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Interferons and Interleukin-6 Suppress the DNA-Binding Activity of E2F in Growth-Sensitive Hematopoietic Cells

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Transcription factor E2F binds to cellular promoters of certain growth- and cell cycle-controlling genes and forms distinct heteromeric complexes with other nuclear proteins. We show here that alpha and beta interferons (α, β) and interleukin-6 abolished the E2F-containing DNA-binding complexes in Daudi Burkitt lymphoma cells and in M1 myeloblastic cells, which responded to the cytokines by suppression of c-*myc* transcription. Time kinetics studies showed that the abolishment of E2F complexes coincided with reduction of c-*myc* expression and that both molecular events preceded the cell cycle block in G_0/G_1 phase. In contrast, the pattern of E2F complexes remained unchanged in an interferon-treated growth-resistant Daudi cell mutant that displayed relaxed regulation of c-*myc*. All of the DNA-binding E2F complexes, including those containing the retinoblastoma protein (pRB), cyclin A-$p33^{cdk2}$, and the free forms of E2F, were reduced by interferons or interleukin-6. Their abolishment was unperturbed by pharmacological treatments that alleviated the cyclin A and pRB responses to interferon. Thus, changes in cyclin A expression and pRB phosphorylation are not primary events that influence the pattern of E2F responses to cytokines. Addition of EDTA to cell extracts of interferon-treated Daudi cells restored the DNA-binding activity of E2F, resulting in the appearance of a single E2F complex that exclusively contained pRB. It is suggested that the regulation of E2F by growth-inhibitory cytokines that induce cell cycle exit takes place at the level of the DNA-binding activity, and by that mean it differs basically from the phase-specific regulation of E2F in cycling cells.

A fruitful approach to the study of the antiproliferative mode of action of cytokines is to measure whether the ligand-receptor interactions modify the expression or the function of cell cycle-controlling nuclear proteins. This approach has been applied in recent years to the study of the growth-suppressive effects of a few cytokines, including interferons (IFNs) and interleukin-6 (IL-6) (reviewed in reference 30). Cell lines that arrest in response to the cytokine in the G_0/G_1 phase of the cell cycle, such as human Daudi Burkitt lymphoma and murine M1 myeloblastic cells (14, 31, 48, 49, 54), were specifically chosen for these studies. The first genes that were identified as downstream targets along the IFN- and IL-6-triggered molecular pathways were c-*myc* and the cyclin A and retinoblastoma (RB) genes (14, 31, 48, 49). While c-Myc and cyclin A responded by reduction in the mRNA and protein expression, RB gene product (pRB) responses occurred at the posttranslational level and consisted of conversion of the protein into the underphosphorylated functional forms (49). Genetic or pharmacological manipulations that rescued only part of the molecular responses were performed to determine the functional role of these events in mediating the cytokine antiproliferative effects (48, 49, 54). It turned out that the rescue of a single gene response (e.g., that of c-*myc*) generated partial growth resistance to the cytokines, manifested by an aberrant proliferation arrest that was less restrictive to the G_0/G_1 phase. This finding further provided the first hallmark for the existence of multiple parallel pathways in the mode of action of IFN or IL-6. Thus, the inhibition of c-*myc* transcription on one hand and the suppression of cyclin A expression and of pRB phosphorylation on the other hand were functionally classified into two independent and complementary pathways that must collaborate in order to arrest cell cycle progression exclusively in the G_0/G_1 phase (48, 49).

In this study, we investigated whether transcription factor E2F is a downstream target activity that participates in one of the above-mentioned negative growth signaling pathways of IFNs or IL-6. E2F was first identified as a DNA-binding cellular factor that interacts with sequences of the adenovirus E2 promoter (32). It was shown to be one of the major targets for the adenovirus E1A-dependent transactivation of E2 transcription that is released by the viral oncoprotein from preexisting nonfunctional complexes with other cellular proteins (3, 40, 47). Except for the E2 viral gene promoter, few cellular promoters that contain E2F-binding sites have been identified as targets for E2F, some of which drive genes that code for transcription factors and enzymes that control growth and cell cycle progression (9, 22, 24, 27, 39, 40, 44). Recent studies of E2F have focused on its association with other cellular proteins in multimeric DNA-binding complexes at specific time points during the cell cycle. For example, E2F was found to associate with pRB (5, 6, 11, 12, 20, 41, 55). In addition, a pRB-related protein, p107, was found in two other distinct complexes that also contain $p33^{cdk2}$ and cyclin A or cyclin E (10, 13, 33, 38, 41, 42, 52). Recently, two genes possessing E2F properties, representing the first members of most probably an extended gene family, have been cloned (16, 21, 28, 51). The protein encoded by one of these genes (E2F-1) has a specific DNA-binding domain at the N-terminal region and a pRB-binding domain that resides within the C-terminal region of the protein required for transcriptional activation (21, 28, 41, 51). Binding of pRB to the C terminus of E2F-1 has been implicated in the suppression of E2F transactivation properties in light of previous reports showing that overexpression of wild-type pRB selectively suppressed expression from few E2F-containing promoters (1, 18, 23, 56). In addition, the DNA-binding region of E2F-1 consists of a basic helix-

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loop-helix domain, which suggests the involvement of dimerization as part of E2F function (21, 28, 41, 51).

A few basic observations prompted our interest in studying whether E2F may be a target for regulation by IFNs or IL-6. First, some of the proteins that form stable DNA-binding complexes with E2F, i.e., pRB and cyclin A, were found to be modulated at the phosphorylation and protein expression levels, respectively, by IFNs and IL-6 (49). These cytokine-induced modifications of the E2F partners could change the pattern of E2F complexes and by that means could modify some of the transactivating properties of E2F. Second, IFNs and IL-6 suppress the transcription of c-*myc* (14, 31), one of the target genes for E2F transactivation. Two E2F-binding motifs were identified between the two promoters of c-*myc*, one of which (the P2 distal site) was found crucial for efficient transcription from the P2 promoter (24). This E2F-binding site was also shown to be a target for transactivation of c-*myc* expression by E1A (24, 34, 53) and to be suppressed by overexpression of pRB (18). This finding raised the possibility that the previously described c-*myc* suppression by growth-inhibitory cytokines may be mediated by modulations of E2F activity. Altogether, these data prompted us to test whether IL-6 or IFNs modify the pattern of E2F complex formation or, alternatively, change the DNA-binding activity of E2F itself as part of the cytokine antiproliferative mode of action. To test these possibilities, we analyzed the effects of cytokines on E2F activity by mobility gel shift assays, using the c-*myc*-derived E2F-binding sequences as the labeled DNA probe. We found that IFN-α+β and IL-6 abolished all of the DNA-binding E2F-containing complexes in growth-sensitive cell lines as a result of selective inhibition of the DNA-binding activity of E2F. The loss of E2F binding was observed even when the normal effect of IFNs on two of the E2F regulators, cyclin A and pRB, was disrupted. The suppression of E2F binding was reversible and could be alleviated by addition of EDTA to the cell extracts or by removal of Mg^{2+} cations. The reduction in E2F DNA-binding activity was correlated with the cytokine-induced suppression of c-*myc* transcription. It represents another mode of E2F regulation, used during cell cycle exit, that basically differs from the phase-specific regulation of E2F in cycling cells.

MATERIALS AND METHODS

Cell lines, culture conditions, and flow cytometric analysis. Daudi Burkitt lymphoma cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Bio-Lab, St. Paul, Minn.). M1 mouse myeloblastic cells (26) were grown in Eagle's minimal essential medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (Bio-Lab). In this study, we used a clone of M1 cells, designated S6, that can be growth arrested by IFN-α+β, IL-6, or transforming growth factor β1 (TGF-β1). This clone was described in detail elsewhere (48). Both Daudi and M1 cells were treated with the cytokines at a density of 2×10^5 cells per ml. Cell cycle distribution was determined by analyzing samples of 10^6 cells stained with propidium iodide in a fluorescence-activated cell sorter (FACScan; Becton Dickinson) as previously described (48).

Cytokines. Human IFN-α was purified to 5×10^8 U/mg by affinity chromatography with monoclonal antibodies as described previously (14). Murine IFN-α+β (2×10^8 U/mg) was purchased from Lee Biomolecular Laboratories (San Diego, Calif.). Human recombinant IL-6 purified to 2×10^7 U/mg was provided by Interpharm Inc., Rehovot, Israel. TGF-β1 prepared from human platelets was purchased from R & D Systems (Minneapolis, Minn.).

WCE preparation and band shift assay. Whole cell extracts (WCE) were prepared as described by Mudryj et al. (38). Cells were washed twice with cold phosphate-buffered saline (PBS), and a pellet of approximately 4×10^7 cells was suspended in 70 μl of cell lysis buffer (10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.6], 5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM NaF, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM $NaVO_4$) and incubated at 4°C for 15 min. The cells were broken in a Dounce homogenizer (30 strokes), and then 78 μl of extraction buffer (20 mM HEPES [pH 7.6], 1.6 M KCl, 20% glycerol, 0.1 mM EDTA, 0.5 mM NaF, 0.5 mM $NaVO_4$, 0.5 mM PMSF, 1 mM DTT) was added. The extracts were rocked for 30 min at 4°C and then centrifuged for 60 min in 50 Ti rotor (38,000 rpm). The supernatants were removed and dialyzed at 4°C for 2 h on VSWP filters (0.025-μm pore size; Millipore) against dialysis buffer (20 mM HEPES [pH 7.6], 50 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.1 mM NaF, 0.1 mM $NaVO_4$).

For E2F assays, band shift analysis was performed essentially as described before (38). Briefly, 4 μg of WCE was preincubated for 10 min at room temperature in a reaction mixture containing 20 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 0.1 mM EDTA, 40 mM KCl, 0.5 mM DTT, 0.1 μg of poly(dI-dC), 1 μg of sonicated salmon sperm DNA, 60 μg of bovine serum albumin, and 1% Ficoll. Then the ^{32}P-labeled probe was added, and incubation was continued for 20 min. EDTA treatments were performed by adding the chelating agent to the reaction mixtures at the preincubation step to a final concentration of 5 mM unless otherwise indicated. When indicated, the $MgCl_2$ was omitted from the reaction mixture. The probe was an E2F oligonucleotide (0.3 ng) representing the P2-distal E2F-binding site of the c-*myc* promoter (−65 to −77 from the P2 start site of the mouse gene; tgcaCGCTTGGCGGGAA). When indicated, 10 ng of unlabeled competitor DNA was added. AP1 (gatcCGTTGCT GACTAATTGAGAG) is a nonrelevant oligonucleotide from the simian virus 40 (SV40) early promoter; E2-WT (gatcTT GAGAAAGGGCGCGAAACT) is an oligonucleotide containing an E2F-binding site derived from the adenovirus E2 promoter; E2-MUT (gatcTTGAGAAAGGGATCGAAACT) contains a mutated E2F site.

For nuclear factor 1 (NF-1) assays, the binding buffer contained 110 mM KCl, 4 mM $MgCl_2$, 4 mM Tris (pH 7.6), 0.05 μM $ZnCl_2$, 2 μg of poly(dI-dC), and 4% glycerol. The probe was an oligonucleotide (gatcCTGGTCTGTGCCAAG TGTT) corresponding to the NF-1b element of the hepatitis B virus enhancer (8). The DNA-protein complexes were resolved on a 5% polyacrylamide gel in 0.25× Tris-borate-EDTA (TBE) buffer.

Antibodies. DNA-binding reaction mixtures were preincubated for 30 min at room temperature with 1 μl of either of the following antibodies prior to addition of the ^{32}P-labeled E2F probe. Anti-cyclin A and anti-cyclin B antibodies are affinity-purified polyclonal antisera raised against cyclin A and cyclin B, respectively; the anti-CDK2 antibody is an affinity-purified polyclonal antiserum raised against $p33^{cdk2}$ (43); PAb421 is a monoclonal antibody that reacts with p53 (19); XZ55 is a monoclonal antibody directed against pRB (25); anti-peptide 5 is an affinity-purified polyclonal antibody raised against peptide 5 of pRB (residing between amino acids 248 and 262 of human pRB), prepared as previously described (57).

DOC treatment. Reaction mixtures containing WCE were incubated at 4°C in the presence of 0.8% sodium deoxycholate (DOC) for 30 min and then incubated with 1.5% Nonidet P-40 for 10 min prior to the band shift assay as described previously (38).

E1A dissociation of E2F complexes. E1A was expressed either in reticulocyte lysate or in bacteria as a glutathione *S*-transferase (GST)–E1A fusion protein. WCE (4 μg) were preincubated with 2 μl of 1:5 dilution of a reticulocyte lysate programmed with transcripts of the 13S E1A mRNA. After incubation for 30 min, the extracts were assayed for DNA binding. GST and GST-E1A proteins were expressed in bacteria and purified on glutathione beads by standard procedures (29, 50). Extracts were preincubated for 20 min with approximately 100 ng of each protein and subjected to gel shift analysis.

Immunoblot analysis. Samples of WCE (40 μg) were fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Immunoblots were reacted with the appropriate antibodies followed by anti-mouse immunoglobulin G or protein A coupled to peroxidase and the enhanced chemiluminescence detection system as previously described in detail (49). The antibodies used were anti-c-Myc (1:10 dilution of the hybridoma MYC1-3C7 supernatant [15]) and anti-pRB (G3-245; 500 ng/ml; PharMingen) monoclonal antibodies and an anti-cyclin A polyclonal antibody (1:500 dilution [43]).

Transient transfections and CAT assays. Two procedures for transfecting Daudi cells were used. The DEAE-dextran procedure was performed as previously detailed (36). Briefly, exponentially growing cells (10^7 cells per transfection) were incubated in 10 ml of RPMI containing 250 μg of DEAE-dextran per ml and 10 μg of plasmid DNA for 2 h at 37°C. Cells were diluted in RPMI containing 10% fetal calf serum to a density of 2×10^5 cells per ml and divided into two equal portions, one of which received IFN-α (250 U/ml). Extraction was performed after 48 h, and chloramphenicol acetyltransferase (CAT) activity was determined by standard procedures (17), using 250 μg of protein in an overnight incubation.

For electroporation, Daudi cells (2×10^7 cells per transfection) were washed with PBS, resuspended in 0.5 ml of PBS containing 30 to 60 μg of linearized plasmid DNA, and electroporated at 1,500 V as described previously (48). Ten minutes later, the cells were equally split into two flasks, each containing 10 ml of RPMI, and IFN-α was added 3 h later to one of the flasks. Extracts were prepared 40 h later, and 100-μg protein samples were tested for CAT activity.

The constructs E2-CAT (also referred to as pE2 W.T. CAT) and E2F-CAT (E2F-) were a gift from J. Nevins and were described previously (23, 35). pSNmyc-CAT was constructed as follows. A 2.4-kb *Hin*dIII-*Nae*I fragment, excised from the 12.7-kb *Eco*RI-*Eco*RI fragment of the human c-*myc* gene (7) and comprising of the two promoters and 5′ flanking sequences, was cloned into the *Bgl*II site of pSVO-CAT (17). Subsequently, the *Hin*dIII-*Hin*dIII fragment containing the *myc*-CAT fused gene was subcloned into the *Hin*dIII site of pGemIII. Finally, *myc* sequences were truncated by *Sma*I cleavage and religation (see Fig. 4A). The construct pSV2CAT was described before (17).

RESULTS

IFN reduces all E2F-containing DNA-binding complexes in Daudi cells. Initially, control WCE, prepared from exponentially growing Daudi cells, were tested for E2F activity by band shift assays. The pattern of E2F-protein complexes that bind to a ^{32}P-labeled oligonucleotide probe corresponding to the P2-distal E2F-binding site within the c-*myc* promoter (24) was determined. Multiple E2F-containing complexes (designated A, B, and C in Fig. 1A and B) were detected in these cell extracts. E2F specificity was demonstrated by elimination of all three complexes following the addition of excess unlabeled E2F oligonucleotides, while no competition was obtained by an unrelated oligonucleotide (AP1). According to these competitions, the fastest-migrating complex was nonspecific (Fig. 1A). It should be noted that complexes A, B, and C were also eliminated by the addition of excess of unlabeled oligonucleotide that corresponds to the E2F site within the adenovirus E2 promoter and not by a mutated E2F site (Fig. 1B). Complex C always appeared in Daudi cell extracts as two separated bands; in some experiments, the levels of complex C were hardly detectable (Fig. 1B). In repeated gel shift assays, complexes A and B also frequently appeared to resolve into two species migrating as a close doublet (Fig. 1A and B and 2A).

The IFN responses were then measured in these cells. Daudi cells were treated with IFN-α (250 U/ml), a concentration that is sufficient to trigger an efficient block of cellular

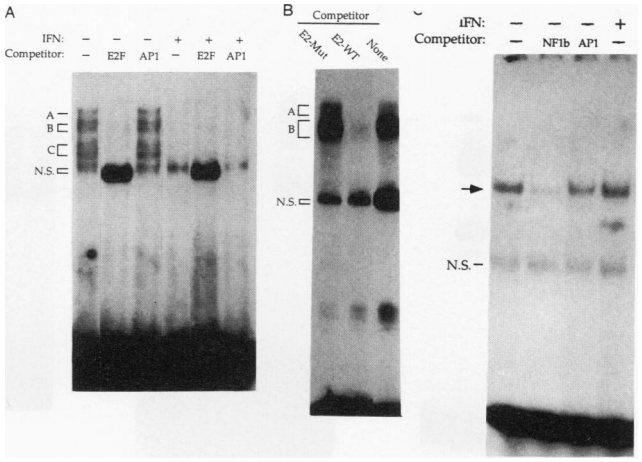

FIG. 1. E2F complexes detected in Daudi cell extracts are specifically abolished by IFN-α. (A and B) Specificity of E2F complexes as determined by competition studies and analysis of the IFN responses. (A) Band shift analysis was performed with WCE prepared from either control exponentially growing Daudi cells or cells that were exposed for 48 h to IFN-α (250 U/ml). A ^{32}P-labeled E2F oligonucleotide probe, representing the P2-distal E2F binding site of the c-*myc* promoter, was incubated with the WCE either alone or together with unlabeled competitor oligonucleotides. E2F is the same oligonucleotide as the probe. AP1 is a nonrelevant oligonucleotide derived from the SV40 early promoter. (B) WCE from control Daudi cells were incubated with the E2F probe either alone or together with competitor oligonucleotides. E2-WT is an oligonucleotide containing an E2F-binding site derived from the adenovirus E2 promoter. E2-MUT contains a mutated E2F site. A, B, and C, specific E2F complexes; N.S., nonspecific complexes; + and −, presence and absence, respectively, of IFN or competitor. (C) NF-1 binding is not affected by IFN-α. The same WCE from control and IFN-treated cells as in panels A and B were subjected to band shift analysis using an oligonucleotide corresponding to the NF-1b element of the hepatitis B virus enhancer as a probe. Specificity of the complexes was determined by competition with either the same NF-1b or the nonrelevant AP-1 unlabeled oligonucleotide. The arrow points to the NF-1 specific complex. N.S., nonspecific complex.

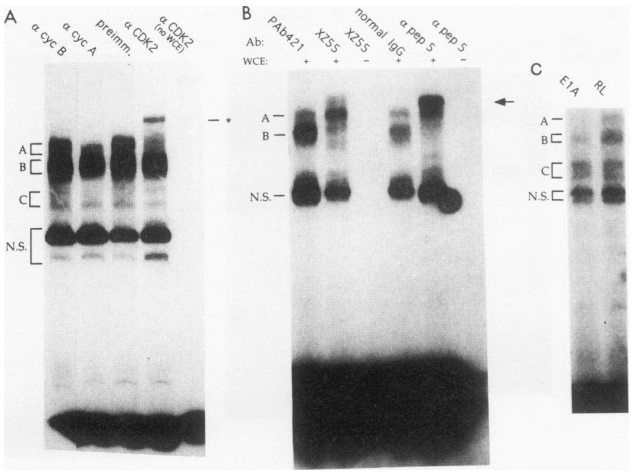

FIG. 2. Analysis of E2F complexes detected in Daudi cell extracts. (A) Effects of anti-cyclin A and anti-p33^{cdk2} antibodies on E2F complexes. Reaction mixtures containing WCE from control Daudi cells were preincubated with the indicated antibodies prior to addition of the ^{32}P-labeled E2F probe and subjected to band shift analysis. α cyc A and α cyc B, antisera raised against cyclin A and cyclin B, respectively; α CDK2, an antiserum raised against p33^{cdk2}; preimm., preimmune rabbit serum. The last lane corresponds to incubation of the anti-p33^{cdk2} antibody with the labeled DNA probe without addition of WCE. The asterisk indicates a nonspecific supershifted band. (B) Effects of anti-pRB antibodies on E2F complexes. Preincubation with the indicated antibodies was performed as for panel A. PAb421 is a control antibody that reacts with p53. XZ55 is a monoclonal antibody directed against pRB; α pep 5 corresponds to an affinity-purified polyclonal antibody raised against peptide 5 of pRB; normal immunoglobulin G (IgG) was added as a control at the same final concentration as the anti-peptide 5 antibody. XZ55 and the anti-peptide 5 antibody were also incubated with the ^{32}P-labeled probe without WCE to ensure the specificity of the supershifted bands. The arrow indicates the position of the E2F-pRB complex after being supershifted by the anti-pRB antibody. (C) Daudi WCE was preincubated with a control reticulocyte lysate (RL) or equal amounts of a reticulocyte lysate programmed with transcripts of the 13S E1A mRNA (E1A) prior to band shift analysis.

proliferation at the G_0/G_1 phase of the cell cycle (14, 31, 54) (see Fig. 7E and F). It was found that extracts prepared from IFN-α-treated Daudi cells displayed strong reduction of all the E2F-specific DNA-protein complexes. The levels of complexes A, B, and C were reduced below the detection limits, and only the rapidly migrating nonspecific band remained unchanged after IFN treatment (Fig. 1A). For some unexplained reason, the behavior of this nonspecific band was not consistent; in some other extract preparations, its intensity was increased by IFN-α (see Fig. 9A) or, alternatively, decreased (see Fig. 9B). After long exposure of the gels, some residual levels of complex B could be detected in the IFN-treated cell extracts (see Fig. 7A, second lane).

Assays of the same WCE for DNA-binding activity of another cellular transcription factor, NF-1, revealed that the binding to this element remained constitutive and did not decline after treatment of Daudi cells with IFN-α (Fig. 1C). Thus, the abolishment of DNA binding in extracts of IFN-α treated cells seems to be specific for E2F complexes.

To identify the composition of the E2F complexes in extracts prepared from growing Daudi cells, we used several antibodies, chosen according to previous information concerning the E2F partners in other cells. Complex A was eliminated by preincubation of the control Daudi cell extracts with an anti-cdk2 antiserum, whereas preimmune rabbit serum had no effect (Fig. 2A). The more slowly migrating band (marked with an asterisk in Fig. 2A) is a nonspecific supershifted band that resulted from direct interaction of the antibodies with the probe, since it also appeared when no extract was added. Incubation of the Daudi cell extracts with affinity-purified anti-cyclin A antibodies exclusively eliminated the upper portion of the doublet in complex A. The affinity-purified anti-cyclin B antibodies had no effect on the E2F-containing complexes, and the pattern was identical to that of the control lane treated with preimmune serum (Fig. 2A). These findings suggest that the upper member of the doublet in complex A contains cyclin A-p33^{cdk2}. The lower member of the doublet in complex A, which was also eliminated by the anti-cdk2 antiserum, may consist of cyclin E-p33^{cdk2} (33). The use of monoclonal or affinity-purified polyclonal antibodies against pRB (XZ55 and anti-peptide 5, respectively), shown to interact exclusively with pRB (25, 35a, 57), resulted in a specific supershift of complex B, not appearing in the absence of cell extract (Fig. 2B). Neither normal immunoglobulin G nor the anti-p53 monoclonal antibody used as a control affected the E2F complexes. These results demonstrate that E2F complex B contains pRB. Finally, band C corresponds to what has been previously described as free E2F because of its resistance to treatment with DOC (see Fig. 8A), a detergent known to disrupt protein-protein interactions, including the heteromers formed by E2F (3). Furthermore, complexes A and B were also suppressed upon addition to the control cell extracts of in vitro-translated E1A (Fig. 2C), shown before to sequester E2F from the heteromeric complexes and release the free forms of E2F (3, 6, 12, 38, 47). Altogether, these findings imply that IFN-α abolishes all previously described forms of E2F, including the free E2F and E2F that is complexed with pRB and with cyclin-cdk2.

Abolishment of E2F-containing complexes by IFNs and IL-6 is linked to suppression of the c-*myc* promoter and is implicated in the antiproliferative mode of action of these cytokines. Temporal kinetics analysis performed at various time intervals after exposure of Daudi cells to IFN-α demonstrated that all of the E2F-containing complexes were reduced with similar kinetics (Fig. 3A). The reduction started between 5 and 8 h after treatment with IFN-α and was almost maximal at the 12-h point, which is before any change in cell cycle distribution is observed (Fig. 3C). It is noteworthy that the time kinetics of E2F responses coincided with the suppression of c-*myc* (Fig. 3B). The c-Myc protein levels were measured in the same cell extracts used for the band shift assay and may reflect the timing of transcriptional responses due to the short half-life of c-Myc protein (30 min).

In vivo functional assays were performed to test whether a region of the c-*myc* promoter that contains the E2F-binding sites can be suppressed by IFN-α. For this purpose, Daudi cells were transiently transfected with a *myc*-CAT construct designated pSNmyc-CAT. This construct consists of a *Sma*I-*Nae*I fragment of the human c-*myc* gene containing the P1 and P2 promoters and flanking sequences fused to the CAT reporter gene (Fig. 4A). The cells were transfected by electroporation, IFN-α was added 3 h after transfection, and extracts were prepared 40 h later and assayed for CAT enzymatic activity. As shown in Fig. 4B, IFN-α reduced by 70% the CAT gene expression driven by the c-*myc* promoters. In contrast, expression from constructs in which the CAT gene was fused to the SV40 promoter (pSV2CAT)

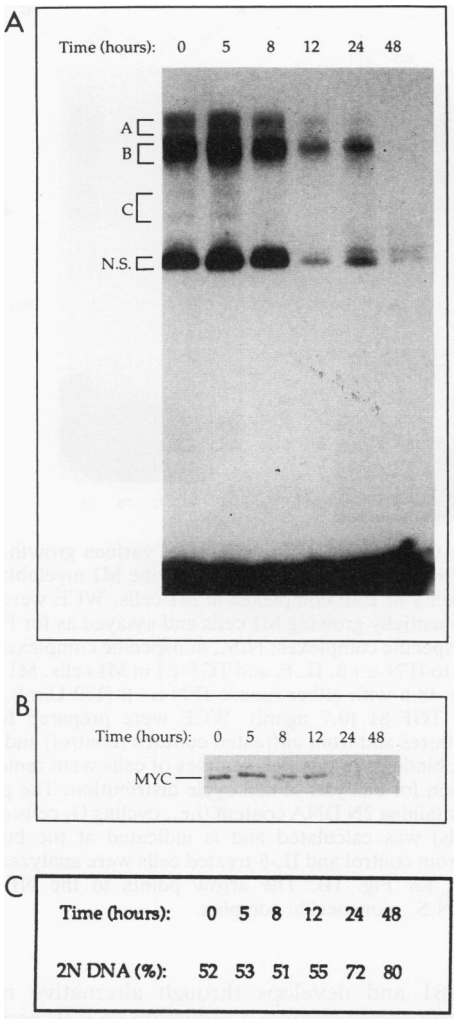

FIG. 3. Temporal kinetics of E2F and c-*myc* responses to IFN-α. (A) WCE were prepared at the indicated time intervals after exposure of Daudi cells to IFN-α and subjected to E2F binding assays by gel shift analysis. N.S., nonspecific band. (B) Equal amounts of proteins from the same extracts were resolved by SDS-polyacrylamide gel electrophoresis, electroblotted, and reacted with antibodies against c-Myc. (C) The percentage of cells containing 2N DNA (either cycling G_1 cells or G_0/G_1-arrested cells) was determined by cytofluorimetric analysis.

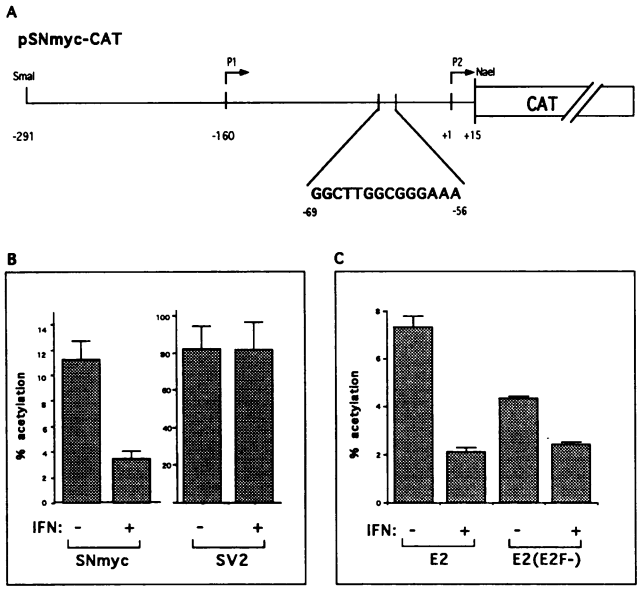

FIG. 4. IFN inhibits c-*myc*- and E2F-dependent transcription. (A) pSNmyc-CAT. The construct pSNmyc-CAT contains the CAT gene under the control of human c-*myc* P1 and P2 promoters and 5′ flanking sequences. The sequence of the P2-distal E2F-binding site is depicted. Numbering is with respect to the transcriptional start site of P2. (B) Daudi cells were transiently transfected by electroporation with pSNmyc-CAT (SNmyc) or pSV2-CAT (SV2) and split into two subpopulations, one of which was treated with IFN-α. The cells were harvested 40 h later, and CAT activity was determined. (C) Transient transfections of Daudi cells by DEAE-dextran were performed with either E2-CAT (E2) or E2-CAT (E2F-). Half of the transfectants were treated with IFN-α for 48 h and processed as for panel B. CAT activity in panels B and C is expressed as percent conversion of chloramphenicol into acetylated forms, and each value represents the average of two independent transfections.

remained constitutive after IFN-α treatment, consistent with previous data concerning other SV40-driven genes (48). This region of the c-*myc* promoter is therefore negatively regulated by IFN-α. To test whether the cytokine-mediated reduction of E2F complexes is responsible, at least in part, for this negative regulation, we tested the effects of IFN-α on the E2F-dependent transcription from the well-studied adenovirus E2 promoter. We used for these studies the previously described wild-type E2 promoter fused to the CAT gene (E2-CAT) and its derivative which is mutated in both E2F sites [E2-CAT (E2F-)] (23). IFN-α reduced the expression of the CAT gene driven by the intact E2 promoter by 71% (Fig. 4C). CAT expression from the mutated E2 promoter was consistently lower and less susceptible to the effect of IFN-α. These results therefore suggest that the E2F-dependent transcription may be suppressed by IFN in Daudi cells.

In an attempt to analyze further the functional role of the E2F inhibition by IFN-α, a Daudi cell variant that is specifically defective in the growth responses to IFN was used. The growth-resistant cell variant was previously isolated in our laboratory by a direct selection (continuous growth in IFN-α-containing medium). Previous molecular characterization of this clone revealed that it expresses functional IFN-α receptors and retains the ability of responding to IFNs by elevation of one of the IFN-induced enzymes, the 2-5-adenylate synthetase (14, 31). However, this cell variant carries an unknown genetic deregulation in the growth signaling pathways that prevents c-*myc* suppression by IFN-α (14, 31) see Fig. 5B). Gel shift assay done with cell extracts prepared from this IFN-resistant clone (designated DR) revealed that IFN-α failed to reduce the E2F-binding activity in these cells. All of the three main complexes, A, B, and C, remained constitutively high at 48 h after IFN-α treatment (Fig. 5A). In the same experiment, wild-type Daudi cells showed the typical decline of the different E2F-containing complexes. Thus, the relaxed regulation of E2F-binding activity in the growth-resistant cell variant further supports the involvement of E2F responses in the growth signaling of IFN and in the negative control of c-*myc* expression.

At this stage, we wished to determine whether the reduction in the DNA-binding activity of the E2F complexes also takes place in response to other growth-inhibitory cytokines

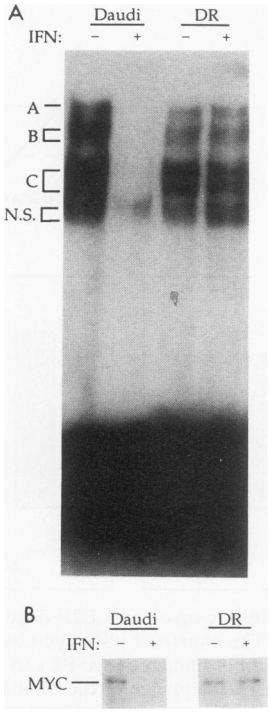

FIG. 5. IFN-α fails to abolish the E2F-containing complexes in an IFN growth-resistant derivative clone of Daudi. (A) The parental Daudi cell line (Daudi) and the IFN-resistant clone (DR) were treated with IFN-α (250 U/ml) for 48 h or left untreated. WCE were prepared and assayed for E2F DNA binding (B). Samples of the same WCE were subjected to Western blot analysis using antibodies against c-Myc. N.S., nonspecific band.

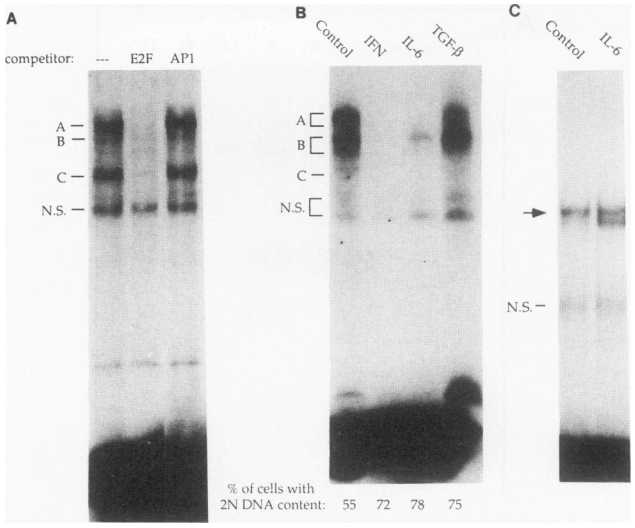

FIG. 6. Comparison of the effects of various growth-inhibitory cytokines on E2F-binding activity in murine M1 myeloblastic cells. (A) Specificity of E2F complexes in M1 cells. WCE were prepared from exponentially growing M1 cells and assayed as for Fig. 1A. A, B, and C, specific complexes; N.S., nonspecific complexes. (B) E2F responses to IFN-α+β, IL-6, and TGF-β1 in M1 cells. M1 cells were treated for 48 h with either mouse IFN-α+β (150 U/ml), IL-6 (250 U/ml), or TGF-β1 (0.7 ng/ml). WCE were prepared from these treated cultures and from untreated cultures (control) and tested for E2F DNA binding. In parallel, samples of cells were removed prior to extraction for analysis of cell cycle distribution. The percentage of cells containing 2N DNA content (i.e., cycling G_1 cells or arrested G_0/G_1 cells) was calculated and is indicated at the bottom. (C) Extracts from control and IL-6-treated cells were analyzed for NF-1 binding as for Fig. 1B. The arrow points to the NF-1-specific complex. N.S., nonspecific complex.

and in other cell systems. Murine M1 myeloblastic cells were chosen for this purpose since they can be growth arrested in G_0/G_1 phase by three different cytokines: IFN-α+β, IL-6, and TGF-β1 (49) (Fig. 6B). Also, we have previously reported that each of these three cytokines reduces the c-Myc protein levels in M1 cells (49), but while IFNs and IL-6 suppress c-*myc* at the transcriptional level, TGF-β1 regulates the gene posttranscriptionally (58). In general, the pattern of E2F-containing complexes in the nontreated M1 cells resembled that of the Daudi cells and included all three specific complexes (Fig. 6A). The only exception was that the free E2F (complex C) appeared as a single band. Exposure of M1 cells to each of the aforementioned cytokines revealed that IL-6 and IFNs had similar effects, and they both suppressed the three E2F-containing DNA-binding complexes. In sharp contrast, TGF-β1 failed to change the DNA-binding activity of these complexes (Fig. 6B), consistent with the failure of this cytokine to transcriptionally suppress the c-*myc* gene in these myeloid cells. Assays of the DNA-binding activity of the nonrelevant NF-1 in these extracts demonstrated that NF-1 complexes did not decline after IL-6 treatment (Fig. 6C), suggesting selectivity in the effects of this cytokine toward E2F, as shown above for IFN.

Altogether, these results link the suppression of the c-*myc* promoter to the abolishment of DNA binding of E2F complexes. They also demonstrate that these E2F responses do not simply reflect the new position of cells along the cell cycle but rather are part of the signaling pathway of IFNs and IL-6, since the G_0/G_1 arrest that is induced in these cells by TGF-β1 and develops through alternative molecular pathways does not involve a reduction of E2F complexes.

Reduction of the DNA-binding E2F complexes is not affected by the modulation of pRB and cyclin A in the system. Two of the major proteins present in complexes A and B, i.e., cyclin A and pRB, are selectively modified by IFNs and IL-6 in the cell systems that were studied in this work (49) (Fig. 7C and D). The question, then, is whether the cyclin A- and pRB-induced changes in expression and function, respectively, may dictate the nature of E2F responses to the cytokines. To address this question in a direct way, we measured E2F responses after two different pharmacological manipulations that specifically interfere with pRB and cyclin A responses to cytokines. One way to prevent the cyclin A and pRB responses without interfering with the suppression of c-*myc* is to block cells in the S phase of the cell cycle before their exposure to the cytokines (49). Exponentially growing Daudi cells were therefore treated with hydroxyurea (HU) prior to the addition of IFN-α. The efficiency of the treatment was examined by cytofluorimetric analysis. Figure 7E shows that most of the cells were blocked in S phase by HU. The effect of the drug was dominant over the cell cycle effects of IFN-α, since the cells remained in S phase following the treatment with HU plus IFN-α, while IFN-α by itself induced the typical G_0/G_1 block. The immunoblot in Fig. 7C clearly illustrates that the pRB and cyclin A responses to IFN-α were completely prevented in the S-phase-blocked cells. It is shown that the classical IFN-induced shift in pRB migration on gels, which reflects the conversion of these

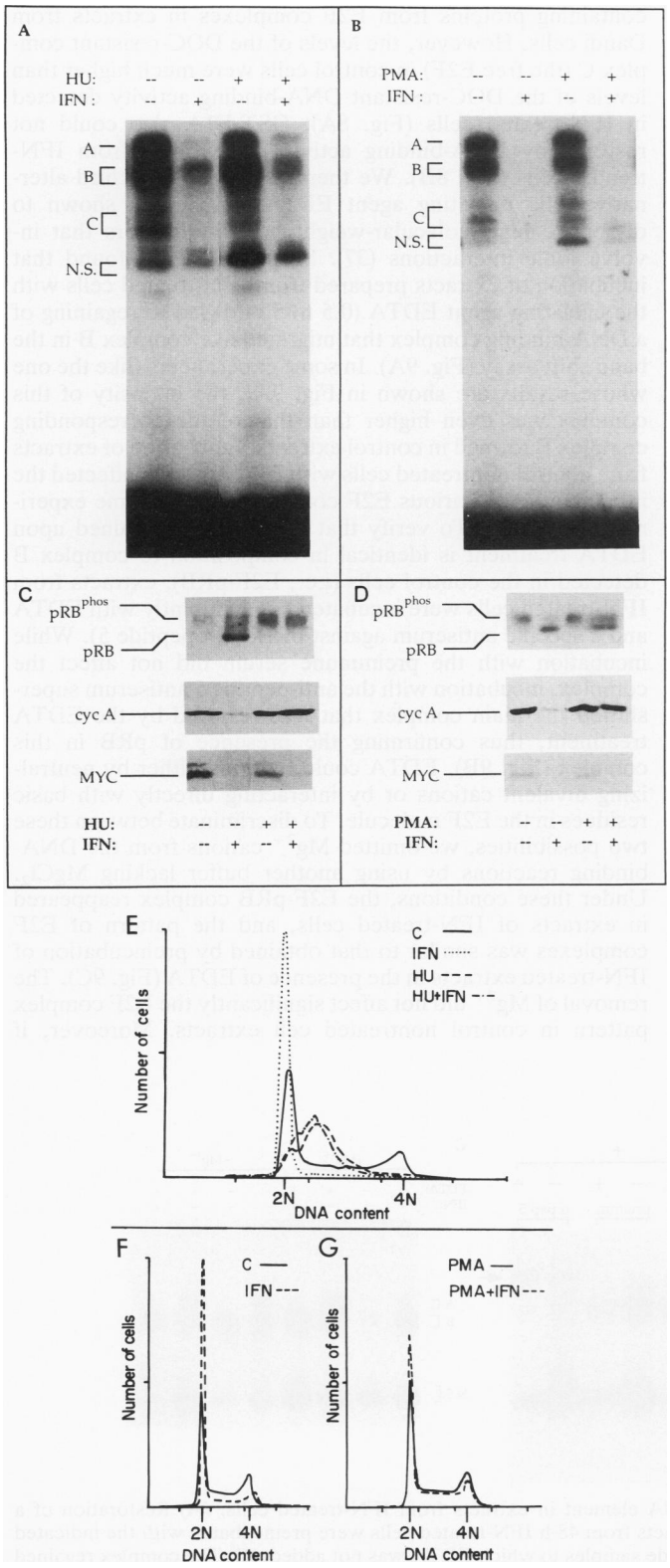

FIG. 7. Abolishment of E2F-containing complexes is not dependent on the IFN-α-induced changes of pRB phosphorylation and cyclin A levels. (A and C) HU experiment. Exponentially growing Daudi cells (4×10^5 cells per ml) were treated with 0.7 mM HU for 24 h, and then IFN-α (250 U/ml) was added to a portion of the culture for additional 48 h. In parallel, a fresh culture of Daudi cells (not pretreated with HU) was exposed to IFN-α or left untreated. (A) WCE were analyzed for E2F DNA binding in band shift assays. (C) Samples of these WCE were subjected to Western blot (immunoblot) analysis using an anti-pRB, anti-cyclin A, or anti-c-Myc antibody. Positions of the slowly migrating hyperphosphorylated (pRB^{phos}) and the rapidly migrating underphosphorylated (pRB) forms of pRB and position of the cyclin A (cycA) and c-Myc (MYC) proteins are indicated. (B and D) Analysis of the effect of PKC depletion on E2F responses to IFN-α. Exponentially growing Daudi cells were exposed to PMA for 24 h, and then IFN-α was added for additional 48 h to a portion of the culture (at a density of 2×10^5 cells per ml). In parallel, naive cells (cells not exposed to PMA) were also treated for 48 h with IFN-α or left untreated. WCE were tested for E2F DNA binding (B) and for pRB, cyclin A, and c-Myc signals on immunoblots (D) as for panels A and C. N.S., nonspecific complex. (E) Cell cycle responses to IFN-α in HU-treated Daudi cells. The cytofluorimetric analysis was performed on samples of naive or HU-treated cells that were either exposed to IFN-α or were not treated by the cytokine as detailed for panel A. C, control exponentially growing Daudi cells; IFN, naive cells that were treated for 48 h with IFN-α; HU, culture maintained in HU for 72 h; HU+IFN, culture maintained in HU for 72 h and also with IFN-α during the last 48 h. (F and G) Cell cycle responses to IFN-α in PKC-depleted cells. The cytofluorimetric analysis was performed on samples of naive or PMA-treated cells that were either exposed to IFN-α or left untreated. C, control culture; IFN, 48 h of IFN treatment; PMA, culture maintained in PMA for 72 h; PMA+IFN, culture maintained in PMA for 72 h and also with IFN-α during the last 48 h.

molecules into rapidly migrating underphosphorylated pRB forms, was completely abrogated in IFN-treated cells that were preexposed to HU. In addition, the strong IFN-mediated reduction in cyclin A protein levels was also completely alleviated when S-phase-blocked cells were exposed to IFN-α. The binding of nuclear proteins to the ^{32}P-labeled E2F oligonucleotide was then tested in these cells. Interestingly, IFN-α continued to reduce by a similar extent all of the E2F-containing complexes in the S-phase-blocked cells in spite of the relaxed regulation of pRB and cyclin A (Fig. 7A). Also, the down regulation of c-*myc* by IFN-α was not impaired in these HU-blocked cells (Fig. 7C).

The second pharmacological manipulation consisted of depleting protein kinase C (PKC) (isoforms α, β, and γ) from Daudi cells by chronic exposure to phorbol 12-myristate 13-acetate (PMA). We showed previously that this treatment exclusively rescued the IFN-α-induced molecular events that depend on active PKC and by that mean caused partial growth resistance to IFN-α (49, 54). Exponentially growing Daudi cells were exposed to PMA (5 ng/ml; Sigma) for 24 h. Then part of the culture received IFN-α (250 U/ml), and the rest was left untreated. Cytofluorimetric analysis demonstrated that, as previously reported, pretreatment with PMA abrogated the selectivity of the IFN effects on the G_0/G_1 phase, and instead the cells stopped to divide in all phases of the cell cycle (Fig. 7G; the normal responses to IFN-α in the same experiment are illustrated in Fig. 7F). At the molecular level, PKC-desensitized cells continued to reduce c-*myc* transcription in response to IFN-α (49, 54) (Fig. 7D), while they clearly failed to reduce the cyclin A protein levels and displayed only partial suppression of pRB phosphorylation (Fig. 7D). Interestingly, consistent with the unperturbed c-*myc* suppression, this manipulation did not prevent development of the E2F responses to IFN-α (Fig. 7B). It is concluded from the two pharmacological manipulations that the IFN-α-induced changes in the phosphorylation of pRB or in the steady-state levels of the cyclin A protein are defi-

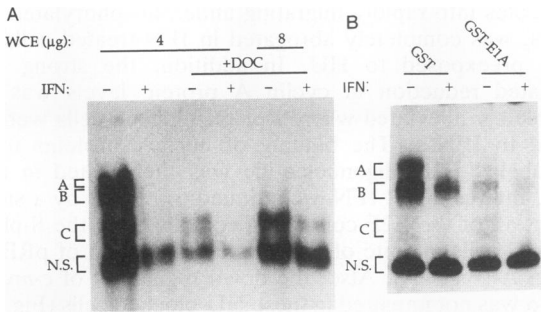

FIG. 8. DOC and E1A fail to restore E2F-binding activity in extracts from IFN-treated cells. (A) Extracts from control and IFN-treated cells (4 and 8 μg) were preincubated with DOC as described in Materials and Methods and analyzed by gel shift assay alongside samples (4 μg) which were not preincubated with the detergent. (B) Extracts from control and IFN-treated cells were preincubated with equivalent amounts of GST or GST-E1A and subjected to gel shift analysis. N.S., nonspecific complex.

nitely not primary events that influence the nature of E2F responses to IFN-α.

EDTA restores the E2F DNA-binding activity in extracts of IFN-α-treated cells. Cytokines may interact with E2F in various ways to prevent the binding of E2F complexes to DNA. They could either suppress E2F mRNA or protein expression, modify the E2F protein in a way that will prevent DNA binding, or induce or activate an inhibitory protein that may interfere with the DNA binding of E2F. To discriminate among these possibilities, extracts from IFN-treated cells were subjected, prior to the band shift assays, to different agents known to dissociate protein-protein interactions. Restoration of DNA-binding activity by such treatments will exclude reduction of E2F expression and support the other possible mechanisms. We found that incubation with DOC and Nonidet P-40 failed to restore E2F DNA-binding activity in the IFN-treated cell extracts. Under the same conditions, DOC was able to dissociate the pocket-containing proteins from E2F complexes in extracts from Daudi cells. However, the levels of the DOC-resistant complex C (the free E2F) in control cells were much higher than levels of the DOC-resistant DNA-binding activity detected in IFN-treated cells (Fig. 8A). GST-E1A also could not restore any DNA-binding activity in extracts from IFN-treated cells (Fig. 8B). We therefore used, as a third alternative, the chelating agent EDTA, previously shown to dissociate high-molecular-weight hetero-oligomers that involve ionic interactions (37). Surprisingly, we found that incubation of extracts prepared from IFN-treated cells with the chelating agent EDTA (0.5 to 5 mM) led to regaining of a DNA-binding complex that migrated like complex B in the band shift assay (Fig. 9A). In some experiments (like the one whose results are shown in Fig. 9A), the intensity of this complex was even higher than that of the corresponding complex B formed in control extracts. Incubation of extracts from control nontreated cells with EDTA hardly affected the intensity of the various E2F complexes in the same experiment (Fig. 9A). To verify that the complex regained upon EDTA treatment is identical in composition to complex B detected in the control cells (i.e., E2F-pRB), extracts from IFN-treated cells were incubated concomitantly with EDTA and a specific antiserum against pRB (anti-peptide 5). While incubation with the preimmune serum did not affect the complex, incubation with the anti-peptide 5 antiserum supershifted the main complex that was restored by the EDTA treatment, thus confirming the presence of pRB in this complex (Fig. 9B). EDTA could function either by neutralizing divalent cations or by interacting directly with basic residues in the E2F molecule. To discriminate between these two possibilities, we omitted Mg^{2+} cations from the DNA-binding reactions by using another buffer lacking $MgCl_2$. Under these conditions, the E2F-pRB complex reappeared in extracts of IFN-treated cells, and the pattern of E2F complexes was similar to that obtained by preincubation of IFN-treated extracts in the presence of EDTA (Fig. 9C). The removal of Mg^{2+} did not affect significantly the E2F complex pattern in control nontreated cell extracts. Moreover, if

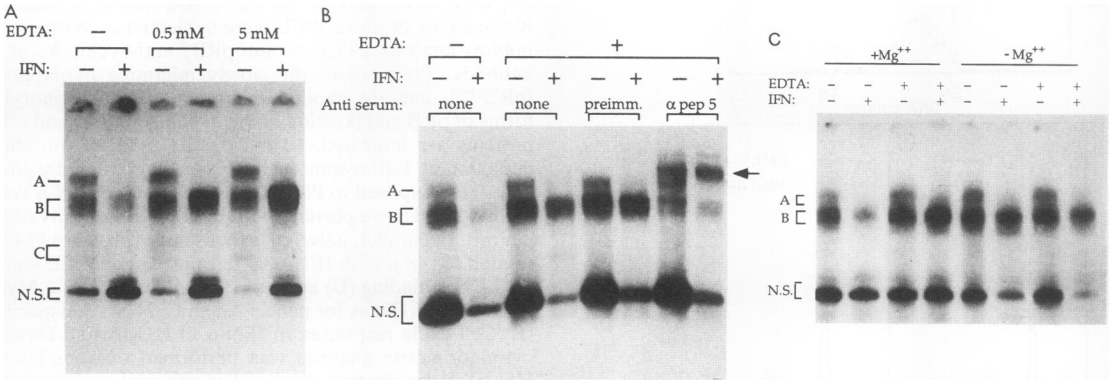

FIG. 9. EDTA restores the binding of complex B to the E2F DNA element in extracts from IFN-treated cells. (A) Restoration of a DNA-binding complex. Control untreated Daudi WCE as well as extracts from 48-h IFN-treated cells were preincubated with the indicated concentrations of EDTA and then analyzed by band shift assay alongside samples to which EDTA was not added. (B) The complex regained upon EDTA treatment contains pRB. WCE from untreated and IFN-treated Daudi cells (extract preparations different from those used for panel A) were preincubated with 5 mM EDTA either alone or with preimmune serum (preimm.) or anti-pRB (α pep 5) antiserum. The DNA-binding reaction samples as well as samples which were not preincubated with EDTA were assayed for band shift. The arrow points to a supershifted complex. (C) Removal of Mg^{2+} cations restores E2F-binding activity. Cell extracts from control untreated and IFN-treated Daudi cells were preincubated and reacted with the labeled DNA probe in binding buffer lacking Mg^{2+} cations. Equivalent samples of extracts were preincubated in the same buffer supplemented with 1 mM $MgCl_2$. When indicated, EDTA (5 mM) was added at the preincubation step. N.S., nonspecific complex.

EDTA was added in the absence of Mg^{2+}, it did not stimulate further complex B formation in the IFN-treated cell extracts, thus ruling out the possibility that restoration depends on direct interactions of EDTA with proteins and supporting the other possibility of neutralizing Mg^{2+} cations (Fig. 9C).

DISCUSSION

In this work, we demonstrate that E2F regulation in response to external signals that induce cell cycle exit and growth arrest differs basically from the previously described cell cycle regulation of E2F in growing cells. The cyclic characteristics of E2F in exponentially dividing cells consisted of phase-specific fluctuations in the pattern of E2F-containing heteromeric complexes, which were dictated by the other protein partners. In this respect, an E2F-pRB complex was seen in G_1 and S phases, an E2F-p107-cyclin A-cdk2 complex was exclusively detected in S phase, and an E2F-p107-cyclin E-cdk2 complex as well as another, less well characterized complex (38, 52) appeared in G_1 phase, suggesting a regulation of E2F function that is translated into an orderly cell cycle progression. In contrast, the E2F responses to IFNs and IL-6 are not primarily influenced by the different protein partners, even though at least some of these proteins (i.e., cyclin A and pRB) are selectively modified by the cytokines at the expression or posttranslational levels (Fig. 7C and D) (49). Recent results indicate that cyclin E mRNA levels are also suppressed by IFN-α in Daudi cells (53a). If the effects of the cytokines on the E2F protein partners were the rate-limiting primary events that dictate E2F responses, then one would expect, for example, that elimination of the upper part of complex A by IFN-α would strictly depend on cyclin A suppression. Moreover, the cytokine-induced underphosphorylated forms of pRB, previously shown to display high affinity to E2F (11, 21, 28, 51), may compete with all of other E2F partners, leading to strong enhancement of complex B at the expense of other E2F complexes. Instead, the results reported here indicate that the dominant regulation by the growth-inhibitory cytokines leads to the simultaneous abolishment of all E2F complexes, including the E2F-pRB complex, irrespective of whether the above-mentioned protein partners were modulated by the cytokine. Thus, the abolishment of E2F-containing DNA-binding complexes continued to take place even if Daudi cells were pretreated with HU (Fig. 7A and C) or with PMA (Fig. 7B and D) before IFN-α addition, despite the fact that each manipulation interfered with cyclin A and pRB responses to the cytokine.

Lack of E2F-containing DNA-binding complexes was also previously observed in serum-starved growth-arrested fibroblasts (39), which could suggest that the effects seen in this work reflect simply the new position of cells in a quiescent phase of the cell cycle. However, it should be emphasized that the pharmacological manipulations described above changed the pattern of cell cycle responses to the cytokines. Under these conditions, the cells either were blocked in S phase as a result of the dominant effect of HU (Fig. 7E) or were blocked in all cell cycle phases (Fig. 7F and G) as a result of the partial responsiveness of PKC-depleted cells to IFN-α (54). Thus, the unperturbed E2F responses to IFN-α in these manipulated cells indicate that abolishment of DNA-binding E2F complexes is not the indirect consequence of maintenance of cells in the G_0/G_1 phase by the cytokine but rather is part of the IFN-α-induced signal transduction pathways. A detailed time kinetics study performed in Daudi cells confirmed that the abolishment of E2F complexes by IFN-α preceded the cell cycle block in the G_0/G_1 phase (Fig. 3). This finding further suggests that suppression of E2F DNA-binding activity leads to the growth arrest rather than reflecting the cell cycle change. This type of E2F regulation is correlated in this work with the behavior of the c-*myc* promoter. First, it failed to develop in response to IFN-α in a growth-resistant, receptor-positive Daudi cell variant that displays relaxed regulation of c-*myc* (Fig. 5). Second, in the PMA- and HU-treated cells, which lost part of the gene responses to IFN-α, both E2F and c-*myc* responses continued to develop (Fig. 7A to D); hence, the two functions may be linked. Third, the functional assays demonstrate that transcription of a minimal promoter of c-*myc* containing E2F sites, as well as the E2F-dependent transcription from the adenovirus E2 promoter, was suppressed by IFN-α. Taken together, these results suggest that E2F regulation by IFN and IL-6, shown in this work, has a central role in the mechanisms that suppress c-*myc* expression. We have previously shown, by introducing a deregulated version of c-*myc*, that the suppression of this gene by IFN and IL-6 is one out of a few essential events that induce the cell cycle exit into G_0/G_1 phase (48). The link between c-*myc* and E2F regulation shown here further suggests that the reduction in E2F complexes may lead to G_0/G_1 arrest via c-*myc* suppression, providing it functions in concert with the other cytokine-induced molecular events that develop independently. Obviously, there could be alternative ways to reduce the c-*myc* mRNA and protein expression which do not involve E2F regulation. One example, involving the TGF-β1 responses of M1 myeloblastic cells, is demonstrated in this study. Consistent with our previous finding that in these target cells, TGF-β1 reduces c-*myc* expression at the posttranscriptional level (58), all E2F-containing complexes remained constitutive in response to this cytokine (Fig. 6B). It will be of interest to test the TGF-β1 effects on E2F complexes in mouse and human keratinocytes, since in those cell systems TGF-β1 suppresses transcription of the c-*myc* promoter (45).

How do IFNs and IL-6 suppress the E2F-containing complexes that bind to DNA? In this work, we have excluded the simple possibility that a reduction in the expression levels of E2F protein could be responsible for the elimination of these complexes. This is concluded since an E2F-containing complex reappeared upon EDTA addition to cell extracts prepared from IFN-α-treated cells. Therefore, the results support the alternative possibility in which the cytokines reduce the DNA-binding activity of E2F protein. However, certain mechanisms may reduce the DNA binding of E2F; some were suggested in the past. For example, it was shown in cell-free systems that the DNA-binding activity of E2F could be modulated by phosphorylation (2). Similarly, IFN-α and IL-6 could change the phosphorylation state of E2F or induce other posttranslational modifications that reduce the DNA-binding activity of the protein. An alternative mechanism may involve induction or activation by the cytokine of an inhibitory protein that interferes with DNA-binding properties by complexing with E2F. The existence of an inhibitory activity, designated E2F-I, that prevented the binding of E2F to DNA has been proposed (4). However, upon fractionation, this DNA-binding inhibitory activity turned out to be pRB. The authors suggested that an excess of activated (i.e., dephosphorylated) pRB could indirectly inhibit E2F binding to DNA by depleting another component necessary for DNA binding, the accessory RBP60 protein, to which pRB associates (46). It was there-

fore not surprising that the inhibitory function of this previously described activity was relieved upon addition of E1A to the cell extracts, known to dissociate the pRB-E2F type of interactions (46). Interestingly, the inhibitions detected in the extracts of IFN-α-treated cells were resistant to DOC or to E1A. In contrast, addition of EDTA to the DNA-binding reaction mixtures or removal of Mg^{2+} cations from the binding buffer resulted in regaining of binding of the E2F-pRB complex to the E2F DNA element in extracts from IFN-treated cells. No pronounced change could be detected in control extracts. EDTA is known to disrupt protein-protein ionic interactions that are different from those which are dissociated by some detergents (37). It is possible that by neutralizing Mg^{2+} cations, EDTA releases an inhibitory protein which binds to a different region of the molecule, e.g., the basic helix-loop-helix DNA-binding domain of E2F, without affecting E2F association with pRB. Since extracts prepared from IFN-treated cells are depleted of cyclin A protein and contain only the dephosphorylated forms of pRB, it is not surprising that upon EDTA-induced restoration of the DNA-binding activity, the E2F-pRB complex predominates (Fig. 9B). In HU-pretreated cells, in which IFN-α failed to modify cyclin A and pRB, EDTA treatment resulted in the regaining of both complexes, A and B (data not shown). However, it should be stressed that the EDTA experiment does not exclude the previously discussed possibility of posttranslational modifications of E2F. Along this line, the removal of Mg^{2+} cations from extracts may counteract the reduced affinity of E2F to DNA that could be caused by its posttranslational modifications. In another scenario, the cytokines may act by modifying the putative RBP60 accessory protein in an EDTA-reversible manner. Biochemical fractionation of extracts from IFN-treated cells should indicate whether a novel inhibitory protein or, alternatively, a posttranslational modification of E2F is the major cause for these reductions in DNA binding.

In summary, these results demonstrate that cytokines reduce E2F-containing complexes by interfering with the DNA-binding properties of E2F. Thus, in contrast to the phase-specific modulation of E2F that affects the transactivating properties of this transcription factor in cycling cells, the growth-inhibitory cytokines suppress the DNA-binding activity of E2F via a mechanism that can be abrogated or counteracted by the simple addition of EDTA or by the removal of Mg^{2+} divalent cations.

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