by glucocorticoid hormones. Several lines of evidence suggest that hormone-dependent repression of octamer activity occurs via a mechanism that does not involve protein-DNA interaction but rather appears to involve interference due to physical interaction between the hormone-activated glucocorticoid receptor and OTF-1. In similar fashion, the glucocorticoid receptor has been reported to antagonize Fos/Jun activity, possibly via formation of an abortive complex with these proteins (12, 29, 37, 55, 70). A similar negative cross-coupling of regulatory pathways has recently been observed between Jun and the helix-loop-helix factors MyoD and myogenin (4, 36). OTF-1, in turn, has been demonstrated to

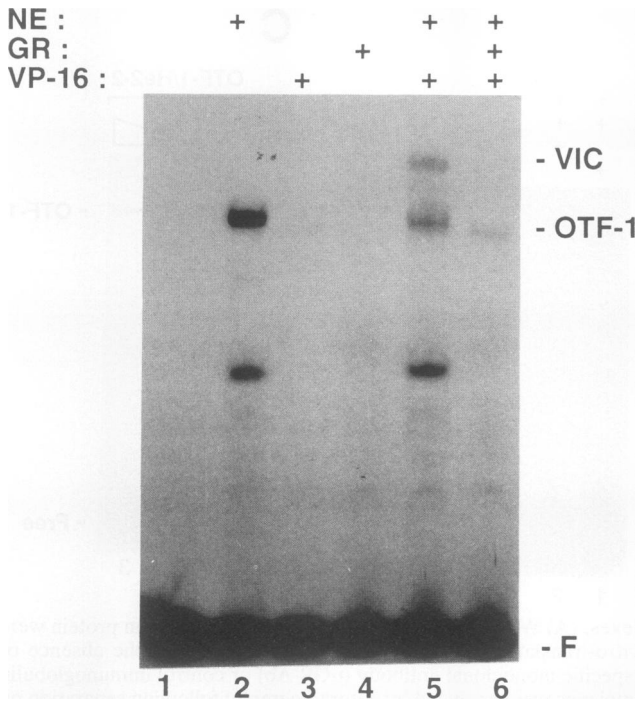

FIG. 5. The glucocorticoid receptor interferes with DNA-binding of the multiprotein complex containing the viral transactivator VP16 and OTF-1. A probe spanning the TAATGARAT motif of the hepes simplex virus ICP0 promoter was used in gel mobility shift experiments to monitor formation of the VP16-induced complex with OTF-1 (VIC). VP16 was in vitro translated in reticulocyte lysate and incubated with crude nuclear extract (NE; 1 μl, 5 mg of protein per ml) from hepatoma cells. To these incubation mixtures purified glucocorticoid receptor (8 ng) was added as indicated. DNA-binding reactions performed with only in vitro-translated VP16 and purified glucocorticoid receptor are shown in lanes 3 and 4, respectively. Lane 1 shows the mobility of the free (F) ICP0 probe.

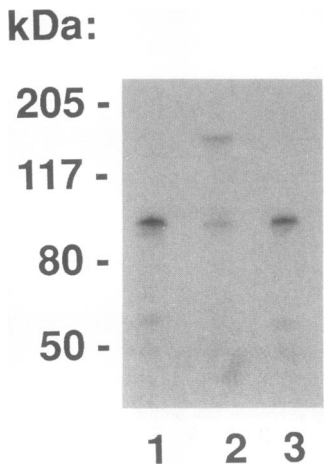

FIG. 6. Chemical cross-linking of OTF-1 and the glucocorticoid receptor. In vitro-translated OTF-1 (10 μl) and purified full-length glucocorticoid receptor (~100 ng) were incubated in the absence (lane 1) or presence (lanes 2 and 3) of a homobifunctional cross-linking reagent. These reactions were immunoprecipitated by using the monoclonal anti-receptor antibody. Precipitated complexes were separated on an SDS–10% polyacrylamide gel and analyzed by autoradiography. In lane 3 the precipitated material was reduced in 15% β-mercaptoethanol prior to electrophoretic analysis.

participate in selective protein-protein interactions with the viral transactivator VP16 resulting in positive transcriptional regulation of target promoters (for a review, see reference 58). Thus, OTF-1 appears to be the target for both positive and negative regulation by protein-protein interaction and thus offers an excellent model system to study the mechanisms of these regulatory pathways.

Our in vitro data argue that OTF-1 activity may be repressed by direct association with the glucocorticoid receptor. Importantly, it was possible to use receptor-specific antibodies to immunoprecipitate partially purified OTF-1 after incubation with purified receptor, and we could chemically cross-link a complex which exhibited the molecular weight of a putative glucocorticoid receptor–OTF-1 heterodimer. However, these in vitro results do not rule out the possibility that additional, auxiliary factors stabilize or catalyze the formation of the complex in vivo. Such factors appear to facilitate the formation of the VP16-induced complex with OTF-1 (20, 32, 33). The VP16-induced complex, however, is assembled on a specific DNA target sequence (20, 40). In contrast, association of the glucocorticoid receptor with OTF-1 did not appear to require the presence of DNA.

Mechanism of repression of target gene expression by the glucocorticoid receptor. Modulation of Fos/Jun activity by the glucocorticoid receptor appears to be very complex (for a review, see reference 53) and involves at least two distinct mechanisms: (i) a complex pattern of protein-DNA interactions at bifunctional (composite) elements involving both the receptor and Fos/Jun and effecting either positive or negative hormonal regulation (12) and (ii) functional antagonism between the glucocorticoid receptor and Fos/Jun at an AP-1 site (such as the AP-1 site in the collagenase promoter) that is not recognized by the receptor (29, 37, 55, 70). Although some efforts to obtain direct evidence for receptor-Fos/Jun heteromeric complexes proved unsuccessful (55), interference at the non-receptor-binding AP-1 site has been proposed to involve the formation of nonproductive, heterodimeric complexes between the receptor and Fos/Jun (29, 70). A similar mechanism of repression has been postulated for transcriptional interference between Fos/Jun and the estrogen (14), thyroid hormone (11, 71), and retinoic acid (11, 56) receptors. These data, together with the present observations on glucocorticoid receptor–OTF-1 interaction, indicate that this type of functional interference (or cross-coupling [53]) may represent a general mechanistic pathway of nuclear hormone receptors. It is noteworthy, however, that the plethora of cell type- and promoter-specific positive or negative effects of c-Jun/c-Fos on nuclear receptors has been used to argue against heterodimer formation as a mechanism to explain these effects (57). Interestingly, retinoic acid has recently been reported to down-regulate interleukin-2 expression via an octamer site (17), suggesting that the retinoic acid receptor may interfere with OTF-1 function by a mechanism similar to that observed here between OTF-1 and the glucocorticoid receptor. Moreover, while this work was in progress, overexpression of glucocorticoid receptor was reported to interfere with function of the lymphoid-specific OTF-2 factor (67). It was postulated that receptor-mediated repression of this factor is due to the exhaustion of unidentified rate-limiting coactivators. In contrast to this model, we provide evidence that the glucocorticoid receptor can produce a nonproductive complex by

direct interaction with the structurally related OTF-1 factor. We do not yet know whether this direct interaction also occurs with OTF-2. Given the considerably lower affinity of VP16 for OTF-2 versus OTF-1 (20, 32, 59), it will be important to elucidate whether a similar difference in affinity exists between the glucocorticoid receptor and OTF-1 or OTF-2.

In any case the fact that antagonism of Fos/Jun activity by the glucocorticoid receptor in vivo and in vitro (29, 37, 55, 70), as well as in vivo or in vitro repression of OTF-1 activity, requires excess amounts of receptor indicates that the possible interaction between the receptor and either Fos/Jun or OTF-1 may be of a rather weak nature. Thus, the balance between the cellular levels of glucocorticoid receptor relative to those of the OTF proteins may be critical in determining a positive versus a negative transcriptional response. In line with this model, certain host cells appear to direct a functional synergism between the glucocorticoid receptor and OTF-1 on promoters containing an octamer site in close proximity to the glucocorticoid response element (5, 54, 67).

Although the glucocorticoid receptor was overexpressed in CHO cells, it did not antagonize OTF-dependent transcriptional or DNA-binding activities in the absence of hormone. This observation may be simply explained by an extranuclear partitioning of the latent, nonactivated (non-DNA-binding) form of the receptor. Alternatively, this result could be interpreted to indicate that the structure mediating interference with OTF-1 may be masked in the latent receptor form. This form of receptor appears to be associated with the 90-kDa heat shock protein, the role of which is believed to involve both masking of the intrinsic DNA-binding activity (10 [and references therein]) and maintenance of a hormone-responsive configuration of the receptor (44). In any case, this observation opens up the interesting possibility that interference of the hormone-activated receptor is related to the release of hsp90. It should be possible to address this issue in in vitro-reconstituted experiments.

Positive and negative regulation of OTF-1 activity by protein-protein interaction: implications for OTF-1 function. Although the exact mechanism for interaction between the glucocorticoid receptor and OTF-1 is unclear, we have shown that the minimal DNA-binding domain spanning amino acids 394 to 519 of the human glucocorticoid receptor (9) exhibited a very reduced efficiency to repress the DNA-binding activity of OTF-1 in vitro. Since the glucocorticoid receptor is phosphorylated at multiple sites (25 [and references therein]) it is formally possible that the bacterially expressed DNA-binding domain of the receptor interacts with OTF-1 because of the lack of a similar posttranslational modification. However, given the interference between bacterially expressed retinoic acid receptor and c-Jun/c-Fos (56), this alternative does not appear very likely. On the other hand, our results could indicate that sequences outside the DNA-binding domain are required for repression. The role of the DNA-binding domain of the glucocorticoid receptor in mediating interference with the c-Jun/c-Fos complex has been debated, since in one study the DNA-binding domain has been reported not to be required for *trans*-repression by the receptor (29), whereas other investigators have implied that it is necessary but not sufficient for strong repression of Fos/Jun activity (55, 70). In similar fashion, the retinoic acid receptor apparently requires both the DNA-binding domain and C-terminal sequences for functional interference with c-Jun/c-Fos activity (56). In line with the observed low activity of the minimal DNA-binding domain of the glucocorticoid receptor to antagonize c-Jun/c-Fos function in vivo (55), this receptor fragment exhibited a similarly low activity to repress the DNA-binding activity of OTF-1 in vitro.

Immunoprecipitation experiments indicated that the homeodomain of OTF-1 may be important in mediating repression by the glucocorticoid receptor. Interestingly, the homeobox region which directs formation of the VP16-induced complex (59) also appears to be important for interaction with the receptor, since a mutated OTF-1 derivative which fails to generate a complex with VP16 did not associate with the purified receptor protein. Moreover, the DNA-binding activity of this mutant fusion protein was not repressed by the full-length glucocorticoid receptor in vitro. Although a detailed mutational analysis is required to delineate sequences mediating interaction with the receptor, our preliminary results raise the possibility that VP16 and the glucocorticoid receptor interact with an overlapping structural motif of the OTF-1 protein to regulate cellular transcription. It is not established whether VP16 mimics the activity of an as yet unidentified cellular analog. It has been proposed that OTF-1 does not activate target promoters independently (62) but may be modulated by as yet unidentified host cell factors. In analogy to VP16, these factors may physically interact with OTF-1. In fact, a cellular factor which selectively interacts with OTF-1 has been identified in T lymphocytes (64), and the OTF-1 POU domain has recently been shown to mediate protein-protein interaction with other OTFs (65). In conclusion, these observations suggest that OTF-1, most notably the OTF-1 POU domain and homeo subdomains, may be the site of a complex pattern of protein-protein interactions determining positive or negative regulation of OTF-1 activity.

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