Coordinate Regulation of Glucocorticoid Receptor and c-*jun* Gene Expression Is Cell Type-Specific and Exhibits Differential Hormonal Sensitivity for Down- and Up-Regulation†

Thomas J. Barrett,‡ Eva Vig, and Wayne V. Vedeckis*

Department of Biochemistry and Molecular Biology and Stanley S. Scott Cancer Center, Louisiana State University Medical Center, New Orleans, Louisiana 70112-1393

Received January 10, 1996; Revised Manuscript Received May 20, 1996⊗

ABSTRACT: We have previously proposed a novel mechanism for the coupled regulation of glucocorticoid receptor (GR) and c-*jun* transcription in triamcinolone acetonide (TA)-treated AtT-20 cells. This involved transcriptional interference of AP-1 (Fos/Jun)-driven gene transcription by the formation of inactive GR/Jun heterodimers. To further elucidate the molecular mechanism for GR autoregulation, the expression of GR and c-*jun* mRNA and protein levels were examined in both mouse L929 fibroblast cells and human CEM-C7 acute lymphoblastic leukemia cells. A rapid down-regulation of both GR and c-*jun* mRNA and protein levels occurs in TA-treated L929 cells. All-*trans*-retinoic acid (RA) treatment of Jun-deficient, mouse F9, teratocarcinoma cells causes the induction of c-*jun* expression. The increased expression of both c-*jun* mRNA and protein is accompanied by the induction of GR expression. These data further suggest that functional cJun is needed for the expression of the GR and c-*jun* genes in F9 cells. CEM-C7 cells undergo apoptosis after exposure to glucocorticoids. There is a parallel up-regulation of GR and c-*jun* mRNA levels in TA-treated CEM-C7 cells. This is accompanied by a concomitant increase in GR and cJun protein levels. Dose−response analyses reveal the expected coordinate regulation of both GR and c-*jun* mRNA and protein in L929 cells (decreasing) and in CEM-C7 cells (increasing). However, ∼20-fold less TA is required for the inhibition of GR and c-*jun* expression as compared to that required for the stimulation of these two genes. These data demonstrate that the coordinate regulation of GR and c-*jun* gene expression is dose-dependent and cell type-specific. These results, along with previously reported data, suggest that GR complex formation with itself or with another transcription factor is important for the coordinate up- and down-regulation, respectively, of the GR and c-*jun* genes.

Gene transcription is regulated by a complex hierarchy of *trans*-acting factors interacting with each other and with distinct regulatory elements (Dynan, 1989; Lin et al., 1990). One of the major signal transduction pathways results from the membrane receptor-mediated extracellular stimulation of cells. The generation of second messengers, inositol 1,4,5-triphosphate, *sn*-1,2-diacylglycerol, and Ca^{2+}, results in the subsequent activation of the protein kinase C (PKC) system [reviewed in Nishizuka (1988) and Cantley et al. (1991)]. PKC enhances activator protein-1 (AP-1,[1] Fos/Jun) activity at the posttranscriptional level by modification of Jun and Fos phosphorylation levels (Boyle et al., 1991; Adler et al., 1992). Either Jun homodimers or Fos/Jun heterodimers bind to 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-responsive elements (TRE, or AP-1 binding sites) to enhance gene transcription [reviewed in Ransone and Verma (1990)]. Another major signal transduction pathway involves the ligand-activated nuclear steroid receptor superfamily, of which the glucocorticoid receptor (GR) is a member. When bound to glucocorticoid response elements (GREs), the GR can regulate gene transcription in the positive or negative direction [reviewed in Carson-Jurica et al. (1990)].

The cellular effects of glucocorticoids are dependent upon the intracellular levels of their receptors (Bourgeois & Newby, 1977; Vanderbilt et al., 1987; Dong et al., 1990). Therefore, the factors that control the number of glucocorticoid receptors within the cell will govern its responsiveness to glucocorticoids. Evidence for GR autoregulation is quite extensive. However, the exact mechanism for GR autoregulation is unknown. Transcriptional (Okret et al., 1986, 1991; Dong et al., 1988; Rosewicz et al., 1988; Burnstein et al., 1990, 1991; Alksnis et al., 1991; Bellingham et al., 1992), posttranscriptional (Vedeckis et al., 1989; Alksnis et al., 1991), and posttranslational (McIntyre & Samuels, 1985; Dong et al., 1988; Hoeck et al., 1989) mechanisms have been implicated in autologous GR regulation. Our laboratory has proposed that GR and cJun (both potent transcription factors) are coordinately regulated and that this regulation is due to cross-talk between the GR and AP-1 signaling pathways (Vig et al., 1994). Some of the evidence that supports this hypothesis is as follows. Cross-talk between the GR and the Fos/Jun signaling pathways can occur through transcrip-

†This research was supported by NIH Grant DK47211 (W.V.V.), the LSUMC Stanley S. Scott Cancer Center (W.V.V.), and a Summer Student Research Fellowship from the Cancer Association of Greater New Orleans (T.J.B.).

* Corresponding author: Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, 1901 Perdido Street, New Orleans, LA 70112-1393. Phone: (504) 568-8175. Fax: (504) 568-6997. E-mail: WVEDEC@LSUMC.EDU.

‡ Present address: Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: GR, glucocorticoid receptor; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); RA, all-*trans*-retinoic acid; AP-1, activator protein-1; GRE, glucocorticoid response element.

S0006-2960(96)00058-X CCC: $12.00

tional interference. That is, overexpression of cJun blunts GR-mediated gene expression and overexpression of GR can block phorbol ester-mediated AP-1 gene activation (Jonat et al., 1990; Yang-Yen et al., 1990; Schüle et al., 1990). In later studies, Maroder et al. (1993) demonstrated that phorbol ester-mediated interference of GR-dependent gene transcription is cell type-specific. Northern blot and nuclear run-on analyses of AtT-20 cells treated with TA, a potent glucocorticoid analog, showed that alterations in the transcript levels for GR and c-*jun* closely paralleled each other (Vig et al., 1994). This hormone-mediated down-regulation was due to a decrease in the transcription rate of both the GR and c-*jun* genes, and it did not require new protein synthesis (Vig et al., 1994). It was proposed that the coordinate expression was a result of transcriptional regulation through a nonconsensus AP-1 site in the c-*jun* promoter (Angel et al., 1988) and a putative AP-1 site in the GR promoter (Zong et al., 1990). These data suggest that cross-talk occurs between the two signaling pathways at physiological levels of these two effector proteins and is cell type-specific.

The goal of the present study was to further explore the molecular mechanism of the coordinate regulation of GR and c-*jun* gene expression using various cell systems. In particular, these studies demonstrate that coupled expression is a fundamental event. This is true whether these genes are either down- or up-regulated. Further, we propose that the sensitivity of this coupled regulation to hormone levels is different and suggest molecular mechanisms to explain this.

MATERIALS AND METHODS

Cell Culture. AtT-20 cells were grown in Dulbecco's modified Eagle's medium F12 (DMEM F12, GIBCO, Grand Island, NY) supplemented with 10% newborn calf serum (Bio Whittaker, Walkersville, MD) as previously described (Vedeckis, 1981). L929 mouse fibroblast cells were grown in DMEM/high glucose (GIBCO) supplemented with 10% fetal bovine serum (FBS, Irvine Scientific, Santa Ana, CA). The human CEM-C7 acute lymphoblastic leukemia cells (a generous gift from Dr. E. Brad Thompson) were grown in DMEM/low glucose (GIBCO) supplemented with 10% dialyzed, heat-inactivated FBS (Irvine Scientific). F9 mouse teratocarcinoma cells were grown in gelatin-coated flasks (Grover & Adamson, 1986) with DMEM/high glucose (GIBCO) supplemented with 15% dialyzed, heat-inactivated FBS (Irvine Scientific). Cells were treated with 1 μM TA, 1 μM all-*trans*-retinoic acid (RA), or vehicle (ETOH; 0.01% final concentration) alone. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Cell viability was determined by trypan blue exclusion. For all experiments, cells were treated while in log phase growth and exhibited greater than 90% viability.

Western Blot Analysis. Total cellular proteins were isolated by suspending the cells in 10 volumes of 1 X Laemmli sample buffer [0.062 M Tris/HCl (pH 6.8), 5% glycerol, and 2% sodium dodecyl sulfate] minus β-mercaptoethanol and bromophenol blue and then sonicating the cell lysate on ice to shear the DNA. Protein was estimated using a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine γ-globulin as a standard. The lysates were flash frozen in liquid nitrogen and then stored at −80 °C.

β-Mercaptoethanol and bromophenol blue tracking dye were added to final concentrations of 1% and 5 μg/mL, respectively, prior to electrophoresis. Total cellular proteins (20 μg/lane) were separated by discontinuous sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using an 8% separating gel and a 5% stacking gel (all chemicals were from Bio-Rad). Prestained molecular weight markers (Sigma Chemical Co. SDS-7B) were loaded in adjacent lanes. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH, BA 85) and stained with Ponceau S (Sigma) to confirm uniformity of transfer. To assay for GR, the blots were incubated with 5% BSA (Sigma; A-7906) in PBS (phosphate-buffered saline) overnight at 4 °C, rinsed in PBS, and then incubated at room temperature for 1 h in the monoclonal antibody BuGR2 (a gift of Drs. R. W. Harrison and B. Gametchu) [1:100 dilution in PBS/T (phosphate-buffered saline containing 0.05% Tween-20)]. The blots were washed six times over 1 h in PBS/T, followed by incubation at room temperature for 1 h in 5% nonfat dry milk dissolved in PBS/T containing 1:2000-diluted, horseradish peroxidase-labeled rabbit anti-mouse IgG (H+L) (Zymed Laboratories, South San Francisco, CA). Blots were washed as previously described and briefly rinsed three times in Tris-buffered saline [TBS; 20 mM Tris/HCl (pH 7.4) and 0.15 M NaCl]. Monospecific, anti-peptide, polyclonal antibodies [1 μg/mL for both cFos (#sc-52) and cJun (#sc-45), Santa Cruz Biotechnology, Santa Cruz, CA; 1 μg/mL for GR (PA1-512), Affinity BioReagents Inc., Golden, CO] were used to detect cFos, cJun, and GR. Variations from the protocol above included blocking in 5% nonfat dry milk for 1 h at room temperature and incubating the polyclonal antibodies overnight at 4 °C. We also used horseradish peroxidase-labeled goat anti-rabbit IgG (H+L) (Zymed) secondary antibody at a 1:2500 dilution for all polyclonal antibodies. All blots were developed with an Enhanced Chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) using Hyperfilm TM (Amersham).

RNA Purification and Northern Blot Analysis. Total cellular RNA was isolated from L929 and CEM-C7 cells by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987). Cells were suspended in 4 M guanidinium thiocyanate, 25 mM sodium citrate, (pH 7.0), 0.5% sodium N-lauroylsarcosine, and 0.1 M β-mercaptoethanol (lysis buffer). To the lysate was added a $^1/_{10}$ volume of 2 M sodium citrate (pH 4.0), an equal volume of water-saturated phenol, and $^1/_{20}$ volume of a chloroform: isoamyl alcohol mixture (49:1) with mixing by inversion after each addition (all chemicals were from Sigma Chemical Co.). The final suspension was vigorously shaken for 10 s. The lysate was centrifuged at 10000*g* for 20 min at 4 °C and the aqueous phase transferred to a new tube. An equal volume of 2-propanol was added, and the samples were placed at −20 °C for 1 h. The samples were centrifuged at 10000*g* for 20 min at 4 °C, and the RNA pellets were resuspended in 0.3 mL of lysis buffer. The RNA was then precipitated by adding an equal volume of 2-propanol and allowing the RNA to sit at −20 °C for at least 1 h. The RNA samples were then centrifuged at 12000*g* for 20 min at 4 °C. The RNA pellets were washed with 75% ethanol, allowed to dry, and resuspended in 50 μL of 0.5% SDS. RNA preparations were flash frozen and stored at −80 °C until they were needed.

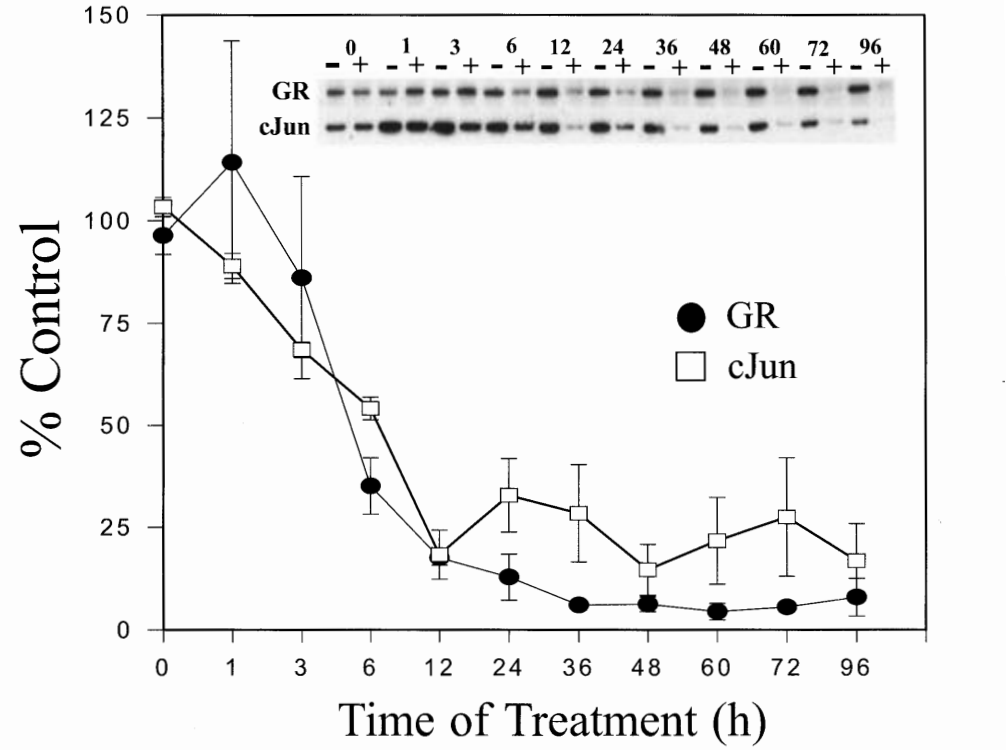

FIGURE 1: TA-dependent down-regulation of GR and cJun protein levels in L929 cells. L929 cells were treated with 1 μM TA (+) or with an ethanol vehicle (−) for the indicated times. Total cellular protein was extracted and subjected to Western blotting using the monoclonal antibody BuGR2 to detect GR and the polyclonal antibody #sc-45 for cJun. The Western blots (insets) are from a representative experiment. The graph is the average ± SEM of three experiments in which the percent of the signal obtained in the TA-treated versus vehicle-treated lanes was determined after densitometric scanning of the ECL films.

F9 total cellular RNA was isolated by suspending the cells in 4 M guanidine thiocyanate, 5 mM sodium citrate (pH 7.0), 5 mM EDTA, 0.5% sodium *N*-lauroylsarcosine, and 0.1 M β-mercaptoethanol. The lysate was layered over a cushion of 5.7 M CsCl (Sigma) and 25 mM EDTA (pH 7.0) and spun at 35 000 rpm in a Beckman SW50.1 (Beckman Instruments, Fullerton, CA) rotor for 18−20 h.

RNA samples (45 μg/lane) were separated on 1.2% agarose formaldehyde gels and transferred onto a Gene Screen *Plus* nylon membrane (Dupont/NEN, Boston, MA). Immobilized RNA samples were hybridized with random-primed (Ambion Inc., Austin, TX), α-^{32}P-labeled cDNA probes corresponding to GR-, c-*jun*-, and β-actin-specific mRNA sequences. The following probes were prepared by enzymatic digestion of host plasmid DNAs: a 1 kbp *Hin*dIII fragment of the pN10, mouse GR cDNA clone (a gift from Dr. M. Danielsen); a 1.6 kbp *Eco*R1 fragment of the pGC-R, human GR cDNA clone (a gift from Dr. P. Chambon); a 1.0 kbp *Pst*I-*Kpn*I fragment of pHJ, c-*jun* plasmid (a gift from Dr. R. Tijan); and a 1.65 kbp *Pst*I fragment of the pA1, β-actin plasmid (a gift from Dr. D. W. Cleveland). Blots were prehybridized at 42 °C in 6 X SSPE buffer containing 50% formamide, 3 X Denhardt solution, 10% dextran sulfate, and 0.5% SDS for 6−24 h. Hybridization was carried out in the same buffer but containing 100 μg/mL denatured salmon sperm DNA and 10^6 dpm/mL radiolabeled probe for 24 h. After hybridization, membranes were rinsed at 42 °C in 2 X SSPE/1% SDS and washed at 42 °C in 1 X SSPE/1% SDS (30 min). Membranes were exposed to Hyperfilm TM (Amersham). Multiple exposure times, followed by densitometric scanning and quantitation, were performed to ensure that the relative signals obtained indicated actual changes in the mRNA levels. Final results were obtained by densitometric scanning of the X-ray films with a BioMed soft laser densitometer.

Image Analysis. Reproductions of the autoradiographs were produced by scanning the images at 400 dpi resolution with a Hewlett Packard ScanJet IIcx instrument (HP DeskScan II Microsoft Windows Version). The figures were compiled and annotated using Serif PagePlus 3.0 desktop publishing software. The figures were printed using a Lexmark Optra R laser printer at 1200 dpi resolution.

RESULTS

Triamcinolone Acetonide (TA) Causes Down-Regulation of GR and cJun Protein Levels in L929 Cells. We chronically treated the mouse L929 fibroblast cell line with a potent glucocorticoid analog, triamcinolone acetonide (TA), and used the BuGR2 antibody (Gametchu & Harrison, 1984) to assay the GR protein levels and a polyclonal antibody (Santa Cruz) to assay the cJun protein levels. Dramatic decreases in both GR and cJun protein levels occurred (Figure 1). cJun protein reached its nadir at 12 h, while GR reached its minimum at 24 h; both proteins maintained their new levels (~5% of control levels) for the remainder of the experiment (84 h). Thus, chronic treatment of L929 cells caused a dramatic, time-dependent down-regulation in both GR and cJun protein levels. In contrast, cJun protein levels increased in TA-treated AtT-20 cells (Vig et al., 1994; Figure 7). This indicates that cJun protein levels are regulated differently in different cell types and points to relevant differences in the mechanisms used by these two cell types to regulate cJun levels.

Coordinate Regulation of GR and c-jun mRNA Levels Occurs in TA-Treated L929 Cells. The effects of 1 μM TA

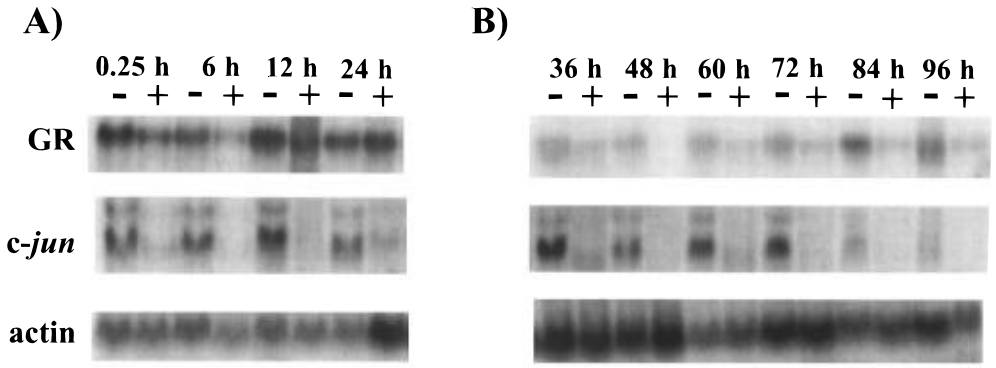

FIGURE 2: TA-dependent down-regulation of GR and c-*jun* mRNA levels in L929 cells. L929 cells were treated with 1 μM TA or with an ethanol vehicle for the indicated times. Total RNA was extracted from the cells and subjected to Northern blot analysis. The blots were cut into two portions and hybridized separately with the GR and c-*jun* cDNA probes, as described in Materials and Methods. The c-*jun* blot was stripped and rehybridized with the β-actin probe. About 15 min was required to process each sample; hence, the first time point obtained is labeled 0.25 h of treatment time. One of two experiments with similar results is shown.

treatment on the levels of GR and c-*jun* mRNA were studied using Northern blot analysis. There was a rapid decrease in both GR and c-*jun* mRNA levels (Figure 2A). Both GR and c-*jun* mRNA levels appear to remain low for the duration of TA treatment (Figure 2B). The initial coordinate down-regulation of both GR and c-*jun* mRNA levels in TA-treated L929 cells is similar to that of TA-treated AtT-20 cells (Vig et al., 1994). GR and c-*jun* mRNA levels oscillate in TA-treated AtT-20 cells (Vig et al., 1994). Due to technical problems, attempts to explore the kinetics (oscillatory or not; e.g., between 12 and 24 h of treatment) of GR and c-*jun* mRNA expression in chronically TA-treated L929 cells were inconclusive.

All-trans-retinoic Acid (RA) Induces c-jun and GR Expression in F9 Cells. To assess the potential importance of functional cJun in the regulation of GR expression, the mouse F9 teratocarcinoma cell line was treated with all-*trans*-retinoic acid (RA). F9 cells contain very little c-*jun* and GR mRNA and protein (Angel & Karin, 1991) under basal cell culture conditions. RA treatment of F9 cells causes both cellular differentiation into parietal endoderm cells and the induction of c-*jun* mRNA levels (Angel & Karin, 1991). F9 cells were treated with 1 μM RA, and the levels of GR and c-*jun* mRNA were assayed at various times. cJun protein levels were determined by Western blotting using the polyclonal antibody previously described. The 8-fold induction of c-*jun* mRNA, along with the 5-fold increase of cJun protein, was accompanied by a 2.5-fold induction of GR mRNA levels (Figure 3). Changes in GR protein levels could not be detected by Western blotting with the BuGR2 antibody or polyclonal antibody PA1-512 (data not shown). The eventual decreases in both GR and c-*jun* mRNA and protein are most likely due to the cells becoming confluent in culture and/or terminally differentiating. These data suggest that functional cJun protein may be involved in the coordinate regulation of GR and c-*jun* expression.

TA Causes Coordinate Up-Regulation of both GR and c-jun mRNA and Protein in CEM-C7 Cells. To determine if the coordinate regulation was limited to cells that either down-regulated GR (AtT-20 and L929 cells) or induced cJun (F9 cells), we selected the human CEM-C7 acute lymphoblastic leukemia cell line, which, in the presence of glucocorticoids, up-regulates GR mRNA and protein levels (Eisen et al., 1988) and undergoes apoptosis (Harmon et al., 1979). CEM-C7 cells were treated with 1 μM TA, and the levels

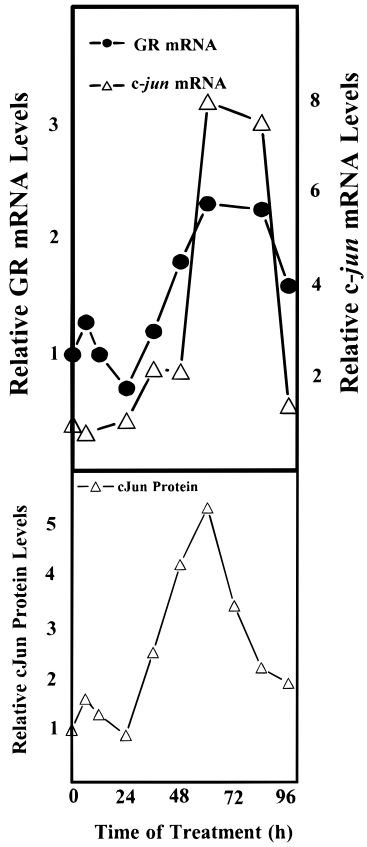

FIGURE 3: RA-mediated induction of c-*jun* and GR expression in F9 cells. F9 cells were treated with 1 μM RA or with an ethanol vehicle for the indicated times. Total RNA was extracted from the cells and subjected to Northern blot analysis (top panel) as described in Materials and Methods and the legend to Figure 2. Western blot analysis (bottom panel) and quantitation were performed as described in Materials and Methods and the legend to Figure 1.

of both GR and c-*jun* mRNA and protein levels were assayed at various times thereafter. TA treatment caused a coordinate up-regulation of both GR and c-*jun* mRNA (Figure 4) and protein levels (Figure 5A,B). The more robust induction of c-*jun* mRNA (~50-fold) versus cJun protein (~3-fold) may indicate an autoinhibitory translational arrest mechanism to counterbalance the autostimulatory effect of cJun on its own gene transcription (Angel et al., 1988). Subsequent decreases in both GR and c-*jun* mRNA (Figure 4) and protein levels (data not shown) occurred after 30 h of hormone treatment. This decrease in mRNA and protein may reflect the fact that

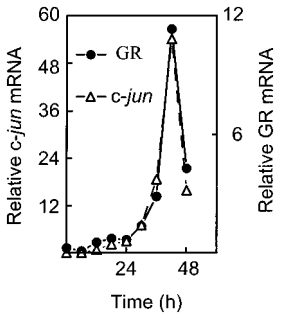

FIGURE 4: TA-dependent coordinate up-regulation of both GR and c-*jun* mRNA in CEM-C7 cells. CEM-C7 cells were treated with 1 μM TA or with an ethanol vehicle for the indicated times. Total RNA was extracted from the cells and subjected to Northern blot analysis as described in Materials and Methods and the legend to Figure 2. One of two experiments with similar results is shown.

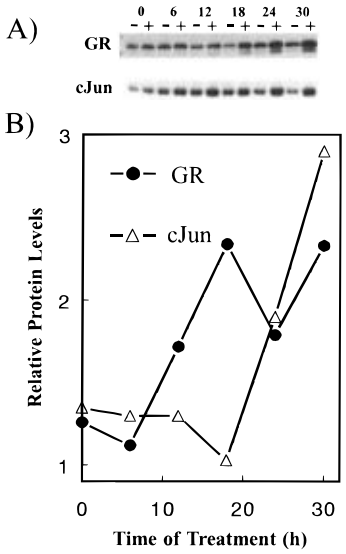

FIGURE 5: TA-dependent up-regulation of both GR and cJun protein in CEM-C7 cells. Total cellular protein was extracted and subjected to western blotting using the polyclonal antibody PA1-512 to detect GR and the polyclonal antibody #sc-45 for cJun. The Western blots (A) were densitometrically scanned and the data plotted as the percent of the signal obtained in the TA-treated versus vehicle-treated lanes (B). Panel A represents the raw Western blot data. In panel B, a nonspecific band that is not regulated by hormone was scanned in each lane, and this was used to normalize the values to account for slight variations in protein load from lane to lane. One of two experiments with similar results is shown.

TA kills CEM-C7 cells after prolonged treatment (data not shown). These data show that the coordinate regulation of GR and c-*jun* occurs in TA-treated CEM-C7 cells and that it is not limited to the process of hormone-mediated down-regulation.

TA Causes a Dose-Dependent Regulation of both GR and c-jun mRNA and Protein in L929 and CEM-C7 Cells. Dose−response experiments were performed to determine if GR down-regulation in L929 cells and GR up-regulation in CEM-C7 cells were dependent upon the dose of the glucocorticoid analog, TA. L929 and CEM-C7 cells were treated with varying doses of TA for 24 and 18 h, respectively. Each set of curves represents both GR and c-*jun* mRNA or GR and cJun protein levels assayed in the respective cell lines (Figure 6). As expected, TA caused a dose-dependent coordinate down-regulation of both GR and c-*jun* mRNA and protein levels in L929 cells (Figure 6A,C). Repeated experiments showed that half-maximal down-regulation occurred at 1−5 nM TA. In CEM-C7 cells, a dose-dependent coordinate up-regulation of both GR and c-*jun* mRNA and protein (Figure 6B,D) also occurred. Repeated experiments showed that half-maximal stimulation occurred at 20−100 nM TA. This 20-fold difference in dose response suggests that there is a fundamental difference in the molecular mechanisms used by these cells to coordinately regulate GR and c-*jun* expression (see Discussion).

Heterogeneity of GR and cJun Regulation in Different Cell Lines. Although cross-talk between GR and c-*jun* gene expression is evident, variations occur dependent upon the particular cell type. Thus, Western blot analysis (Figure 7) using monospecific, anti-peptide, polyclonal antibodies to GR (Affinity BioReagents, PA1-512) and cJun (Santa Cruz, #sc-45), which cross-react with both human and mouse forms of the proteins, were used to investigate GR and cJun protein expression in TA- and vehicle-treated AtT-20, L929, and CEM-C7 cells. Upon hormone treatment (24 h), there is a 50−60% decrease in GR protein levels in AtT-20 and L929 cells, whereas GR protein levels increased by 50% in TA-treated CEM-C7 cells. cJun protein levels undergo a 2-3-fold induction during hormone treatment in AtT-20 and CEM-C7 cells, while hormone treatment caused a 60% decrease in cJun protein levels in L929 cells. cFos protein levels did not change in these cells (data not shown). An uncloned glucocorticoid-resistant population of cells, NRCEM (nonresponsive CEM-C7), which was derived from parental CEM-C7 cells, shows little or no change in both GR and cJun levels, indicating that a functional hormonal response is required for the induction of GR and cJun levels. These studies indicate that cell type-specific differences for GR/cJun cross-talk occur. Further studies are required to determine if, as suggested previously (Maroder et al., 1993), the relative ratios of the GR, cJun, and perhaps other AP-1 family members are reponsible for the observed differences seen in various cell types.

DISCUSSION

The present study examines ways in which GR gene expression is regulated. We have previously proposed that the tightly coupled regulation of GR and c-*jun* gene expression in TA- or RA-treated AtT-20 cells is mediated by the AP-1 sites located in the promoters of their respective genes (Vig et al., 1994). The present studies show that the coordinate regulation of GR and c-*jun* gene expression also occurs in TA-treated L929 and CEM-C7 cells, although the interactions between AP-1 and the hormone−receptor signaling pathways show cell type specificity. In the case of TA-treated L929 cells, there is a comparable down-regulation of GR protein levels to that observed in TA-treated AtT-20 cells [Figures 1 and 7 and Vig et al. (1994)]. There appears to be a similar initial down-regulation of GR and c-*jun* mRNA levels in TA-treated L929 cells (Figure 2) to that of TA-treated AtT-20 cells (Vig et al., 1994). The difference in the regulation of cJun protein levels (up in AtT-20 cells but down in L929 cells) could be due to a lack of release of translational inhibition in TA-treated L929 cells, as compared to the proposed TA-mediated augmentation of cJun translation in AtT-20 cells [Figure 7 and Vig et al. (1994) and Angel et al. (1988)]. Sharp decreases in cJun protein levels in TA-treated L929 cells could cause a sustained decreased GR and c-*jun* gene expression, while the increase of cJun protein in hormone-treated AtT-20 cells could result in the rebound in

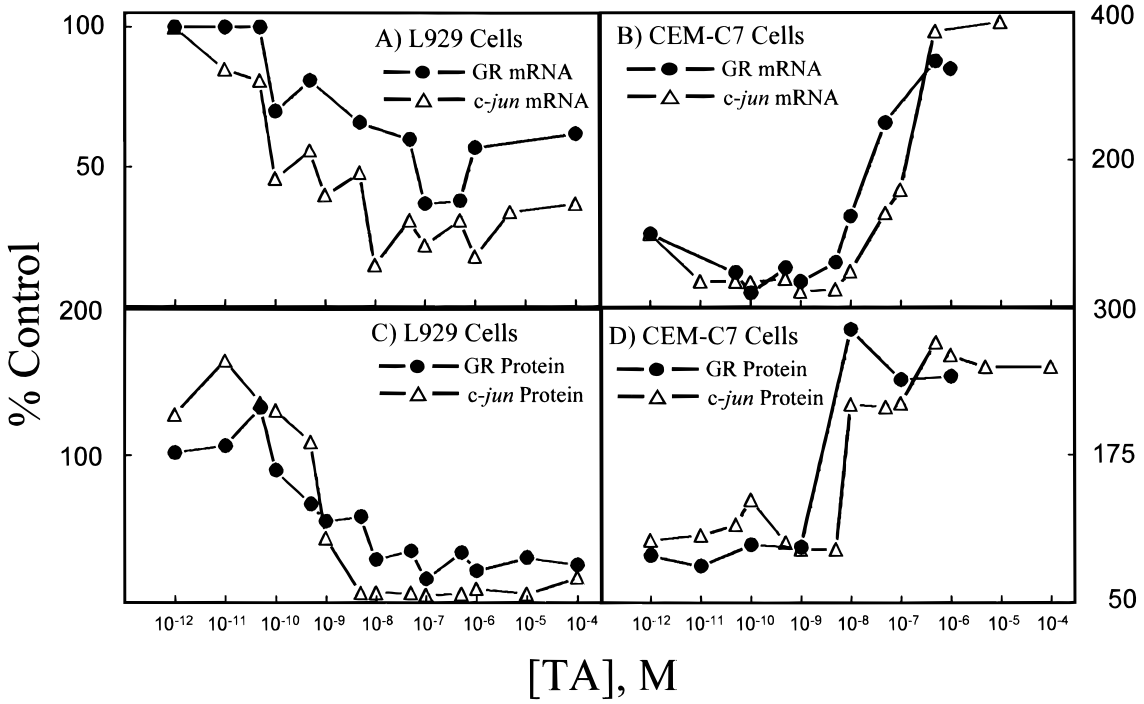

FIGURE 6: Dose-dependent regulation of both GR and c-*jun* mRNA and protein in L929 and CEM-C7 cells. L929 and CEM-C7 cells were treated for 24 and 18 h, respectively, with the indicated concentrations of TA. Total RNA was extracted from both L929 and CEM-C7 cells and analyzed by Northern blotting (panels A and B) for GR and c-*jun* as described previously in the legend to Figure 2 and Materials and Methods. Western blot analysis (panels C and D) of GR (BuGR2 antibody for L929 cells; PA1-512 for CEM-C7 cells) and cJun and quantitation were performed as described in Materials and Methods and the legend to Figure 1. Each curve is a representative experiment (from three total) where either both GR and c-*jun* mRNA levels were analyzed or both GR and cJun protein levels were determined in the same cell population.

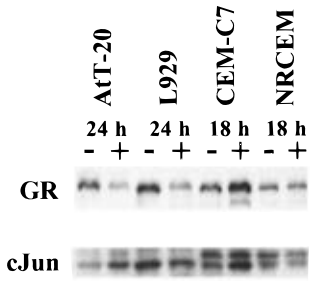

FIGURE 7: Relative GR and Jun protein levels in TA-treated AtT-20, L929, CEM-C7, and NRCEM cells. Western blot analyses using polyclonal antibodies to GR (Affinity BioReagents, PA1-512) and cJun (Santa Cruz, #sc-45) that cross-react with both human and mouse forms of the proteins were used to compare the immunogenic levels of protein in TA-treated AtT-20, L929, CEM-C7, and NRCEM cells. These experiments were performed using an enhanced chemiluminescence kit (Amersham). Shown are the relative protein levels for GR and cJun at 1 min of exposure under identical conditions.

GR and c-*jun* mRNA levels at later times after TA treatment (Vig et al., 1994). However, in both cases, alterations in cJun protein levels correlate with changes in GR and c-*jun* mRNA levels. This supports the contention that functional AP-1 complex binding to the AP-1 sites in the promoters of both genes is involved in the coupled regulation that is observed.

RA treatment of mouse F9 cells causes an increase in functional intracellular AP-1 protein complexes. This could then drive the coordinate increased transcription of both the GR and cJun genes, again, because of the binding of AP-1 sites in the promoters of both genes. Furthermore, recent studies indicate that serum stimulation of serum-starved NIH 3T3 cells, which causes a rapid transient induction in c-*jun* and c-*fos* mRNA and protein levels, causes a concomitant or slightly delayed increase in GR mRNA levels (P. Wei and W. V. Vedeckis, unpublished observations). This further supports the concept that the induction and/or activation of AP-1 activity results in increased GR gene expression.

The data presented here show a coordinate increase in GR and c-*jun* mRNA and protein levels in CEM-C7 cells after glucocorticoid treatment. The c-*jun* mRNA results contradict observations made by Maroder et al. (1993). The reasons for this discrepancy can be twofold. First, the tissue culture and hormone treatment conditions are not identical [see Materials and Methods in Maroder et al. (1993) and this paper]. Second, the increases in c-*jun* mRNA and protein reported here occur at later times than those investigated by Maroder et al. (1993). Increases in hGR mRNA and protein levels appear to precede c-*jun* mRNA and protein increases. The observation that increased GR protein levels did not decrease GR and c-*jun* gene expression would appear to contradict the proposed transcriptional interference model. This can be explained as a cell-specific variation in promoter utilization, where the increase in cJun levels results in the formation of active AP-1 complexes to such an extent that the potential interference caused by hGR is overridden. For example, the increase in c-*jun* expression could be maintained by a positive effect exerted through the putative GRE located 5′ of position −1600 of the c-*jun* promoter (Jonat et al., 1990). Alternatively, there may be a GRE in a human GR, T cell-specific promoter or in one of the more than seven human GR gene promoters that may be used (Denton et al., 1993). A T cell-specific promoter has previously been demonstrated in mouse T lymphoma cells (Strähle et al., 1992). Human GR could then feed-forward stimulate hGR expression which could then stimulate c-*jun* expression from

the GRE in the c-*jun* promoter. Increased cJun could then feed-forward stimulate c-*jun* and hGR expression through the AP-1 sites in both promoters. Overall, differential promoter utilization for both the GR and c-*jun* genes in T lymphocytes (resulting in increases in hGR and cJun protein levels) would insure that CEM-C7 cells undergo their proper physiological response, glucocortieoid-mediated apoptosis. cJun expression appears to be critical for glucocorticoid-mediated apoptosis in CEM-C7 cells (Zhou & Thompson, 1996).

Dose-response studies by Jonat et al. (1990) suggest that the hormone dose required for AP-1-mediated reporter plasmid inhibition was 1 order of magnitude below that necessary to stimulate expression of a reporter gene driven by the MMTV-LTR. Our data show that half-maximal inhibition of both GR and c-*jun* mRNA and protein levels in L929 cells occurs at 1−5 nM TA, while half-maximal stimulation of both hGR and c-*jun* mRNA and protein levels in CEM-C7 cells occurs at 20−100 nM TA. Denton et al. (1993) showed that a progressive increase in hGR gene expression occurred with progressively higher concentrations of dexamethasone in 6TG1.1 cells (a subclone of the dex^s human leukemic cell line CEM-C7), with a half-maximal response observed between 10−100 nM dexamethasone. There was a similar dose response observed for hGR protein (Denton et al., 1993). Thus, our results on CEM-C7 cells are similar to those in 6TG1.1 cells with respect to hGR dose-dependent up-regulation.

Because the down-regulation of GR and c-*jun* expression is about 20-fold more sensitive to TA than GR and c-*jun* up-regulation, the molecular mechanism involved in these two processes appears to be fundamentally different. Ours and other data (Jonat et al., 1990; Yang-Yen et al., 1990, 1991; Schüle et al., 1990; Kerppola et al., 1993; Pfahl, 1993) suggest that the formation of the inhibitory GR/AP-1 complexes occurs at a lower hormone concentration because GR hormone-mediated AP-1 interference does not require the activation of two molecules of GR. Conversely, the GR-mediated induction of gene expression (e.g., that of c-*jun* in TA-treated CEM-C7 cells) requires two activated GR molecules to form homodimers to stimulate gene transcription from a positive GRE [reviewed in Wright et al. (1992)]. Liu et al. (1995) demonstrated that transrepression by GR/AP-1 (using mutant or normal GR) of the AP-1 inducible collagenase promoter is 100-fold more sensitive to dexamethasone than transactivation of a MMTV promoter by normal GR. This suggests that inhibition of AP-1 by GR/AP-1 interactions (pseudo-first-order) kinetically occurs at concentrations of activated GR that are far below the levels of activated GR necessary to form stimulatory GR/GR complexes (a second-order reaction).

Numerous studies have demonstrated that some activated GR is necessary for both hormone-mediated transrepression of AP-1 and transactivation of GRE, but the absolute number of receptors required for either one of these functions is unclear. Studies by Jonat et al. (1990), Liu et al. (1995), and Maroder et al. (1993) suggest that the absolute number of receptors is not as important as the cell-specific differential balance of Fos/Jun and GR levels. Heck et al. (1994) have demonstrated that repression of AP-1 activity is a function of GR monomers, while DNA binding and activation of glucocorticoid-regulated promoters require GR dimerization. cJun is important for mediating AP-1 activity, and this balance can be upset upon hormone treatment, resulting in the variable regulation of AP-1 activity. Alternatively, the formation of DNA-bound GR/Jun heteromeric complexes incapable of recruiting the proper components necessary to promote transcription could also occur (König et al., 1992).

Undoubtedly, there are additional levels of complexity that regulate GR and c-*jun* expression. Other heteromeric complexes could potentially form between the nuclear receptors and other transcription factors, such as Fos-related antigens (Fra's), JunB, JunD, and CREB [reviewed in Angel and Karin (1991) and Hill and Treisman (1995)]. Further studies are needed to address the potential importance, if any, of these transcription factors in the coordinate regulation of GR and c-*jun* expression. Although we have focused on a potential mechanism involving GR/AP-1 heterodimers and transcriptional interference, these direct interactions are not the only ones possible. For example, binding of the GR to a coactivator of AP-1 could remove an essential component necessary to efficiently assemble the important basal transcriptional machinery. The recent discovery of a coactivator (steroid receptor coactivator-1, SRC-1) for the steroid hormone receptors, including the GR (Oñate et al., 1995), argues that crucial undiscovered transcription factors and coactivators remain to be found.

A detailed analysis of promoter function of the GR and c-*jun* gene and studies on physiological, intracellular, GR/Jun protein/protein interactions are necessary and are ongoing. However, the studies presented here indicate that the GR and AP-1 signaling pathways are coupled whether these factors are down-regulated or up-regulated by various effectors, in different cell types. Recent studies in our laboratory showing that the putative AP-1 site in the GR promoter binds the AP-1 transcription factor (Breslin & Vedeckis, 1996) further support the crucial role of GR/AP-1 cross-talk in regulating the coordinate regulation of gene expression.

In conclusion, these studies are the first to analyze the differential regulation of GR expression in cells that either down- or up-regulate the GR after glucocorticoid treatment. They also are the first to show that GR and c-*jun* expression are coordinately regulated irrespective of the direction of regulation. The differential sensitivity of glucocorticoid-mediated down-regulation may be significant. Thus, at low hormone concentrations (∼1−5 nM), gene repression (e.g., anti-AP-1; inhibitory) via transcriptional interference may be favored, while at higher concentrations (20−100 nM), gene activation (glucocorticoid-responsive; stimulatory) may predominate. A sequential inhibition of expression of one gene set, followed by a stimulation of a second gene set, is one way in which a gene expression program could be controlled. Finally, together with results presented elsewhere (Zhou & Thompson, 1996), these studies are the first to implicate the importance of the coordinate up-regulation in both GR and c-*jun* gene expression in the physiologically relevant processes of apoptosis in T cell leukemic blasts.

NOTE ADDED IN PROOF

During typesetting, a paper appeared [Kamei, Y., et al. (1996) *Cell 85*, 403−414] which indicated a crucial role for CREB-binding protein (CBP) in GR-mediated up-regulation of gene expression and in down-regulation of AP-1 activity. This does not change the fundamental observations or

conclusions of the present paper. Rather, they confirm the alternative hypothesis presented in the Discussion, that is, GR binding of a coactivator of AP-1 (CBP) is likely to be the mechanism for GR-mediated transcriptional interference of AP-1 activity. The kinetic argument for the different dose responsiveness of down- and up-regulation by glucocorticoids still is valid, although GR/CBP (rather than GR/cJun) complex formation probably is responsible for the observed effect.

REFERENCES

Adler, V., Franklin, C. C., & Kraft, A. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5341−5345.

Alksnis, M., Barkhem, T., Strömstedt, P.-E., Ahola, H., Kutoh, E., Gustafsson, J-Å., Poellinger, L, & Nilsson, S. (1991) *J. Biol. Chem. 266*, 10078−10085.

Angel, P., & Karin, M. (1991) *Biochim. Biophys. Acta 1072*, 129−157.

Angel, P., Hattori, K., Smeal, T., & Karin, M. (1988) *Cell 55*, 875−885.

Bellingham, D. L., Sar, M., & Cidlowski, J. A. (1992) *Mol. Endocrinol. 6*, 2090−2102.

Bourgeois, S., & Newby, R. F. (1977) *Cell 11*, 423−430.

Boyle, W. J., Smeal, T., Defize, L. H., Angel, P., Woodgett, J. R., Karin, M., & Hunter, T. (1991) *Cell 64*, 573−557.

Breslin, M. B., & Vedeckis, W. V. (1996) *Endocrine 4* (in press).

Burnstein, K. L., Jewell, C. M., & Cidlowski, J. A. (1990) *J. Biol. Chem. 265*, 7284−7291.

Burnstein, K. L., Bellingham, D. L., Jewell, C. M., Powell-Oliver, F. E., & Cidlowski, J. A. (1991) *Steroids 56*, 52−58.

Cantley, L. C., Auger, K. A., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Solotoff, S. (1991) *Cell 64*, 2281−2302.

Carson-Jurica, M. A., Schrader, W. T., & O'Malley, B. W. (1990) *Endocr. Rev. 11*, 201−220.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156−159.

Denton, R. R., Eisen, L. P., Elsasser, M. S., & Harmon, J. M. (1993) *Endocrinology 133*, 248−256.

Dong, Y., Poellinger, L., Gustafsson, J.-Å., & Okret, S. (1988) *Mol. Endocrinol. 2*, 1256−1264.

Dong, Y., Cairns, W., Okret, S., & Gustafsson, J.-Å. (1990) *J. Biol. Chem. 265*, 7526−7531.

Dynan, W. S. (1989) *Cell 58*, 1−4.

Eisen, L. P., Elsasser, M. S., & Harmon, J. M. (1988) *J. Biol. Chem. 263*, 12044−12048.

Gametchu, B., & Harrison, R. W. (1984) *Endocrinology 114*, 274−279.

Grover, A., & Adamson, E. D. (1986) *In Vitro Cell. Dev. Biol. 22*, 280−284.

Harmon, J. M., Norman, M. R., Fowlkes, B. J., & Thompson, E. B. (1979) *J. Cell. Physiol. 98*, 267−278.

Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P., & Cato, A. C. B. (1994) *EMBO J. 13*, 4087−4095.

Hill, C. S., & Treisman, R. (1995) *Cell 80*, 199−211.

Hoeck, W., Rusconi, S., & Groner, B. (1989) *J. Biol. Chem. 264*, 14396−14402.

Jonat, C., Rahmsdorf, H. J., Park, K.-K., Cato, A. C. B., Gebel, S., Ponta, H., & Herrlich, P. (1990) *Cell 62*, 1189−1204.

Kerppola, T. K., Luk, D., & Curran, T. (1993) *Mol. Cell. Biol. 13*, 3782−3791.

König, H., Ponta, H., Rahmsdorf, H. J., & Herrlich, P. (1992) *EMBO J. 11*, 2241−2246.

Lin, Y. S., Carey, M., Ptashne, M., & Green, M. R. (1990) *Nature 345*, 359−361.

Liu, W., Hillmanag, A. G., & Harmon, J. M. (1995) *Mol. Cell. Biol. 15*, 1005−1013.

Maroder, M., Farina, A. R., Vacca, A., Felli, M. P., Meco, D., Screpanti, I., Frati, L., & Gulino, A. (1993) *Mol. Endocrinol. 7*, 570−584.

McIntyre, W. R., & Samuels, H. H. (1985) *J. Biol. Chem. 260*, 418−427.

Nishizuka, Y. (1988) *Nature 334*, 661−665.

Okret, S., Poellinger, L., Dong, Y., & Gustafsson, J.-Å. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5899−5903.

Okret, S., Dong, Y., Brönnegård, M., & Gustafsson, J.-Å. (1991) *Biochimie 73*, 51−59.

Oñate, S. A., Tsai, S. Y., Tsai, M. J., & O'Malley, B. W. (1995) *Science 270*, 1354−1357.

Pfahl, M. (1993) *Endocr. Rev. 14*, 651−658.

Ransone, R. J., & Verma, I. M. (1990) *Annu. Rev. Cell Biol. 6*, 539−557.

Rosewicz, S., McDonald, A. R., Maddux, B. A., Goldfine, I. D., Miesfeld, R. L., & Logsdon, C. D. (1988) *J. Biol. Chem. 263*, 2581−2584.

Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M., & Evans, R. M. (1990) *Cell 62*, 1217−1226.

Strähle, U., Schmidt, A., Kelsey, G., Stewart, A. F., Cole, T. J., Schmid, W., & Schütz, G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6731−6735.

Vanderbilt, J. N., Miesfeld, R., Maler, B. A., & Yamamoto, K. R. (1987) *Mol. Endocrinol. 1*, 68−74.

Vedeckis, W. V. (1981) *Biochemistry 20*, 7237−7245.

Vedeckis, W. V., Ali, M., & Allen, H. R. (1989) *Cancer Res. (Suppl.) 49*, 2295s−2302s.

Vig, E., Barrett, T. J., & Vedeckis, W. V. (1994) *Mol. Endocrinol. 8*, 1336−1346.

Wright, K. D., Wright, A., Carlstedt-Duke, J., & Gustafsson, J.-Å. (1992) *J. Steroid Biochem. Mol. Biol. 41*, 249−272.

Yang-Yen, H.-F., Chambard, J. C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J., & Karin, M. (1990) *Cell 62*, 1205−1215.

Yang-Yen, H.-F., Zhang, X.-K., Graupner, G., Tzukerman, M., Sakamoto, B., Karin, M., & Pfahl, M. (1991) *New Biol. 3*, 1206−1219.

Zhou, F., & Thompson, E. B. (1996) *Mol. Endocrinol. 10*, 306−316.

Zong, J., Ashraf, J., & Thompson, E. B. (1990) *Mol. Cell. Biol. 10*, 5580−5585.

BI960058J