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The Macromolecular State of the Transcription Factor E2F and Glucocorticoid Regulation of c-*myc* Transcription*

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Glucocorticoids inhibit transcription of the proto-oncogene c-*myc* in lymphoid cells of thymic origin. To determine if this effect is associated with changes in the properties of the transcription factor E2F, extracts were prepared from control and glucocorticoid-treated P1798 murine T lymphoma cells, and the macromolecular state of E2F was assessed by gel-mobility shift. Control extracts exhibit two predominant gel-mobility shift entities of which one corresponds to "free" E2F. A second entity, complex C, has properties similar to those described for the complex containing E2F, p107, cyclin A, and Cdk2. Complex C disappears after addition of dexamethasone and is replaced by complex D. The mobility of this complex and its sensitivity to SV40 T antigen suggest that complex D corresponds to an E2F·p105$^{Rb\text{-}1}$ complex. Extracts from control and glucocorticoid-treated cells yield identical DNase I protection patterns on the c-*myc* P2 promoter. Furthermore, such extracts transcribe the c-*myc* P2 promoter *in vitro* with equal activity. The relative abundance of the E2F complexes was measured after addition of dexamethasone. Complex C disappears as cells withdraw from S phase, and complex D appears at this time. The genes encoding thymidine kinase (*Tk-1*) and p34^{cdc2} (*cdc2*) are regulated with kinetics similar to those observed for changes in the macromolecular state of E2F. However, regulation of c-*myc* expression occurs long before any change in E2F. The macromolecular state of E2F may regulate expression of genes at the G_1/S boundary. However, the data are not consistent with the hypothesis that association of E2F with tumor suppressor gene products such as p107 or p105$^{Rb\text{-}1}$ is relevant to glucocorticoid regulation of c-*myc* transcription.

Glucocorticoids inhibit proliferation and, in some cases, cause death of lymphoid cells of thymic origin (reviewed in Refs. 1–4). The proto-oncogene c-*myc* is involved, at least in part, in this response (5–7). The mRNA encoding p64^{myc} decreases in glucocorticoid-treated leukemia and lymphoma cells (5–9). The decrease is due to inhibition of transcription (6), which can be observed within a few minutes after addition of dexamethasone to mid-log cultures of P1798 murine T-lymphoma cells (9). We have studied this P1798 cell line extensively because, unlike most T cell lymphoma and leukemia lines, P1798 cells do not die when treated with glucocorticoids (6, 9). One can study glucocorticoid regulation of gene expression without fear that the observations that are made reflect manifestations of cell death.

Our immediate goal is to determine the mechanism that accounts for glucocorticoid inhibition of c-*myc* transcription. It has recently been suggested that the products of certain tumor suppressor genes, in concert with a c-*myc* transcription factor, may regulate cell cycle-dependent c-*myc* activity (10, 11). The c-*myc* promoter contains binding sites for the cellular transcription factor E2F (12). The human promoter contains two E2F-binding sites and the mouse promoter one site located between the two major promoters P1 and P2. Mutations in the E2F site inhibit basal transcription of c-*myc* and obviate regulation (12, 13).

Regulation of E2F activity has become a topic of great interest since the initial discovery that this transcription factor binds to the product of the tumor suppressor gene *Rb-1* (for review, see Refs. 14 and 15). E2F associates with p105$^{Rb\text{-}1}$ in a cell cycle-dependent manner (16–18). It has also been determined that E2F will bind to a complex that contains the Rb-related p107 protein plus cyclin A and the cyclin-dependent kinase Cdk2 (16–19). In co-transfection assays, it has been shown that ectopic expression of p105$^{Rb\text{-}1}$ will block the ability of E2F to activate the adenovirus E2e promoter (13, 20). Overexpression of p105$^{Rb\text{-}1}$ will also inhibit c-*myc* expression (13), suggesting that association between E2F and p105$^{Rb\text{-}1}$ may play a physiological role in regulation of c-*myc* transcription. This proposition led us to wonder to what extent glucocorticoids might effect the association between p105$^{Rb\text{-}1}$ and E2F and what role such macromolecular associations might have in glucocorticoid regulation of gene expression in T cells.

EXPERIMENTAL PROCEDURES

Materials—Radioactive nucleotides were purchased from DuPont NEN. Fetal bovine serum and RPMI 1640 medium were obtained from Flow Laboratory (McLean, VA). Reagent-grade chemicals were obtained from Sigma. ω -Aminohexyl-agarose was purchased from Sigma.

The plasmid pD305neo, containing the SV40 large T antigen gene under the control of the mouse mammary tumor virus long terminal repeat plus the *neo* selectable marker (21), was obtained from Jerry Shay of the University of Texas Southwestern Medical Center. All synthetic oligonucleotides were obtained from Genesys (Houston, TX). The sequence of the E2F oligonucleotide was as described (19). The thymidine kinase oligonucleotide corresponds to −55 to −30 bp[1] (relative to the translational start site) of the mouse *Tk-1* promoter (22). The se-

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[1] The abbreviations used are: bp, base pair(s); *tk*, thymidine kinase; in the absence of any systematic nomenclature, we will adhere to the following convention for mammalian genes, irrespective of the species: the names of all genes will be given in italics with the first letter lower case (*e.g.* c-*myc*). The products of the genes will bear the same name as the gene, but not in italics with the first letter capitalized (*e.g.* c-Myc mRNA or c-Myc protein). In some cases, proteins are identified by molecular mass and gene name is superscript. For example, p105$^{Rb\text{-}1}$ is the 105-kDa product of the retinoblastoma susceptibility gene *Rb-1*.

quence of these oligonucleotides is as follows: thymidine kinase, 5′-TCTGTCGAAATTTTCCACCACGGACTCT; E2F, 5′-GATCCACTAGTTTCGCGCCCTTTCTAG.

Cell Culture—Murine P1798 lymphoma cells were grown in suspension, as previously described (23), and mid-log phase cultures containing about 5×10^5 cells/ml were used for all experiments. Labeling with [^{3}H]thymidine was carried out as previously described (6). The SV40 T antigen expression vector was introduced in P1798 cells by electroporation. Exponentially growing cells (10^7) were washed with HBS (containing 20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 5 mM glucose). The cells were suspended in 0.5 ml of HBS with 10 μg of linearized plasmid DNA. After incubation on ice for 15 min, the cells were electroporated at 1,200 V and 25 microfarad using a Bio-Rad Gene Pulser. The cells were chilled on ice for 15 min and transferred to T25 tissue culture flasks containing 10 ml of medium with 5% fetal bovine serum. Two days after transfection, G418 (Sigma) was added to 400 μg/ml. G418-resistant populations emerged after 6–8 weeks.

E2F Binding Assay—Both nuclear and whole cell extracts were used for E2F binding assays. Both techniques give extracts with similar E2F-binding properties, and the whole cell extraction procedure was convenient for preparation of large numbers of extracts from small numbers of cells (as in time course experiments). Nuclear extracts were prepared according to the protocol described by Dignam *et al.* (24). Whole cell extracts were prepared by the freeze-thaw techniques described originally by Kumar and Chambon (25). Briefly, about 3×10^6 cells were washed with phosphate-buffered saline and suspended in 50 μl of an hypertonic buffer containing 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 25% glycerol. The cells were mixed by vortex for 15 s and frozen at −70 °C. The samples were thawed on ice, mixed by vortex for 30 s, and insoluble material was sedimented by centrifugation at 20,000 × *g* for 30 min at 4 °C. The protein content of the supernatant was about 10 mg/ml, as determined by the Bradford assay (26).

The binding reaction mixture contained 50 mM KCl, 10 mM HEPES, pH 8.0, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 μg of salmon sperm DNA, 12% glycerol, 5% Ficoll (M_r 400,000), about 20,000 ^{32}P cpm of 5′-end-labeled oligonucleotide (20 fmol), and 5–10 μg of protein in 25 μl. Reactions were carried out for 15 min at room temperature and the mixture was loaded on a 5% acrylamide gel. Electrophoresis was carried out in 0.25 × TBE (1 × TBE contains 50 mM Tris, pH 8.2, 1 mM EDTA, 50 mM boric acid) at 10 V/cm. Electrophoresis was carried out for 1 h before the samples were loaded. After electrophoresis, the gels were dried and exposed to Kodak X-AR film with intensifying screens. Autoradiograms were quantified using the Applied Imaging Lynx Digital Imaging System.

Preparation of Extracts for Transcription and Footprinting—P1798 cells were washed by centrifugation in phosphate-buffered saline. The washed cell pellet was weighed and suspended in 0.5–1 ml of phosphate-buffered saline/g of cells. About 5 ml of ice-cold cell lysis buffer (2.5 mM $MgCl_2$, 0.00008% Nonidet P-40) was added per gram of cells. This suspension was held in ice/H_2O for 2–3 min. The lysate was centrifuged at 3,000 rpm for 3 min at room temperature in a table top centrifuge. The supernatant was carefully decanted and the viscous nuclear pellet was lysed in cold nuclear lysis buffer (about 10 ml/g cells) while the nuclear pellet was being vortexed. The nuclear lysis buffer was prepared by adding 1 volume of saturated $(NH_4)_2SO_4$ at 4 °C to 9 volumes of 20 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM Na_2EDTA, 2 mM Na_2EGTA, 2 mM dithiothreitol, and 25% glycerol (v/v) (27). The nuclear lysate was rocked on a wheel-roller for 20–30 min at 4 °C and sedimented by centrifugation at 4 °C for 20–30 min at 40,000 rpm (Beckman SW40 rotor). The supernatant was transferred to a 13-ml tube. Solid ammonium sulfate (0.4 g/ml) was added to the supernatant and the sample was rocked on a wheel-roller for 15–30 min at 4 °C. The precipitate was collected by centrifugation at 15,000 rpm for 10 min at 4 °C (Beckman JA20 rotor). The pellet was dissolved in about 2 ml/g of pellet of 0.1 M KCl/HGED (HGED contains 20 mM HEPES, pH 7.9, 0.2 mM Na_2EDTA, 0.2 mM Na_2EGTA, 0.5 mM dithiothreitol, and 20% glycerol). The soluble nuclear extract was dialyzed against about 100 volumes of 0.1 M KCl/HGED at least 3 times, for at least 90 min each, at 4 °C. The equilibration was measured by conductivity. The extract was cleared by centrifugation in a table top centrifuge at 14,000 rpm for 10–15 s. Nuclear extracts could be frozen at −70 °C before fractionation on ω-aminohexyl-agarose column.

All fractionation procedures were performed at 4 °C. Crude nuclear extract was applied to an ω-aminohexyl-agarose column (capacity ≥6 mg of protein/ml column) equilibrated in 0.1 M KCl/HGED. The column was washed with 0.1 M KCl/HGED until the flow-through protein peak was completely eluted. Normally, 4 column volumes of buffer were needed. Then the column was eluted with 0.4 M KCl/HGED to collect the active portion (HexII) of the crude nuclear extract. The peak fractions ($OD_{280} \geq 0.1$) of 0.4 M KCl/HGED eluate were pooled and diluted with 1 volume of HGED or dialyzed to reduce the concentration of KCl below 0.25 M before solid ammonium sulfate (0.4 g/ml) was added. The sample was rocked on a wheel-roller for 20–30 min. The precipitate was collected by centrifugation at 15,000 rpm for 10 min (Beckman, JA20 rotor). The pellet was dissolved in 0.1 M KCl/HGED (about 2 ml/g of pellet) and was dialyzed against about 100 sample volumes of 0.1 M KCl/HGED for at least 3 times, at least 90 min each. This fraction was designated as HexII.

Transcription in Vitro—The template plasmid pMYCP2GF4 was constructed by ligation of the mouse *c-myc* promoter-containing fragment of −8 to +155 bp with respect to P1 transcription initiation site into a G-free cassette vector $pRVC_2AT$ which contains an *Eco*RV site at the 5′-end of a G-free cassette of 379 nucleotides. The *c-myc* P2 promoter initiates inside the G-free cassette of pMYCP2GF4 giving rise to an authentic transcript of 369 nucleotides (9). pAdGF was constructed by ligating the *Hin*dIII-*Eco*RI adenovirus major late-G-free containing fragment of $pMLC_2AT$ (28) into the *Hin*dIII-*Eco*RI sites of pBS$^+$. This template gives rise to an authentic transcript of 386 nucleotides.

The transcription reaction (30 μl) contained 66.7 mM KCl, 13.3 mM HEPES, pH 7.9, 6.6 mM $MgCl_2$, 13.3% glycerol, 0.13 mM Na_2EDTA, 0.13 mM Na_2EGTA, 1.3 mM dithiothreitol, 360 μM ATP and CTP, 60 μM 3′-*O-methyl*-GTP, 10–20 units of RNase T_1, 18 μM UTP, and 10 μCi of [α-^{32}P]UTP (800 Ci/mmol). Routinely, the reaction contained 1 pmol of circular DNA template and about 50 μg of HexII protein. The reaction was carried out at 30 °C for 60 min. The reaction was terminated by adding 100 μl of 1.25 M $NH_4CH_3CO_2$ containing about 50 μg/ml tRNA. This mixture was extracted once with phenol/chloroform (1:1, v/v). The phenol was saturated with 10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA. The aqueous phase was mixed with 250 μl of ethanol and RNA was precipitated in a dry ice/ethanol bath. RNA was sedimented by centrifugation in a microcentrifuge for 15 min at 12,000 rpm. The RNA pellet was dissolved in 10 μl of 10 M urea in TBS (containing 90 mM Tris, 90 mM boric acid, 0.05% SDS, 0.025% xylene cyanol, 0.025% bromphenol blue). Samples were heated at 95 °C for 2 min, loaded on a 5% polyacrylamide, 8 M urea gel, and resolved by electrophoresis in 2 × TBE (1 × TBE contains 100 mM Tris, 2 mM Na_2EDTA, 100 mM boric acid). The gels were dried and exposed to x-ray film.

DNase I Footprinting—The binding reaction was carried out under the same condition as described for transcription *in vitro*. The 30-μl reaction buffer contains about 20 fmol of 5′-end-labeled DNA probe (about 5×10^4 cpm) and 4 μg of poly(dI-dC). The protein content was approximately 50 μg. The binding reaction was incubated at room temperature for 20–30 min. Then 0.05–0.5 unit of DNase I was added for 3 min at room temperature. DNase I digestion was terminated by addition of 200 μl of stop buffer (20 mM Tris-HCl, pH 7.5, 20 mM Na_2EDTA, 0.5% SDS, 0.2 mg/ml proteinase K) at 50 °C for 60 min. The labeled DNA was extracted with phenol/chloroform and precipitated with ethanol in the presence of 5 μg of tRNA. The pellet was dissolved in 70% formamide loading buffer (0.025 (w/v) xylene, 0.025 (w/v) bromphenol blue) and heated at 95 °C for 2 min before loading on 6% polyacrylamide sequencing gel. The gels were dried and exposed to x-ray film.

Northern Analysis—Cellular RNA was isolated as described by Chomczynski and Sacchi (29). Total RNA was used and electrophoresis and hybridization analysis were carried out as previously described (6, 9). Autoradiograms were quantified as described for gel-mobility shift assays.

RESULTS

Glucocorticoids Regulate the Macromolecular State of E2F—Nuclear extracts from mid-log phase P1798 cells contain proteins that bind to a synthetic oligonucleotide corresponding in sequence to the E2F-binding site of the adenovirus E2e promoter. As shown in Fig. 1, three major gel shift entities may be observed, labeled C, F, and E. There are also two minor complexes, designated A and B. All of these complexes result from association with proteins of relatively low abundance, as evidenced by displacement with unlabeled oligonucleotide competitor (*lanes 3–5*).

Both qualitative and quantitative differences are observed when one carries out gel-mobility shift assays using extracts from dexamethasone-treated cells (Fig. 1, *lanes 6–9*). The mi-

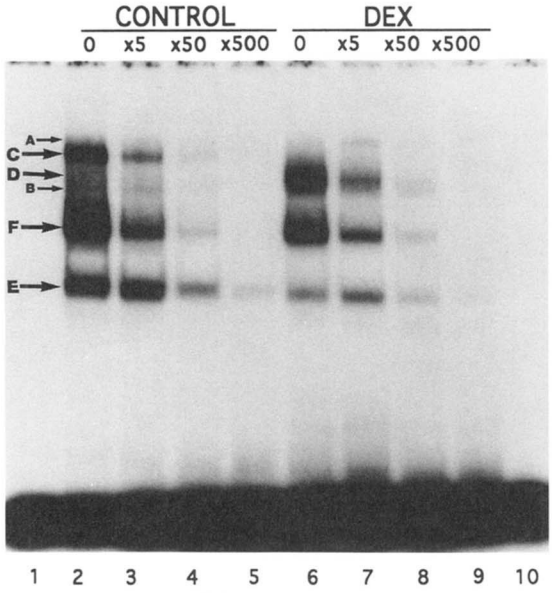

FIG. 1. **E2F binding activity in extracts from control and glucocorticoid-treated cells.** Nuclear extracts were prepared from either control (mid-log) cultures or from cultures that had been exposed to 0.1 μM dexamethasone (*DEX*) for 24 h. *Lanes 2–5* contain control extracts and *lanes 6–9* contain extracts from dexamethasone-treated cells. *Lanes 1* and *10* contain no protein. Gel-mobility shift assays were carried out as described under "Experimental Procedures." Competition was carried out using unlabeled E2F oligonucleotide at 5-, 50-, or 500-fold molar excess (relative to labeled probe) as indicated above the appropriate lanes.

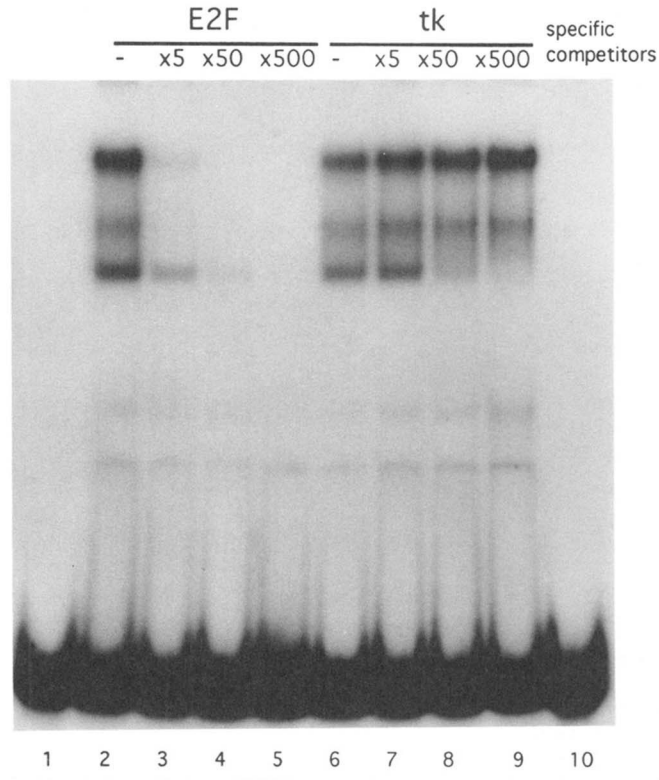

FIG. 2. **Specificity of E2F interactions.** *Lanes 1–10* contain labeled E2F oligonucleotide, and *lanes 2–9* contain nuclear extract from control cells. Specific competition with unlabeled E2F oligonucleotide was carried out at 5-, 50-, or 500-fold molar excess, as shown in *lanes 3–5*, respectively. Nonspecific competition was carried out using the thymidine kinase oligonucleotide that was described under "Experimental Procedures." *Lanes 7, 8,* and *9* contain 5-, 50-, or 500-fold molar excess of the thymidine kinase oligonucleotide.

nor complexes, A and B, are unchanged by dexamethasone treatment. The slight decrease in the complex called E is not reproducibly observed. The most striking effect of dexamethasone is the disappearance of complex C and the appearance of a new complex, called complex D. Complex D can also be displaced by competition with unlabeled E2F oligonucleotide (Fig. 1, *lanes 7–10*).

The complex called E in Fig. 1 does not appear to be a specific E2F complex. Although this complex is displaced by competition with unlabeled E2F oligonucleotide (Fig. 2, *lanes 3–5*), this entity can also be disrupted by competition with unrelated oligonucleotides. *Lanes 7–9* of Fig. 2 illustrate the observation that complex E, but not complexes F and C, can be displaced by an unlabeled oligonucleotide that is pyrimidine-rich (as is the E2F oligonucleotide) but is unrelated in sequence to the authentic E2F-binding site. The nature of this E complex is unknown, but others have observed a similar entity in nuclear extracts from a number of different cell types (12, 16, 30).

Complexes C and D appear to result from macromolecular associations between an E2F-like DNA-binding protein and other proteins. As shown in Fig. 3, both complex C (*lanes 3–5*) and complex D (*lanes 7–9*) are disrupted by addition of deoxycholate. Complex F is not dissociated, which is consistent with the conclusion that this complex results from strong DNA-protein interactions between E2F-like proteins and the E2F-binding site (16, 20, 30).

P1798 cells were transformed with a vector that contains the mouse mammary tumor virus promoter fused to the coding sequence of large T antigen of SV40. Stable transformants express T antigen under the control of glucocorticoids, and immunoblot analysis has indicated that T antigen is expressed only in glucocorticoid-treated cells (data not shown). A clone from these T antigen-producing cells was analyzed for the macromolecular state of E2F in the presence and absence of glucocorticoids. As shown in Fig. 4, the MMTV/T antigen-transformed cells are indistinguishable from untransformed cells in the absence of glucocorticoids. A comparison of *lanes 1* and *3* of Fig. 4 reveals that both cell cultures express predominantly the F and C complexes. When untransformed cells are treated with dexamethasone (*lane 2*) the C complex disappears and the D complex appears. The C complex disappears in dexamethasone-treated MMTV/T antigen-transformed cells (*lane 4*), but the D complex does not accumulate. This observation indicates that expression of T antigen disrupts the D complex. Complexes A and B are not effected by dexamethasone or by T antigen, as can be seen in *lane 4* of Fig. 4.

The properties of complex F indicate that this entity results from association between the E2F oligonucleotide and "free" E2F (16, 20, 30). Complex D is due to protein-protein interactions between E2F and some other protein(s). The observation that this complex cannot be observed in T antigen-expressing cells is consistent with the conclusion that this is the E2F·p105$^{Rb\text{-}1}$ complex (10, 20). The electrophoretic mobility and deoxycholate sensitivity of complex C are consistent with those of a complex, described by several laboratories (16–19), that contains E2F, p107, Cdk2, and cyclin A. There is a temporal association between complex C accumulation and S phase progression (as will be shown below) which also suggests that complex C may contain p107. However, for reasons that will be discussed below, it has been impossible to make a more definitive identification of these complexes.

Structure and Function of DNA-Protein Complexes on the c-myc P2 Promoter—Glucocorticoids cause a dramatic change in the distribution of macromolecular forms of E2F in P1798 cells. DNase I protection studies were carried out to determine if there are corresponding changes in DNA-protein interaction on the c-*myc* P2 promoter. Extracts from control and dexa-

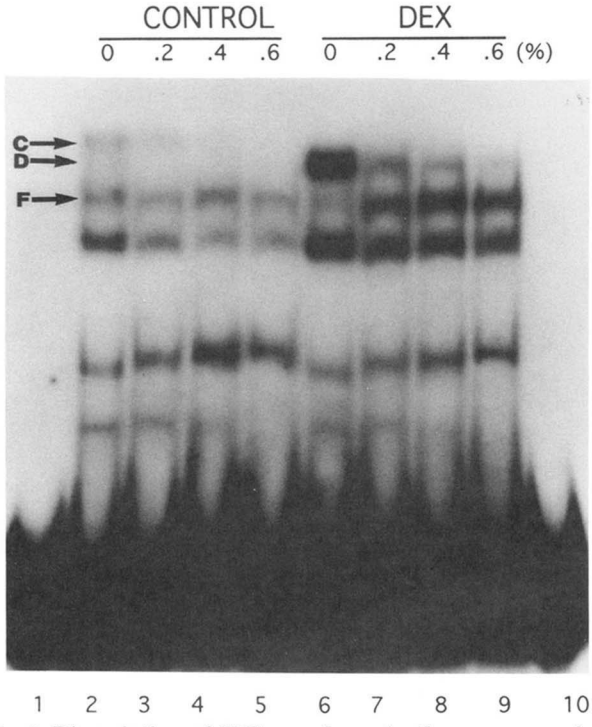

FIG. 3. **Dissociation of E2F complexes in the presence of deoxycholate.** Nuclear extracts from control or dexamethasone (*DEX*)-treated cells were incubated with labeled E2F oligonucleotide, as shown in *lanes 2–5* and *6–9*, respectively. *Lanes 1* and *10* contain no protein. Deoxycholate was added to final concentrations of 0.2, 0.4, or 0.6% (w/v) as indicated above the appropriate lanes.

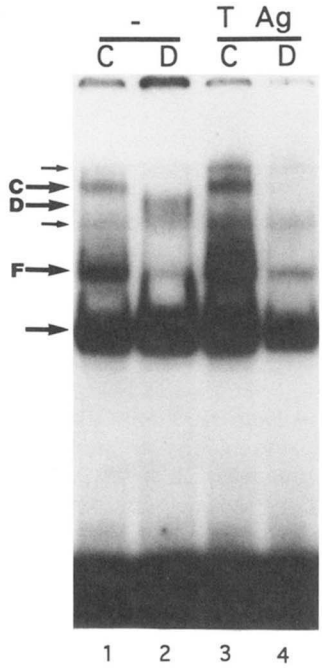

FIG. 4. **E2F binding in cells that express SV40 T antigen under the control of the MMTV promoter.** Binding assays were carried out using nuclear extracts prepared from wild type P1798 (*lanes 1* and *2*) and transformed cells that express T antigen from the MMTV promoter (*lanes 3* and *4*). Extracts were prepared from either control (*C, lanes 1* and *3*) or dexamethasone-treated (*D, lanes 2* and *4*) cells.

methasone-treated cells were incubated with an end-labeled polymerase chain reaction fragment that extends from just upstream of the P1 start site (designated +1) to about 40 bp downstream from the P2 start site (at +162 bp). (Note that all positional assignments are relative to the P1 start site at +1.)

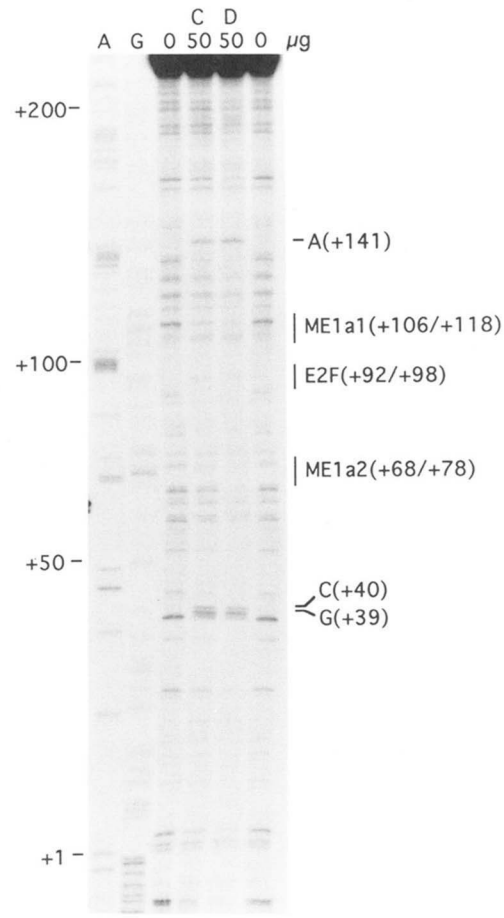

FIG. 5. **DNA-protein interactions with the c-*myc* P2 promoter.** Nuclear extracts were prepared from mid-log phase P1798 cultures and from cultures that had been treated with dexamethasone for 24 h. These were fractionated as described under "Experimental Procedures." Aliquots containing 50 μg of protein were incubated with a 5′-end-labeled polymerase chain reaction product. DNase I digestion was carried out, as described under "Experimental Procedures," and the products were resolved by electrophoresis. *Lanes A* and *G* contain Maxam and Gilbert sequencing reactions carried out for AMP and GMP using the end-labeled polymerase chain reaction product. The lanes designated *0* contain no protein, whereas *lanes C* and *D* contain proteins extracted from control and hormone-treated cells.

The analysis was focused upon this region, which contains the well-characterized E2F-binding site (centered about +96 bp). Optimum protection was obtained using 50 μg of nuclear extract protein, and a representative protection pattern is shown in Fig. 5. Several features of this "footprint" warrant comment. Three nucleotides are rendered hypersensitive upon addition of nuclear extract from control (*lane C*) or dexamethasone-treated (*lane D*) cells. There is a prominent hypersensitive AMP residue at +141 bp, lying between the P2 TATA box and the P2 start site at +162 bp. The A+141-hypersensitive site is generated by extracts from both control and dexamethasone-treated cells, and the P2 TATA box is also protected by both extracts. There are two well-characterized, GC-rich c-*myc* promoter elements called ME1a1 and ME1a2 (10). These are weakly protected by extracts from both control and dexamethasone-treated cells. Gel-mobility shift assays using ME1a oligonucleotides indicate that the amount of ME1a1/a2-binding factor(s) is the same in extracts from control and glucocorticoid-treated cells (data not shown). The E2F-binding site lies between the ME1a1 and ME1a2 sites (10). Both control and dexamethasone-treated extracts protect this region, although the features of this interaction are not as dramatic as are those of the hypersensitive sites at C+40 and G+39.

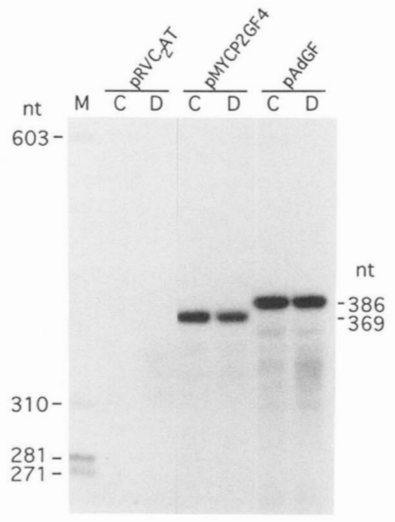

FIG. 6. **Transcription from c-*myc* P2 *in vitro*.** Nuclear extracts were prepared and fractionated as described under "Experimental Procedures" and the legend to Fig. 5. Approximately 50 μg of protein was used to transcribe the promoterless construct $pRVC_2AT$, the P2 promoter-containing construct pMYCP2GF4, or the adenovirus major late promoter-containing plasmid pAdGF. Lanes designated *C* contain control extract, whereas lanes designated *D* contain extracts from dexamethasone-treated cells. Data were quantified using the Lynx Digital Imaging System. *nt,* nucleotide.

The DNase protection patterns observed with extracts from control and dexamethasone-treated cells are indistinguishable from each other and are similar to patterns reported for extracts from other cell types (10). DNase footprinting is not a functional assay, and the data shown in Fig. 5 do not preclude the possibility that the E2F site is occupied by an E2F activator complex (*e.g.* E2F·p107) in control cells and an E2F inhibitory complex (*e.g.* E2F·$p105^{Rb\text{-}1}$) in dexamethasone-treated cells. In order to test this hypothesis, it was necessary to develop an assay for c-*myc* promoter function *in vitro*.

The c-*myc* P2 promoter (from −8 to +155 bp) was ligated to the G-free cassette derivative $pRVC_2AT$. This chimaeric *myc*/G-free construct, which is called pMYCP2GF4, supports initiation 8 bp inside the G-free cassette, giving rise to an authentic transcript of 369 nucleotides, as shown in Fig. 6. Readthrough transcription from adventitious, upstream initiation gives rise to a transcript of 377 nucleotides, which can be observed if very high concentrations of the promoterless vector ($pRVC_2AT$) are added to nuclear extracts. Under the conditions that are routinely used for transcription in this laboratory, nonspecific initiation on the promoterless construct is not detectable (Fig. 6). Transcription was assayed using nuclear extract preparations from control and dexamethasone-treated cells (as shown in Fig. 6, *lanes C* and *D*, respectively). Transcription of the adenovirus major later promoter/G-free construct (pAdGF) is shown for comparison. In more than three different sets of extracts, the activity of the P2/G-free construct in control extracts, when normalized to the activity of the adenovirus major late promoter, is not significantly different from the activity detected in extracts from dexamethasone-treated cells.

Gene Expression and the Macromolecular State of E2F—Glucocorticoids inhibit c-*myc* transcription in lymphoid cells of thymic origin (6, 9). The observation that glucocorticoids alter the macromolecular state of E2F is consistent with the hypothesis that E2F may be involved in glucocorticoid regulation of c-*myc* expression (10–13). The hypothesis predicts that changes in E2F should precede (or be concomitant with) changes in expression of c-Myc mRNA. To test this prediction, extracts were prepared from P1798 cells at intervals following addition of dexamethasone to mid-log cultures. RNA was also extracted and analyzed for abundance of mRNAs transcribed from c-*myc*, *Tk-1* (thymidine kinase), and *cdc2* ($p34^{cdc\,2}$). A parallel set of P1798 cultures was treated with dexamethasone for 24 h. Thereafter, the steroid was removed and nuclear extracts and mRNA were prepared as a function of time of recovery from dexamethasone-mediated arrest. In all cultures, DNA synthesis was monitored by measuring [^{3}H]thymidine incorporation. The results of this experiment are shown in Fig. 7.

The gel-mobility shift data shown in Fig. 7 (*Panel A*) illustrate the changes in DNA-binding states of E2F as a function of time after addition of dexamethasone (*lanes 2–8*). *Lanes 9–19* contain extracts from cells that were rescued from dexamethasone. The *bar graphs* show [^{3}H]thymidine incorporation after addition of dexamethasone to mid-log cells (Fig. 7*B*) or after withdrawals of dexamethasone from glucocorticoid-arrested cells (Fig. 7*E*). Quantitative data from the gel-mobility shift experiment are shown in Fig. 5, *C* and *F*, in which the percent of E2F in any of its macromolecular states is shown as a function of time after addition (Fig. 7*C*) or withdrawal (Fig. 7*F*) of dexamethasone. The amount of complex C (Fig. 7*C*, *open circles*) remains relatively constant for 10–12 h after addition of dexamethasone, decreases rapidly after 12 h, and cannot be detected by 20 h after addition of dexamethasone. Complex D (Fig. 7*C*, *closed circles*) is first observed about 12 h after addition of dexamethasone and accumulates from 16 to 20 h. The data indicate that a critical transition in the macromolecular associations of E2F that occurs 10–12 h after addition of glucocorticoids; the number of S phase cells also decreases at this time (as shown in Fig. 7*B*).

The data from the recovery experiment (Fig. 7*A*, *lanes 9–19*) indicate that as P1798 cells re-enter the cell cycle, the macromolecular state of E2F begins to change within 10–12 h after removal of the steroid. The D complex (Fig. 7*F, closed circles*) disappears between 16 and 20 h and complex C (Fig. 7*F, open circles*) appears rapidly after 16 h. Disappearance of the D complex and appearance of complex C occurs at the time that cells begin to enter S phase, as evidenced by the thymidine incorporation data shown in Fig. 7*E*.

Fig. 5, *D* and *G*, contain the results of the analysis of mRNA abundance in dexamethasone-treated and rescued cells. The abundance of c-Myc mRNA (Fig. 7*D, triangles*) begins to change within minutes (9) and inhibition of c-*myc* expression is complete several hours before any change can be detected in the macromolecular associations that are characteristic of E2F in glucocorticoid-treated cells. Conversely, c-Myc mRNA returns to basal levels within 2–3 h after cells are rescued from the dexamethasone-mediated cell cycle block (Fig. 7*G, triangles*). Glucocorticoids also regulate transcription of *Tk-1* (31) and *cdc2*,[2] as determined by analysis of nuclear run-on transcription, and as evidenced by the decrease in abundance of the corresponding mRNAs in glucocorticoid-treated cells (Fig. 7*D*) and their reappearance following rescue (Fig. 7*G*). Both Tk-1 and Cdc2 mRNA exhibit similar kinetics of inhibition, with 50% inhibition observed about 12 h after addition of glucocorticoid. Fifty percent recovery of Tk-1 and Cdc2 mRNA occurs about 12 h after withdrawal of dexamethasone, just prior to onset of S phase (Fig. 7*E*).

DISCUSSION

There is an E2F-binding site in the c-*myc* P2 promoter (10, 11). Mutation of this element inhibits basal activity and obviates c-*myc* regulation (11, 12). Transient overexpression of *Rb-1* inhibits c-*myc* as the E2F·$p105^{Rb\text{-}1}$ complex accumulates (13).

[2] K. S. Rhee, W. Bresnahan, and E. A. Thompson, manuscript in preparation.

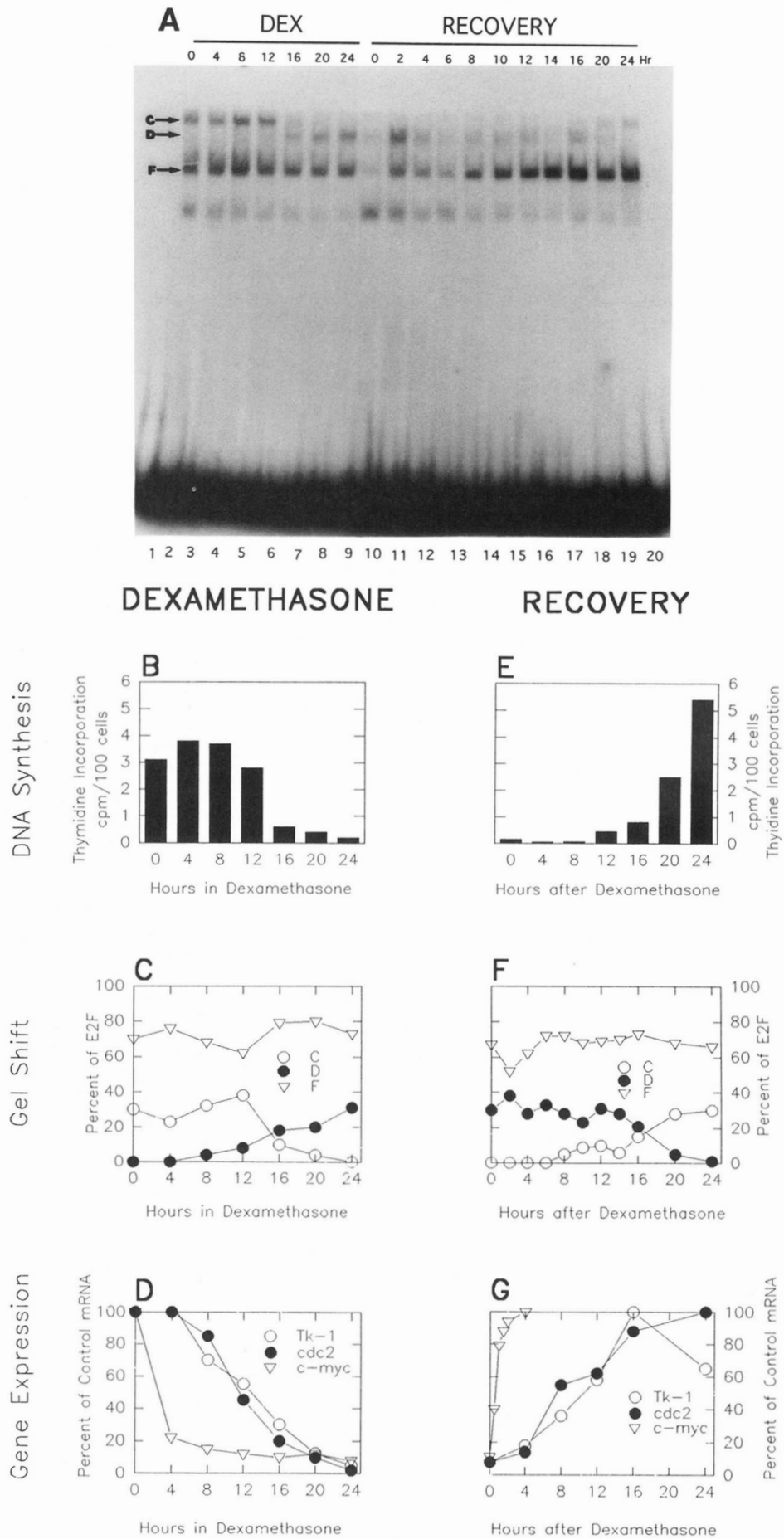

FIG. 7. **The kinetics of appearance and disappearance of E2F forms compared with S phase progression and gene expression.** Gel-mobility shift data were obtained using freeze-thawed extracts from whole cells. *Lanes 2–8* of *Panel A* contain extracts from cells that were treated with dexamethasone for 0–24 h, as indicated above the respective lanes. In a separate experiment, cells were treated with dexamethasone for 24 h. The steroid was removed and extracts were prepared at intervals thereafter. The results obtained with such extracts are illustrated by the gel-mobility shift data in *lanes 9–19* of *Panel A*. Tritiated thymidine labeling was carried out in parallel, and the results of this experiment are shown in the *bar graph* shown in *Panel B*. *Panel C* shows the results of quantitative analysis of the relative contributions of the free form of E2F (*F, open triangles*), complex C (*open circles*), and complex D (*closed circles*). Data from glucocorticoid treatment of exponentially growing cells are shown on the *left*, and data from rescued cells are shown on the *right*. In addition, the abundance of mRNAs encoding thymidine kinase (*Tk-1, open circles*), $p34^{cdc2}$ (*closed circles*), and c-Myc (*open triangles*) was measured, as shown in *Panel D*.

The experiments described above were undertaken to test the hypothesis that glucocorticoid inhibition of c-*myc* transcription is mediated by changes in the macromolecular state of E2F. Three obvious predictions emanate from this hypothesis: 1) glucocorticoids should cause changes in the relatively well-characterized associations between E2F and tumor suppressor gene products such as $p105^{Rb\text{-}1}$ or p107; 2) these changes should be reflected by corresponding changes in the properties of the DNA-protein complexes that form on the P2 promoter; and 3) such changes should precede, or at least occur concomitant with, inhibition of c-*myc* transcription.

Mid-log phase P1798 cells express two major forms of E2F. One of these, complex F, is the so-called free form of E2F, as evidenced by the observation that this entity is resistant to deoxycholate (16, 20, 30). The relative proportion of free E2F (with respect to all specific E2F-containing entities) is variable among different cell lines (30). The amount of complex F that is observed in P1798 cells is relatively high, when compared to mitogen-activated human T cells (18) or to other established cell lines (30). The observation that half or more of the E2F is always free may indicate that P1798 cells express relatively low levels of $p105^{Rb\text{-}1}$ and p107. Alternatively, P1798 cells may express forms of E2F that cannot interact with these tumor suppressor gene products.

The other specific E2F state that is observed in mid-log phase P1798 cells (complex C) is probably the mouse homolog of the E2F·cyclin A·Cdk2·p107 complex described in human cells by several investigators (16–19). This complex is known to be sensitive to deoxycholate (16, 20, 30) and to accumulate as cells enter S phase (16, 18). One would expect that a substantial amount of E2F would exist in this complex in asynchronous P1798 cultures, of which 60–70% are in S phase.[3] When dexamethasone is removed from glucocorticoid-arrested cells, they enter S phase in a synchronous fashion within 12–16 h. The expression of complex C correlates with entry of dexamethasone-rescued P1798 cells into S phase, which is also consistent with the identification of complex C as the E2F·p107 complex. Finally, the electrophoretic mobility of complex C is similar to that described for E2F·p107 (16–19). This is evidenced by the relative mobility of complex C and complex D (which is probably the $E2F{\cdot}p105^{Rb\text{-}1}$ complex, as discussed below).

Definitive identification of complex C requires the use of antibodies to confirm the presence of p107 or cyclin A in this complex. Available antibodies against human p107 react very weakly with mouse p107.[4] Antibodies against human cyclin A[5] react weakly with complex C and will disrupt this complex when added at high concentrations (data not shown). Although this behavior is characteristic of the effect of cyclin A antibodies on the human E2F·p107 complex (16), the amount of antibody that is required to cause dissociation of mouse complex C is so high that we have no confidence in the specificity of the response.

Complex D, found in dexamethasone-treated P1798 cells, is probably the murine homolog of the $E2F{\cdot}p105^{Rb\text{-}1}$ complex. This complex migrates more slowly than free E2F, but faster than E2F·p107, and disappears as cells enter S phase (16–18). The $E2F{\cdot}p105^{Rb\text{-}1}$ complex dissociates in the presence of T antigen (32) and other viral transforming proteins (15, 30), as does complex D. We have tested several antibodies that were raised against human $p105^{Rb\text{-}1}$. A few of these antibodies react weakly with mouse $p105^{Rb\text{-}1}$ on immunoblots, but none that we have tested can immunoprecipitate native mouse $p105^{Rb\text{-}1}$, and none will interact with complex D. Although a number of indirect observations suggest that complexes C and D are the mouse homologues of the E2F·p107 and $E2F{\cdot}p105^{Rb\text{-}1}$ complexes that have been described in human cells, we have been unable to confirm this hypothesis directly. For this reason, we persist in calling these P1798 complexes C and D until we can obtain reagents appropriate to their identification.

Although we have been unable to provide a definitive identification of the macromolecular associates, it is clear that there are two higher order complexes that are representative of the state of E2F in control and dexamethasone-treated cells. This observation is consistent with the hypothesis that changes in the macromolecular state of E2F are involved in glucocorticoid regulation of c-*myc* transcription. However, DNase I footprinting data indicate that these changes in the macromolecular state of E2F are not reflected by corresponding changes in the DNA-protein interactions that can be detected on the P2 promoter. This is consistent with the report that serum starvation, which inhibits c-*myc* transcription, does not effect the occupancy of the E2F site *in vivo* (33).

It might not be expected that changes in protein-protein interactions between E2F and tumor suppressor gene products would alter the occupancy of the E2F site. One could imagine that complexes C and D could bind equally well to the promoter, yet have different effects on promoter function. The footprinting data suggest that this is not the case. The nuclease hypersensitive site near the P2 start site is characteristic of an activated state of the c-*myc* promoter *in vivo* (34). The observation that AMP residue +141 is rendered hypersensitive by both control and hormone-treated extracts suggests that both extracts are contributing to formation of a structure that is similar to that of the activated c-*myc* P2 promoter. The transcription data are consistent with this observation. The chimaeric c-*myc* P2/G-free construct that was assayed in this series of experiments has equivalent activity in extracts from control and dexamethasone-treated cells. This observation, in and of itself, does not preclude the possibility that inhibitory interactions between E2F complexes and c-*myc* do not occur *in vivo*; nevertheless, the data are inconsistent with this conclusion.

The third prediction that emanates from the working hypothesis states that the rates of appearance and disappearance of the presumptive regulatory E2F complexes should correlate with changes in c-Myc mRNA abundance. This is not the case. Complex C disappears as glucocorticoid-treated P1798 cells cease to synthesize DNA, whereas inhibition of c-*myc* transcription can be demonstrated within minutes after addition of glucocorticoids (6, 9). Conversely, complex D disappears at about the time that cells re-enter S phase following withdrawal of dexamethasone, whereas the abundance of c-Myc mRNA returns to basal levels within 2 h after removal of the steroid. Changes in c-*myc* expression are complete hours before any change in E2F can be demonstrated. Similar results have been observed under other regulatory circumstances. Nevins and colleagues observed changes in the macromolecular state of E2F that occurred about 12 h after serum stimulation of quiescent fibroblasts (16). Although c-*myc* transcription was not measured in that series of experiments, it is known that c-*myc* is usually induced within an hour or so after serum stimulation (35). The kinetics of regulation of c-*myc* are inconsistent with a role of E2F complex formation. This does not preclude the hypothesis that some genes may be inhibited by the E2F·p105 complex. Indeed, all E2F-dependent genes (including c-*myc*) may be inhibited if one achieves sufficiently high level expression of $p105^{Rb\text{-}1}$ (13). In P1798 cells, the majority of E2F appears to be free (complex F) under all circumstances. Since the free form of E2F is thought to be fully active in transcription (reviewed in Ref. 14), more complicated models would be required

[3] M. Hirai and A. Thompson, unpublished data.
[4] E. Harlow, personal communication.
[5] The generous gift of Ed Harlow.

to explain how association of a fraction of E2F with either $p105^{Rb\text{-}1}$ or p107 might have regulatory significance.

We conclude that changes in the macromolecular state of E2F probably have little direct relevance for regulation of c-*myc* transcription. This is consistent with a recent report that occupancy of the E2F-binding site of c-*myc* does not change *in vivo* as c-*myc* transcription is inhibited (32). Expression of the genes encoding thymidine kinase and $p34^{cdc2}$ (*Tk-1* and *cdc2*, respectively) were also analyzed. These genes are markers for late G_1 phase of the cell cycle (reviewed in Ref. 14). Furthermore, both *Tk-1* and *cdc2* contain potential E2F-binding sites and are candidates for regulation by changes in the macromolecular state of E2F (36, 37). Expression of these genes changes at about the same time that changes in complex C and complex D are observed. The data are consistent with the hypothesis that these late G_1 genes could be stimulated by complex C or inhibited by complex D.

It is said that kinetic studies can eliminate a mechanism from consideration, although kinetics can never prove a mechanism. The data presented above illustrate this point as it applies to gene regulation by changes in the macromolecular associations of E2F. The data indicate that glucocorticoid regulation of c-*myc* transcription will *not* be resolved in terms of the protein-protein interactions between E2F and the products of tumor suppressor genes and their relatives (such as p107). It has been reported that E2F plays a significant role in regulation of *Tk-1* and *cdc2* (36, 37), and our kinetic studies are consistent with the hypothesis that these late G_1/S phase-specific genes are regulated by changes in the association between E2F and other proteins. The idea that E2F regulates a cohort of S phase-specific genes is compelling (14), but the physiological role of tumor suppressor gene products in this process remains unclear.

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