Regulation of the Human Cyclin-dependent Kinase Inhibitor
p18\(^{\text{INK4c}}\) by the Transcription Factors E2F1 and Sp1*

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The p18\(^{\text{INK4c}}\) cyclin-dependent kinase inhibitor is an important regulator of cell cycle progression and cellular differentiation. We and others found that overexpressed E2F proteins up-regulate p18 expression. To better understand this phenomenon, we performed a functional analysis of the human p18 promoter. Deletion studies revealed that the E2F-responsive elements of the promoter are located within 131 bp upstream of the transcription start site. This region contains putative Sp1- and E2F-binding sites. Mutational inactivation of these elements revealed that the Sp1 sites were important for the basal activity of the promoter but could also mediate the effects of E2F1 on the p18 promoter. Moreover, we found that E2F1 and Sp1 can synergistically enhance the activity of the proximal p18 promoter. Gel shift analyses using p18 promoter-derived probes led to the identification of several multiprotein complexes that were found to contain different combinations of E2F proteins and/or Sp1. Recombinant E2F1 was also capable of binding to the E2F-binding sites. Chromatin immunoprecipitation experiments demonstrated that E2F1 and E2F4 associate with the p18 promoter in unperturbed cells. Based on these findings, we conclude that E2F proteins and Sp1 play an important role in the control of p18 expression.

Progression through the mammalian cell cycle is governed by cyclins, cyclin-dependent kinases, and regulators thereof, whose expression and activity are tightly coordinated through a series of well ordered, but not completely understood, relationships. One event of prime importance during the cell cycle is the passage from the G1 to the S phase, during which a complex intracellular signalization, involving a transient rise in the levels of G1 phase cyclin proteins and a concurrent increase in the activity of the associated kinases, leads to the activation of the E2F transcription factor, an important regulator of cell cycle-dependent gene expression (reviewed in Refs. 1–5).

The E2F transcription factor family (comprising E2F1 to E2F6, DP1, and DP2) is known to modulate the expression of a number of proteins implicated in cell division, accounting for its major role in regulating the cell cycle. By associating with their DP dimerization partner, some E2F transcription factors can modulate the expression of proteins involved in DNA synthesis (thymidylate synthase (6), thymidine kinase (7), and DNA polymerase α (8)), DNA repair (uracil-DNA glycosylase (9, 10)), and cell cycle control (c-Myc (11), c-Myb (12), and cyclin E (13–15)), among others. Although it is known that E2F1 is a powerful promoter of mitosis (16, 17), knock-out experiments in mice have demonstrated a higher incidence of tumor development in mice that lack the E2F1 gene (18). This observation suggests that E2F1-mediated signaling may be part of an anti-proliferative pathway.

One of the cell cycle regulatory genes whose expression is induced by E2F is p18\(^{\text{INK4c}}\), a member of the INK4 sub-family of cyclin-dependent kinase inhibitors. p18 shares sequence homology with p16\(^{\text{INK4a}}\), p15\(^{\text{INK4b}}\), and p19\(^{\text{INK4d}}\) and acts primarily on CDK4 and CDK6 kinases uncomplexed with their cyclin partners (19–21). DeGregori and co-workers (22) have shown that some members of the E2F family of transcription factors were able, when overexpressed by adenoviral infection, to induce expression of the p18 gene. The fact that E2F induces p18 expression is surprising considering that the p18 protein is involved in cell cycle arrest in a variety of physiological processes such as differentiation of adipocytes (23), B-lymphocytes (24), granulocytes (25), osteoblasts (26), neuroblasts (27), and myoblasts (28, 29). p18 is also induced by IL-6 in B-lymphocytes (30) and by progesterone in breast cancer cells (31). On the other hand, p18 expression is down-regulated by agents that stimulate cell proliferation such as genistein in breast cancer cells (32), phorbol esters in HL-60 cells (25), and during HTLV infection of T-cells (33).

Mutations that disrupt the ability of cyclin-dependent kinase inhibitors to bind their target CDK have been discovered in a variety of cancers (34, 35). Our group has shown previously (36, 37) that BT-20 breast cancer cells, as well as three breast tumor biopsies of 35 samples, carry the p18-A72P mutation which decreases the ability of p18 to bind CDK6 and inhibit colony formation in transient transfection assays. Mice whose p18 alleles have been knocked out display a variety of aberrant phenotypes including lymphoproliferative disorders, organomegaly, and pituitary gland hyperplasia. Double knock-out mice for p18 and other members of the cyclin-dependent kinase inhibitor family display more varied and pronounced phenotypes (38–40), indicating that this gene is important for the

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1 The abbreviations used are: CDK, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; BSA, bovine serum albumin; IVT, in vitro translated; pB6, retinoblastoma protein; GFP, green fluorescent protein; GST, glutathione sulftotransferase; HA, hemagglutinin; oligo, oligonucleotide; PBS, phosphate-buffered saline; FCS, fetal calf serum; DTT, dithiothreitol; DMEM, Dulbecco’s modified Eagle’s medium.

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proliferative control of different cell lineages. Despite the increasing knowledge concerning the role of p18 in diverse physiological processes, very little is known regarding the precise molecular mechanisms that regulate the expression of this gene.

The human p18\(^{INK4c}\) gene consists of three exons, with the two last exons containing the entire p18 protein coding sequence. In most cell lines, this gene is transcribed in two predominant mRNA species of 2.1 and 1.0 kb. However, several cDNAs of intermediate length have also been cloned. By Northern blot analysis, we have determined that the longest form is the most abundant form in cancer cell lines of various origins including breast cancer (MCF7, MDA-MB-231, and BT-20), erythroleukemia (K562), and cervix carcinoma (HeLa). The 2.1-kb transcript is also the one the most influenced by E2F expression. The p18 promoter, defined in this study as the genomic sequences located upstream of the transcription start site of the long form cDNA (GenBank™ accession number AF041245 (37)), does not contain a TATA box, and its principal feature, reminiscent of other TATA-less promoters, is a region of high G + C content immediately upstream of the transcription initiation region. There are no evident initiator elements (41, 42) and no downstream promoter elements (DPE (43–45)), which is consistent with the presence of more than one transcription initiation site (37, 42).

An understanding of the fundamental mechanisms whereby E2F regulates p18\(^{INK4c}\) expression would undoubtedly clarify the roles of both E2F and p18 in cell cycle regulation and might eventually lead to a better comprehension of the paradoxical effects of E2F on cell proliferation. In an attempt to achieve this objective, we have isolated p18\(^{INK4c}\) regulatory sequences and identified those that mediate activation by E2F. We report here that both E2F and Sp1 response elements mediate E2F-induced activation of p18\(^{INK4c}\) gene transcription. Furthermore, we have found that some members of the E2F transcription factor family can bind these elements in vitro and induce the transcription of the p18 gene. Moreover, we found that E2F1 and E2F4 associate with the regulatory region of the p18 gene in vivo.

MATERIALS AND METHODS

Cell Culture—The human cell lines MCF7 (breast adenocarcinoma), MDA-MB-468 (breast adenocarcinoma), HeLa S3 (CCL 2.2, cervix carcinoma), WI-38 (lung diploid fibroblast), and the Drosophila melanogaster cell line Schneider-2 (SL2) were obtained from the American Type Culture Collection (Manassas, VA). S-MEM, DMEM-high glucose, and DMEM-F-12 culture media were purchased from Sigma. Schneider Drosophila medium, glutamine, trypsin, and antibiotics were from Invitrogen. Fetal calf serum (FCS) was obtained from Wisent (St-Bruno, Canada); estradiol (E2) was from Steraloids (New Port, RI), and cell culture plasticware was purchased from BD Pharmingen.

MCF7 cells were grown in DMEM-F-12 supplemented with 5% FCS, 2 mM glutamine, 1 × 10\(^{-9}\) M E2, 100 units of penicillin/ml, and 50 µg of streptomycin/ml. HeLa S3 cells were grown in 75-cm\(^2\) flasks as a loosely attached monolayer in high glucose DMEM supplemented with 10% v/v fetal calf serum, 10% v/v fetal calf serum, 2 mM glutamine, 100 units of penicillin/ml, and 50 µg of streptomycin/ml. The cells were split in three 175-cm\(^2\) flasks and cultured for 5 days after the medium to S-MEM supplemented with 10% v/v fetal calf serum, 2 mM glutamine, 50 µg of NaF (Sigma), 50 µg Na2VO3 (Sigma), 50 µg NaF (Sigma), 10 mM ß-glycerophosphate (Sigma). Complete protease inhibitor mixture, 0.7 µg/ml pepstatin, and 1 mM Pefabloc (all three from Roche Molecular Biochemicals). Cells were suspended and incubated on ice for 20 min before being stored at −80 °C for at least 1 day. Prior to use, lysates were thawed, vortexed vigorously, and centrifuged. Protein concentrations were determined using the Bio-Rad DC protein assay.

Western Blots—Total protein extracts were separated by SDS-PAGE on a Mini-Protean II apparatus (Bio-Rad) and electroblotted onto 0.2-µm pore size nitrocellulose membrane (Schleicher & Schuell) using a mini trans-blot apparatus (Bio-Rad). The nitrocellulose membranes were blocked using 5% non-fat dry milk diluted in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) supplemented with 0.05% Nonidet P-40 (Fluka, Switzerland) and 0.05% Tween 20 (Sigma). The antibodies used were anti-p18 (NeoMarkers, Fremont, CA, catalog number Rb-029), anti-cyclin D1 (NeoMarkers, catalog number MS-210), anti-cyclin E (BD PharMingen, catalog number 14591A), anti-pS (BD PharMingen, catalog number 14001A), anti-a-tubulin (Santa Cruz Biotechnology, catalog number SC-8035), and anti-TRADD (Transduction Laboratories, Lexington, KY, catalog number 703520).

Plasmid Constructs—The GST-Sp1−8xHis protein encoded by the pGEX-Sp1−8xHis plasmid contains the sequence encoding the 696 C-terminal amino acids of human Sp1 protein (provided in pCMV-Sp1 by Dr. Tjian, Berkeley, CA) followed by eight histidine residues at the C terminus, cloned in the pGEX-6P-1 plasmid (Amersham Biosciences AB). The pPOX-E2F1−8xHis and pPOX-E2F2−8xHis plasmids that encode full-length human E2F1 and DP1 proteins fused to the C terminus of the GST protein and bearing an eight-histidine residue tag at the C terminus were constructed in a similar manner. The pCDNA3-E2F1-HA plasmid was constructed by cloning the entire coding sequence of the human E2F1 gene (provided in pCMV-E2F1 by K. Haelin), in the expression plasmid pCDNA3-HA, in-frame with sequences encoding a C-terminal HA tag. The pCDNA5-FLAG-DP1 plasmid was constructed by inserting the human DP1-coding sequence, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid, preceded by DNA encoding the FLAG epitope, into the pCDNA3 expression plasmid.
culture was prepared by inoculating 20 ml of LB medium (containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 2% w/v glucose) with a single colony. The culture was grown overnight at 37°C and then diluted 500-fold in the same medium and allowed to grow until A600 = 0.5. Isopropyl-1-thio-β-D-galactopyranoside (Amersham Biosciences) was then added to a final concentration of 0.4 mM. Three hours later, the cells were harvested by centrifugation, washed once in cold PBS, aliquoted in the proper number of tubes, and centrifuged again to obtain pellets originating from 100 ml of broth. The supernatants were thoroughly decanted, and the pellets were stored at −80°C until use.

Unless stated otherwise, all purification steps were carried out at 4°C or in an ice water bath. To purify the overexpressed protein, the cell lysate was resuspended in 10 ml of ice-cold PBS containing 2% Triton X-100 and vortexed vigorously. This suspension was then subjected to two freeze-thaw cycles, sonicated twice (1 min each), and then slowly agitated on a rocking platform for 30 min. The lysate was centrifuged at maximum speed in a microcentrifuge. The supernatant was decanted, and the pellet containing the GST–Sp1–8xHis protein was resuspended in 10 ml of solubilization buffer (50 mM sodium phosphate buffer, pH 6.8, 6 M urea, 1 mM DTT, and 0.5% Triton X-100). The suspension was sonicated and agitated as described above and dialyzed for 4 h in order to re-nature the fusion protein. The dialysis buffer was composed of PBS supplemented with 2 mM urea, 10 μM ZnSO4, 1 mM DTT, 10% v/v glycerol, and 0.5% Triton X-100. The dialysis buffer was then replaced with buffer without urea, and the dialysis was prolonged for an additional 16 h. The dialyzed lysate was centrifuged to remove insoluble matter and allowed to bind to glutathione-Sepharose beads (Amersham Biosciences) at 4°C overnight.

The beads were washed three times in PreScission reaction buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100) and incubated in 1 ml of this buffer supplemented with 20 units of PreScission protease (Amersham Biosciences) for 24 h at 4°C. The beads were centrifuged at 10,000 × g for 5 min, and the supernatant was conserved.

Glycerol was added to the cleaved protein solution to a final concentration of 50% v/v, and the solution was stored in small aliquots at −80°C. At this step, the recombinant protein appeared to have a purity of at least 75% as judged by Coomassie Blue staining of the protein solution separated by SDS-PAGE (data not shown). The GST–E2F1–8xHis and GST–DP1–8xHis proteins were purified by differential solubilization. The induction and purification procedure for the GST–E2F1–8xHis and GST–DP1–8xHis proteins was the same as for the Sp1 protein, with the following modifications. After solubilization of the cell lysates with 2% Triton X-100 and sonication, the solution was centrifuged, and the pellet was resolubilized in STE buffer supplemented with DTT and Sarkosyl (150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.75% N-lauryl sarcosine sodium salt (Sigma)). The solution was vortexed, sonicated briefly, agitated at 4°C for 30 min, and centrifuged at 10,000 × g for 10 min. Glycerol was added to the solubilized protein solutions to a final concentration of 50% v/v, and the solution was stored in small aliquots at −80°C. At this step, the recombinant proteins appeared to have a purity of at least 75% as judged by Coomassie Blue staining of the protein solution separated by SDS-PAGE (data not shown). Contrary to Sp1–8xHis, the E2F1–8xHis and DP1–8xHis proteins were not separated from GST by protease digestion. The presence of Sarkosyl did not alter the DNA-binding properties of the proteins, because these solutions were highly diluted in the gel shift reaction mixtures (400-fold dilution).

Electrophoretic Mobility Shift Assays—Probes encompassing different regions of the promoter were synthesized by annealing complementary synthetic oligonucleotides. The upper strand oligonucleotide of the promoter was 80 bp in length. The probe was labeled with [32P]-dCTP using the Klenow fragment of DNA polymerase and [α-32P]dCTP. Unincorporated radioactive nucleotides were removed by gel filtration on Bio-Spin 30 columns (Bio-Rad). The binding conditions that gave satisfying results for both E2F and Sp1 binding contained 5 μg of total cellular proteins, 500 ng of sheared salmon sperm DNA, 25 mM Hepes, pH 7.6, 25 mM KC1, 1 mM EGTA, 2 mM MgCl2, 0.05% Nonidet P-40, and 10% glycerol, in a final assay volume of 20 μl. The probe was added to the binding reaction mixture, and binding was allowed to occur for 25 min at room temperature. 40,000 cpm of probe (generally representing 5–25 fmol of double stranded probe) were used in each EMSA reaction. For competition experiments, 1 pmol of unlabeled oligonucleotide was added prior to the addition of the labeled probe. When EMSA experiments were performed using bacterially produced or IVT proteins, 50 ng of the Sp1–8xHis protein, 50 ng of GST–DP1–8xHis protein, 5 ng of GST–E2F1–8xHis protein, or 2 μl of each in vitro translated (IVT) protein were used, and 100 ng of sheared salmon sperm DNA and 40 μg of bovine serum albumin (Sigma) were included in the binding mixtures. IVT reactions were performed as specified by the manufacturer (Promega) in 50-μl reactions programmed with 0.5 μg of T7 promoter-driven expression plasmid. An IVT reaction programmed with the empty pcDNA3 plasmid was used as a negative control. For antibody supershift experiments, 1 μg of antibody was added at room temperature 10 min before addition of the probe. The antibodies used are as follows: anti-Sp1 (Santa Cruz Biotechnology, catalog number SC-59), anti-Sp3 (Santa Cruz Biotechnology, catalog number SC-644), anti-E2F1 (NeoMarkers, catalog number MS-878), anti-E2F3 (Geneka, Montréal, Canada, catalog number 32010020, clone PG-30), anti-E2F4 (NeoMarkers, catalog number MS-1057), and anti-DP1 (NeoMarkers, catalog number MS-1056). The binding reactions were loaded on 4% non-denaturing polyacrylamide gels using 1× TGE (25 mM Tris, pH 8.5, 190 mM glycerin, 1 mM EDTA) as the buffering system. The gels were run at 25 mA per gel at 4°C for ~2.5 h. After drying, the gels were autoradiographed on Hyperfilm (Amersham Biosciences) for 24 h with intensifying screens at ~80°C.

Chromatin Immunoprecipitation Assays—Detection of promoter-bound E2F1 and E2F4 proteins was assessed by chromatin immunoprecipitation assays, essentially as described previously (52), except that the chromatin purification step on CsCl gradient was omitted. The chromatin was sonicated in order to obtain fragments of approximately 600 bp in length. E2F1- and E2F4-containing complexes were immunoprecipitated using anti-E2F1 (Santa Cruz Biotechnology, catalog number SC-1093) and anti-E2F4 (Santa Cruz Biotechnology, catalog number SC-1082) antibodies. Antibody against the FLAG epitope (Santa Cruz Biotechnology, catalog number SC-807) was used as a negative control. To detect the p18 gene in protein-DNA complexes, a 170-bp fragment of exon I was amplified by PCR using oligonucleotides 5′-ctctgcagctcttttaact-3′ (nucleotides +1 to +23 of exon I) and 5′-tttggctgaaacatctgctgt-3′ (nucleotides +170 to +148 of exon I). The primers used to detect the actin gene promoter region are the same as used by others (52). The two genes were detected by 34 cycles of PCR, with an annealing temperature of 58°C for the p18 gene and 60°C for the actin gene.

Transient Transfections and Promoter Activity Assays—MCF7 cells were seeded the day before transfection in 24-well plates at a density of 1.2 × 104 cells per well. The DNA (500 ng of reporter plasmid, 10 ng of pCMV-RL, 2 ng of pCMV-LacZ, and a total of 100 ng of expression vector for E2F1 and/or DP1 and/or empty expression plasmid) was diluted in 150 mM NaCl to a final volume of 3 μl. ExGen 500 (MBI Fermentas, Burlington, Canada) was diluted 8-fold in 150 mM NaCl, and 20 μl of this solution was added to the DNA. The DNA/ExGen 500 mixture was then incubated for 20 min at room temperature. The cells were rinsed with serum-free medium, and 250 μl of this medium was added to the cells. The DNA/ExGen mixture was added to the cells and incubated for 3 h at 37°C, after which the DNA/ExGen solution was removed by aspiration, and fresh serum-containing medium was added to the cells. Cells were harvested 24 h later by replacing the growth medium with 150 μl of Passive Lysis Buffer (PLB, Promega), and incubating the cells in PLB on a rocking platform for 15 min at room temperature. For each transfected well, 20 μl of lysate were transferred to a 96-well plate, and firefly and Renilla luciferase activities were measured on an automated computer-assisted luminometer (Berthold, Germany), using the dual-luciferase assay kit (Promega), according to the manufacturer’s instructions. To correct for well-to-well variability in transfection efficiency, the firefly luciferase activity values were divided by those of the Renilla luciferase activity, which are assumed to reflect transfection efficiency. This quotient is referred to as the normalized firefly luciferase activity.

Schneider-2 (SL2) Drosophila cells were seeded at 5 × 104 cells per well in 24-well plates. The next day, the cells were transfected using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals). The DNA (20 ng of reporter plasmid, 20 ng of pRL-null, 5 ng of pAc5/V5-His/LacZ, and a total of 80 ng of expression vector for E2F1, DP1, or Sp1) was diluted in a final volume of 2 μl in TE buffer. FuGENE 6 reagent was diluted 33-fold in Opti-MEM (Invitrogen), incubated 10 min at room temperature, and 18 μl of this dilution was added to the DNA solution. This mixture was further incubated for 20 min and was then added directly to the wells containing 250 μl of fresh culture medium. The DNA was left in contact with the cells until harvest, 48 h later. The procedures for cell harvest and determination of reporter gene activity were as described for MCF7 cells.
involved in apoptosis. Neither were altered by overexpression of E2F1 or E2F2 (Fig. 1, A and B, lower panels). These experiments confirmed that E2F1 or E2F2 overexpression leads to increased p18 mRNA levels and showed that p18 protein levels are also up-regulated.

Putative Regulatory Elements of the p18 Promoter—One important observation from the previous experiment is that the predominant p18 transcript expressed in both cell lines is the long (2.1 kb) mRNA form and that this transcript is highly induced by both E2F proteins. Subsequent studies to determine whether the increased p18 expression observed in E2F-overexpressing cells is a direct effect of E2F therefore focused on regulatory elements of the p18 gene that lie upstream of exon I. The first 150 bp of the promoter contain no TATA box (Fig. 2A) but have a very high G + C content, which is a hallmark of several TATA-less promoters (42). This proximal portion of the p18 promoter constitutes the 3′ end of a CpG island that extends from −640 to +35 relative to the transcription start site, as determined by the CpGPlot software (54). The 131 nucleotides that precede the transcription initiation site contain two elements, located at −121 and −23 relative to the start site, that loosely fit the consensus DNA sequence of E2F-binding sites (TTTSSCGG, where S is G or C (55)). The G + C-rich region also contains numerous putative Sp1-binding sites (GC boxes (56)) that are located at −121 and −98 and in a cluster at −56, −49, −42, −38, and −32. Note that the element at −121 fits the consensus sequence of both transcription factors. The preliminary genomic sequence of the murine p18 gene (Arachne assembly contig_50802) reveals that the putative regulatory elements contained in the human promoter are conserved in the murine promoter (Fig. 2B). This high degree of conservation suggests that these elements are likely to play an important role in the regulation of p18 expression.

E2F1 and Sp1 Transactivate the p18INK4c Gene Promoter—To determine whether the putative E2F- and Sp1-binding elements of the p18 promoter can mediate the constitutive or E2F-induced activity of the promoter, we tested the function of these elements in transient expression assays.

We constructed a reporter plasmid consisting of 1600 bp (construct A) of p18 promoter sequence fused to the luciferase reporter gene and transfected it, as well as a series of 5′-deletion mutants, in MCF7 human breast cancer cells (Fig. 3). We found that promoter construct B, which contains 131 bp of 5′-regulatory DNA, is as active as construct A. On this basis, we concluded that the region from −1600 to −132 is dispensable for basal p18 promoter activity. Moreover, we found that the region from −131 to −32 contains elements that are important for basal transcription because its deletion (construct P) almost completely abrogates luciferase activity. This region includes the putative Sp1 DNA-binding sites identified in Fig. 2. Mutation of the five Sp1-binding sites clustered between −56 and −28 (construct E) abolished all basal transcriptional activity. In the absence of a functional Sp1 cluster, additional mutations (constructs G and I–K) did not further reduce promoter activity. Mutations of the other putative regulatory elements of the promoter, either alone or in combination, were considerably less deleterious than mutation of the Sp1 cluster (see constructs B–D, F, and H). In agreement with these results, deletion of nucleotides −131 to −91 (construct N) did not have a pronounced effect on the basal activity of the promoter, but mutation of the Sp1 cluster (construct O) disrupted the activity of the −90 promoter.

To determine which putative regulatory elements mediate inducibility by E2F, similar experiments were conducted in MCF7 cells using expression vectors for E2F1 and DP1 (Fig. 4). DP1 was included in these experiments to avoid that the lim-

**RESULTS**

**E2F Up-regulates p18 mRNA and Protein Levels—**Previous reports (22, 53) showed that the p18 mRNA could be induced by adenoviral overexpression of E2F1 in rat and murine embryonic fibroblasts. As a preliminary to a detailed analysis of the p18 promoter, we decided to repeat these experiments and extended them to determine whether increased p18 mRNA levels lead to increased p18 protein levels.

To assess the effect of E2F on p18 expression, adenovirus vectors expressing either the green fluorescent protein (GFP), human E2F1, or human E2F2 were introduced into MDA-MB-468 human breast adenocarcinoma cells and into a human cell line of finite life span, namely WI-38 normal lung fibroblasts. Infected cells were harvested after 18 h post-infection, and p18 mRNA and protein levels were determined by Northern blot and Western blot, respectively (Fig. 1).

In MDA-MB-468 cells, E2F1 overexpression caused a 19-fold increase in p18 mRNA levels and a 13-fold increase in p18 protein levels (Fig. 1, A and B, upper panels). In the same cell line, E2F2 overexpression caused a 16-fold increase in p18 mRNA levels and a 7-fold increase in p18 protein levels. In WI-38 cells, E2F1 caused 9-fold increases in p18 mRNA and protein levels, whereas E2F2 caused 5-fold increases in p18 mRNA and protein levels. As controls we assessed the mRNA levels of the housekeeping gene glyceraldehyde-phosphate dehydrogenase, as well as the protein levels of TRADD, a protein involved in apoptosis. Neither were altered by overexpression of E2F1 or E2F2 (Fig. 1, A and B, lower panels). These experiments confirmed that E2F1 or E2F2 overexpression leads to increased p18 mRNA levels and showed that p18 protein levels are also up-regulated.

**FIG. 1. Analysis of p18 mRNA and protein levels following E2F overexpression.** Human MDA-MB-468 and WI-38 cells were infected with adenoviruses encoding GFP, FLAG-E2F1, or FLAG-E2F2, and total RNA and proteins were extracted. A, Northern blot analysis of p18 mRNA (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lower panel) abundance in MDA-MB-468 (lanes 1–3) or WI-38 (lanes 4–6) cells infected with AdCMV-GFP (lanes 1 and 4), AdCMV-FLAG-E2F1 (lanes 2 and 5), or AdCMV-FLAG-E2F2 (lanes 3 and 6). B, Western blot analysis of p18 (lower panel) and TRADD (upper panel) protein levels in MDA-MB-468 (lanes 1–3) or WI-38 (lanes 4–6) cells infected with AdCMV-GFP (lanes 1 and 4), AdCMV-FLAG-E2F1 (lanes 2 and 5), or AdCMV-FLAG-E2F2 (lanes 3 and 6).
E2F1 and Sp1 Regulation of p18^INK4c Expression

Fig. 2. Sequence of the p18^INK4c promoter. A, the 150 bp of p18 promoter sequence preceding the transcription initiation site are presented. The G + C-rich region is underlined, and putative E2F and Sp1 transcription factor-binding sites are indicated. B, alignment of the proximal 170 bp of the human and murine p18 promoters, using the FASTA program (54, 74). The two sequences share 71.196% identity (75.287% ungapped). The putative regulatory elements of the human promoter identified in A are boxed. C, schematic representation of the p18 promoter. The arrow indicates the position of the transcription initiation site (arbitrarily identified as +1), and putative binding sites are designated by their most 5'-nucleotide (e.g. E2F - 23). The black boxes indicate putative E2F-binding sites, and white ovals indicate the position of putative Sp1 DNA-binding sites. The putative E2F/Sp1-binding site at -121 is depicted as a white box. The positions of the four oligonucleotide probes used in EMSA experiments are also depicted.

E2F1 and Sp1 Cooperate to Induce Transcription from the p18^INK4c Promoter—To confirm that the proximal promoter is directly implicated in Sp1-mediated transactivation, we tested the same promoter constructs in SL2 D. melanogaster cells that are devoid of Sp1 activity (58). The promoter constructs were co-transfected with an Sp1 expression plasmid or a control plasmid (Table II; see under "Materials and Methods" for details concerning data treatment and statistical analysis). Sp1 caused a 38-fold increase in the activity of promoter construct B in SL2 cells. This effect is specific to the p18 promoter, because an artificial reporter construct (E2F-BS) consisting of five E2F DNA-binding sites cloned upstream of the E1B-TATA-box and the luciferase gene is not transactivated by Sp1 (Table II, compare constructs B and L). Disruption of the Sp1-binding sites located at -121 (construct C) and at -96 (construct D) diminished the luciferase activity induced by Sp1 by 10 and 4%, respectively. However, it is clear that the major element conferring Sp1 responsiveness to the promoter is the cluster of Sp1 sites, which is contained within -56 to -32, as depicted in Fig. 2C.

In order to evaluate the combined effect of E2F1 and Sp1 on p18 promoter activity, E2F1 and DP1 were co-transfected in combination with Sp1 along with our panel of reporter constructs to confirm that the proximal promoter is directly implicated in Sp1-mediated transactivation, we tested the same promoter constructs in SL2 D. melanogaster cells that are devoid of Sp1 activity (58). The promoter constructs were co-transfected with an Sp1 expression plasmid or a control plasmid (Table II; see under "Materials and Methods" for details concerning data treatment and statistical analysis). Sp1 caused a 38-fold increase in the activity of promoter construct B in SL2 cells. This effect is specific to the p18 promoter, because an artificial reporter construct (E2F-BS) consisting of five E2F DNA-binding sites cloned upstream of the E1B-TATA-box and the luciferase gene is not transactivated by Sp1 (Table II, compare constructs B and L). Disruption of the Sp1-binding sites located at -121 (construct C) and at -96 (construct D) diminished the luciferase activity induced by Sp1 by 10 and 4%, respectively. However, it is clear that the major element conferring Sp1 responsiveness to the promoter is the cluster of Sp1-binding sites, which is contained within -56 to -32, as depicted in Fig. 2C.

In order to evaluate the combined effect of E2F1 and Sp1 on p18 promoter activity, E2F1 and DP1 were co-transfected in combination with Sp1 along with our panel of reporter co-
structs in SL2 cells (Table II). E2F1 and DP1 co-expression caused a 102-fold induction in promoter activity of construct B in SL2 cells. Co-expression of E2F1, DP1, and Sp1 leads to a 2343-fold increase in promoter activity. The effect of E2F1, DP1, and Sp1 together is far more than additive and leads us to suspect a functional cooperation between the two transcription factors. This possible cooperative effect of E2F1, DP1, and Sp1 is specific to the p18 promoter because the co-expression of Sp1...
with E2F1 and DP1 reduced the activity of an artificial reporter construct containing five tandemly repeated E2F-binding sites (construct L).

We also examined the effect of E2F1 and Sp1 on the activity of p18 promoter constructs containing mutations in one or three of the four response elements (constructs C–D). Although the effect of combined expression of E2F1, DP1, and Sp1 on each of these constructs was mathematically more than additive, the cooperativity between E2F1 and Sp1 relied principally on an intact cluster of Sp1 sites and the additional GG dinucleotide are shown. Mutated oligonucleotides are underlined. WT, wild type; M, mutant.

### Table I

| Probe | Promoter region | Sequence |
|-------|-----------------|----------|
| Probe A WT | 30 to 70 | GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
mutated variants thereof. Interestingly, the binding of Sp1 to probe C was unaffected by the E2F-binding site oligo (Fig. 7, lanes 2 and 3), and the binding of GST-E2F1 and GST-DP1 to this probe was unaffected by the Sp1-binding site oligo (Fig. 7, lanes 13 and 14), indicating that the GST-E2F1/GST-DP1 dimer can discriminate between different GC-rich templates. The strength and specificity of binding of GST-E2F1/GST-DP1 to probe C was unaffected by the presence of Sp1 and vice versa (Fig. 7, lanes 17–23).

Taken together, the results of this last experiment support those presented in Fig. 4 and Table II showing that E2F1 can transactivate from the p18\textsuperscript{INK4c} promoter by binding to the GC-rich region located between −56 and −28.

Cellular E2F and Sp1 Interact with the −121 Promoter Element—By having determined in vitro that the p18 promoter contains E2F and Sp1 DNA response elements, we proceeded to verify if these transcription factor-binding sites were recognized by cellular proteins. To do so, we incubated probes A–D with total cellular extracts of HeLa S3 cells.

A number of protein complexes interacted specifically with probe A (Fig. 8A, compare lanes 1, 6, and 7). Competitions using an Sp1 consensus binding site prevented the formation of most of these protein complexes (Fig. 8A, lane 2), indicating that Sp1 is a major component of these complexes. Competitions using an E2F consensus binding site prevented the appearance of the lower molecular weight complexes (Fig. 8A,
Mutants are shown.

The sequences of wild-type probe D and were included in using unlabeled probes A and D or versions mutated at critical sites.

Oligonucleotides containing a consensus wild-type (WT) or mutated (M) were included in EMSA experiments using unlabeled oligonucleotides in order to more easily identify DNA-binding affinities, associate with elements contained between 130 to –101 of the p18 promoter.

To confirm that Sp1 and/or Sp3 are present in complexes B1, B2, and B4, supershift experiments were conducted using anti-Sp1 and/or anti-Sp3 antibodies. These showed that complex B1 contains Sp1, whereas complexes B2 and B4 contain the Sp3 protein. These results confirm those obtained using recombinant Sp1.

Cellular Sp1 Interacts with the –96 to –32 Regulatory Elements—Incubating probe B with cellular proteins yielded four major protein-DNA complexes, designated B1, B2, B3, and B4 (Fig. 9). Three of these complexes (complexes B1, B2, and B4) contain an Sp1-related protein because their formation was prevented by competition with an Sp1 consensus oligonucleotide but not by a mutant thereof (Fig. 9, lanes 1–3).

To observe supershifts using antibodies against Sp1/Sp3 in the absence of excess E2F oligo, but the disappearance of the original complexes was masked by the presence of E2F complexes with similar gel mobilities.

These results confirm those obtained with recombinant proteins, namely that E2F and Sp1 transcription factors can interact with regulatory elements contained in probe A.

Cellular proteins produced four major complexes (denoted C1, C2, C3, and C4) when incubated with probe C (Fig. 10). All four complexes are attributable to an Sp1-like DNA binding activity because they were displaced from the probe by an excess of wild-type Sp1 consensus oligo but not by an excess of mutated Sp1 consensus oligo (Fig. 10, lanes 1–3). Probe C contains up to five putative Sp1-binding sites. We attempted to determine whether only a subset of these is responsible for Sp1 binding. However, under the experimental conditions described here (i.e., EMSA experiments using either recombinant Sp1 or total cellular extracts and a series of mutated probes), it seems that all five putative binding sites have activity because they were displaced from the probe by an excess of wild-type Sp1 consensus oligo but not by an excess of mutated Sp1 consensus oligo (Fig. 10, lanes 1–3).

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Sp3. The appearance of complexes C1 and C2 as a doublet of bands of slightly different gel mobilities is more evident on a shorter exposure of the film (data not shown).

**Cellular E2F Associates with the Proximal E2F-binding Site**—The binding of total cellular proteins on probe D (Fig. 11) yielded four major specific complexes (designated D1, D2, D3, and D4). Competition experiments using an E2F consensus oligo revealed that all four complexes contain an E2F-like DNA site.

**Table II**  
Transactivation of the p18 promoter by exogenous Sp1, E2F1, and DP1 in SL2 cells

*Drosophila melanogaster* SL2 cells were transfected with reporter constructs consisting of a wild-type or mutated p18 promoter fragment cloned upstream of the firefly luciferase reporter gene, as described in the legend of Fig. 3, and different combinations of pAc5 expression vectors for Sp1, FLAG-E2F1, and DP1. The cells were harvested 24 h after transfection and lysed in Passive Lysis Buffer. Firefly and *Renilla* luciferase activities were measured in a luminometer, and the normalized luciferase activity was calculated. The results are presented, for each promoter construct, as the fold increase in normalized luciferase activity relative to that obtained with construct B co-transfected with the empty pAc5 expression plasmid (arbitrarily set to 1), and are the means ± S.D. of three experiments conducted in triplicate.

| Promoter construct | Transcriptional activator / Relative promoter activity (Arbitrary units) |
|--------------------|---------------------------------------------------------------|
|                    | pAc5/V5-His | Sp1 | FLAG-E2F1 + DP1 | FLAG-E2F1 + DP1 + Sp1 |
| B                  | 1.0 ± 0.0 | 37.5 ± 1.3 | 102 ± 5 | 2343 ± 60 |
| C                  | 1.2 ± 0.0 | 33.7 ± 1.1 | 79.8 ± 9.9 | 2530 ± 69 |
| D                  | 1.2 ± 0.0 | 36.0 ± 1.1 | 123.6 ± 4 | 2570 ± 91 |
| E                  | 1.0 ± 0.0 | 6.2 ± 0.2 | 54.8 ± 3.7 | 222 ± 10 |
| F                  | 1.1 ± 0.0 | 54.6 ± 1.3 | 38.1 ± 1.7 | 569 ± 17 |
| G                  | 0.7 ± 0.0 | 1.6 ± 0.1 | 25.0 ± 1.9 | 48.5 ± 4.9 |
| H                  | 0.6 ± 0.0 | 13.0 ± 0.5 | 22.0 ± 1.6 | 366 ± 27 |
| I                  | 0.6 ± 0.0 | 3.0 ± 0.4 | 10.5 ± 0.7 | 263 ± 4.3 |
| J                  | 0.6 ± 0.1 | 2.7 ± 0.2 | 20.6 ± 1.2 | 36.8 ± 4.0 |
| K                  | 0.9 ± 0.0 | 2.0 ± 0.1 | 9.4 ± 0.7 | 23.1 ± 1.6 |
| L E2F-BS           | 3.8 ± 0.2 | 3.9 ± 0.1 | 1669 ± 111 | 941 ± 33 |
| M pGL3-Basic       | 6.1 ± 2.2 | 7.1 ± 1.2 | 13.3 ± 1.8 | 16.1 ± 0.5 |

**Fig. 7.** Binding of E2F1, DP1, and Sp1 to the Sp1 cluster and proximal E2F site. Recombinant GST, GST-E2F1–8xHis, GST-DP1–8xHis, and Sp1–8xHis were produced in *E. coli* and purified as described. The proteins were incubated, alone or in various combinations, with radiolabeled probe C (lanes 1–23) or probe D (lanes 24–29) in an EMSA experiment. Unlabeled oligonucleotides were added as indicated in order to evaluate binding specificity. WT, wild-type; M, mutated.
binding activity because they were abolished when the wild-type oligonucleotide was added but were unaffected by the mutated E2F-binding site (Fig. 11, lanes 2 and 3). Supershift/interference experiments using antibodies raised against different members of the E2F family showed that complexes D1, D3, and D4 did not form when the anti-DP1 antibody was added to the binding mixture, and thus must contain the DP1 protein (Fig. 11, lane 9). Complex D3 was supershifted by the anti-E2F4 antibody and thus contains the E2F4 protein (Fig.
chromatin in the PCR (relevant antibody (anti-FLAG) or no antibody at all. As expected, consisted of performing the immunoprecipitation with an irrelevant antibody (anti-FLAG). The presence of chromatin fragments corresponding to the p18 gene or to the actin gene promoter was assessed by semi-quantitative PCR using gene-specific primers. Recovery of p18 and actin gene fragments from the protein-chromatin extract (prior to immunoprecipitation) is shown in lane 3. Controls include a PCR without DNA (lane 1, H2O), an immunoprecipitation assay with beads and antibody but without chromatin (lane 2, Mock IP), an immunoprecipitation assay performed without antibody (lane 4, No Ab), or with an irrelevant antibody (lane 5, anti-FLAG). The PCRs were separated by electrophoresis on a 2% agarose gel. The low background signal detected in all lanes is attributable to ethidium bromide staining of PCR products (P). B, schematic diagram depicting the fragments of the p18 and actin genes that were amplified. The positions of the PCR primers used to detect p18 promoter fragments relative to the transcription start site are indicated by arrows.

Taken together, these results confirm that the p18\textsuperscript{INK4c} promoter is able to recruit proteins of the Sp1 and E2F families of transcription factors.

In Vivo Association of E2F Proteins with the p18 Gene—To confirm that E2F1 and E2F4 associate with regulatory elements of the p18 promoter in vivo as well as in vitro, we performed chromatim immunoprecipitation experiments in WI-38 cells (Fig. 12). Cells growing in log phase were treated with formaldehyde to create cross-links between transcription factors and chromatin. The chromatin was isolated, sheared, and immunoprecipitated (IP) using antibodies against E2F1, E2F4, or a control antibody (anti-FLAG). The presence of chromatin fragments corresponding to the p18 gene or to the actin gene promoter was assessed by semi-quantitative PCR using gene-specific primers. Recovery of p18 and actin gene fragments from the protein-chromatin extract (prior to immunoprecipitation) is shown in lane 3. Controls include a PCR without DNA (lane 1, H2O), an immunoprecipitation assay with beads and antibody but without chromatin (lane 2, Mock IP), an immunoprecipitation assay performed without antibody (lane 4, No Ab), or with an irrelevant antibody (lane 5, anti-FLAG). The PCRs were separated by electrophoresis on a 2% agarose gel. The low background signal detected in all lanes is attributable to ethidium bromide staining of PCR products (P). B, schematic diagram depicting the fragments of the p18 and actin genes that were amplified. The positions of the PCR primers used to detect p18 promoter fragments relative to the transcription start site are indicated by arrows.

DISCUSSION

Understanding the complex mechanisms that regulate cell cycle progression will contribute to a better comprehension of the control of cell proliferation and differentiation in physiological circumstances, as well as of the pathological disruptions of this growth control pathway that occur in cancer. We have examined the regulation of p18 promoter activity by the cell cycle regulator E2F in order to understand, at the molecular level, how a transcription factor renowned for stimulating cell proliferation can up-regulate a gene whose product serves to prevent passage from G1 to S phase. Previous studies had shown that E2F up-regulates p18 mRNA levels. We have extended these observations by demonstrating that E2F-induced increases in p18 mRNA levels are accompanied by corresponding increases in p18 protein levels. Moreover, we found that the p18 promoter is a direct transcriptional target of E2F, and we have identified promoter elements that mediate the effect of E2F, and we have identified transcription factors that associate with these elements.

To examine the various aspects of the regulation of p18 promoter activity by E2F, we used different experimental models that we deemed most informative. Adenovirus infections and chromatin immunoprecipitation assays were performed in WI-38 cells because these cells represent a “normal” cell line with an intact Rb-E2F pathway. MCF7 cells were chosen for promoter deletion/mutation analyses because they have relatively low endogenous E2F activity, and they are easily transfected. Likewise, SL2 cells were chosen for experiments requiring overexpression of Sp1 because they lack endogenous Sp1.

To determine transcription factors that associate with the E2F-responsive region of the p18 promoter, we performed electrophoretic mobility shift assays using HeLa cell extracts because these have been extensively used to characterize E2F. The sum of these experiments provides a set of consistent data that

**Fig. 11. Binding of cellular proteins to the −30 to −1 region of the p18 promoter.** Probe D was incubated with HeLa S3 high salt cellular protein extracts in EMSA experiments. Competition experiments were performed by adding a wild-type (WT) or mutated (M) version of an E2F consensus oligo or probe D. Antibody supershift experiments were performed by adding 1 μg of antibody against E2F/DP1 in the binding reaction before the addition of the labeled probe. NS indicates nonspecific binding or background signal.

11, lane 8). The E2F1 and E2F3 members are also present in some complexes because the anti-E2F1 and anti-E2F3 antibodies yielded weak but nevertheless detectable supershifts (Fig. 11, lanes 6 and 7, marked with asterisks). As expected from the competition experiment using an E2F consensus oligonucleotide, complex D2 was unaffected by the antibodies used.

Taken together, these results confirm that the p18\textsuperscript{INK4c} promoter is able to recruit proteins of the Sp1 and E2F families of transcription factors.

In Vivo Association of E2F Proteins with the p18 Gene—To confirm that E2F1 and E2F4 associate with regulatory elements of the p18 promoter in vivo as well as in vitro, we performed chromatim immunoprecipitation experiments in WI-38 cells (Fig. 12). Cells growing in log phase were treated with formaldehyde to create cross-links between E2F and associated promoter regions. Chromatin was then isolated, fragmented by sonication, and subjected to immunoprecipitation by using antibodies directed against either E2F1 or E2F4, two of the E2F family members that associate with p18 promoter probes A and D. The presence of the proximal p18 gene promoter in the immunoprecipitated chromatin was detected by amplifying the 5' end of exon I by PCR. A DNA fragment corresponding to the actin gene promoter was amplified as a control. As shown in lanes 6 and 7, a genomic DNA fragment containing p18 exon I co-immunoprecipitated with both E2F1 and E2F4 whereas the actin gene fragments from the protein-chromatin extract (prior to immunoprecipitation) is shown in lane 3. Controls include a PCR without DNA (lane 1, H2O), an immunoprecipitation assay with beads and antibody but without chromatin (lane 2, Mock IP), an immunoprecipitation assay performed without antibody (lane 4, No Ab), or with an irrelevant antibody (lane 5, anti-FLAG). The PCRs were separated by electrophoresis on a 2% agarose gel. The low background signal detected in all lanes is attributable to ethidium bromide staining of PCR products (P). B, schematic diagram depicting the fragments of the p18 and actin genes that were amplified. The positions of the PCR primers used to detect p18 promoter fragments relative to the transcription start site are indicated by arrows.

**Fig. 12. Immunoprecipitation of E2F-associated p18 promoter fragments from WI-38 cells.** A, human WI-38 diploid fibroblasts were treated with formaldehyde to create cross-links between transcription factors and chromatin. The chromatin was isolated, sheared, and immunoprecipitated (IP) using antibodies against E2F1, E2F4, or a control antibody (anti-FLAG). The presence of chromatin fragments corresponding to the p18 gene or to the actin gene promoter was assessed by semi-quantitative PCR using gene-specific primers. Recovery of p18 and actin gene fragments from the protein-chromatin extract (prior to immunoprecipitation) is shown in lane 3. Controls include a PCR without DNA (lane 1, H2O), an immunoprecipitation assay with beads and antibody but without chromatin (lane 2, Mock IP), an immunoprecipitation assay performed without antibody (lane 4, No Ab), or with an irrelevant antibody (lane 5, anti-FLAG). The PCRs were separated by electrophoresis on a 2% agarose gel. The low background signal detected in all lanes is attributable to ethidium bromide staining of PCR products (P). B, schematic diagram depicting the fragments of the p18 and actin genes that were amplified. The positions of the PCR primers used to detect p18 promoter fragments relative to the transcription start site are indicated by arrows.

**DISCUSSION**

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To examine the various aspects of the regulation of p18 promoter activity by E2F, we used different experimental models that we deemed most informative. Adenovirus infections and chromatin immunoprecipitation assays were performed in WI-38 cells because these cells represent a “normal” cell line with an intact Rb-E2F pathway. MCF7 cells were chosen for promoter deletion/mutation analyses because they have relatively low endogenous E2F activity, and they are easily transfected. Likewise, SL2 cells were chosen for experiments requiring overexpression of Sp1 because they lack endogenous Sp1. To identify transcription factors that associate with the E2F-responsive region of the p18 promoter, we performed electrophoretic mobility shift assays using HeLa cell extracts because these have been extensively used to characterize E2F. The sum of these experiments provides a set of consistent data that
paints a clearer picture of the mechanism of p18 promoter regulation by E2F. These data allow us to draw a number of conclusions.

First, the elements of the p18 promoter that are required for induction by E2F reside within the first 131 bp upstream of the transcription initiation site. Transient transfection experiments performed in MCF7 cells showed that the 131-bp promoter was as responsive to E2F as a construct containing 1600 bp of promoter DNA. Although we cannot exclude the possibility that sites upstream of 1600 could be involved in activation by E2F, we believe that the −131 promoter contains the most essential E2F-responsive elements because the magnitude of the response of the −1600 and −131 promoters to E2F in transient expression experiments (38–53-fold) is at least equal to the magnitude of the increase in endogenous p18 mRNA levels induced by E2F in the context of an adenoviral infection (5–19-fold). Similar transient transfection experiments performed in T98G (neuroblastoma), HeLa (cervix carcinoma), and MDA-MB-231 (breast adenocarcinoma) cells, as well as adenoviral infection experiments performed in U-2 OS and Saos-2 (osteogenic sarcoma) cells, yielded comparable results (data not shown).

The −131 promoter contains four regulatory elements that could potentially bind transcription factors. These include a combined E2F/Sp1-binding site at −121, an Sp1-binding site at −98, a cluster of five Sp1-binding sites between −56 and −32, and an E2F-binding site at −23. The specificity of each response element was confirmed by EMSAs by incubating the corresponding labeled DNA probes with excess amounts of oligonucleotides containing E2F or Sp1 consensus binding sites. In addition, as predicted, the −121 and −23 E2F-binding sites could bind to recombinant (synthesized in vitro) E2F1 and DP1, whereas the −98 Sp1 site and the Sp1 cluster could interact with purified Sp1 protein. The physiological relevance of these interactions was further confirmed by performing EMSA antibody interference (supershift) experiments using HeLa cell extracts. These experiments showed that different members of the E2F family (E2F1, E2F3, E2F4, and DP1) and of the Sp1 family (Sp1 and Sp3) interact with the corresponding response elements. These results were confirmed by EMSA experiments performed with protein extracts prepared from T98G and MCF7 cells (data not shown).

One particularly interesting finding of the EMSA experiments was that E2F1 and DP1 (expressed as GST fusion proteins), as well as Sp1, can interact with the Sp1 cluster of the p18 promoter. We could detect a strong interaction between bacterially produced GST-E2F1 and the −70 to −21 region of the p18 gene promoter, and this binding was unaffected by the binding of Sp1 to the probe (i.e. neither mutually exclusive nor cooperative). This observation agrees with data concerning the herpes simplex virus-thymidine kinase gene, and its induction by E2F involves a direct interaction between E2F1 and a GC-rich portion of the promoter of this gene (61).

Additional experiments will be needed to determine whether other transcription factors, particularly the other members of the E2F family, interact with the −131 p18 promoter. For instance, the sequences of the −121 and −23 E2F-binding sites of the p18 promoter are highly similar to the “class I E2F consensus site,” 5′-TCTCCCGC-3′, which is a preferred binding site for the E2F6 protein (62). Although the structure of the E2F6 protein suggests that it could act as a transcriptional repressor or as an inducer of E2F-regulated genes, depending on the cellular context, the function of this protein in cell cycle regulation is not yet well defined. In addition, because there exist different isoforms of some of these proteins which may be differentially regulated or expressed during the cell cycle, it will be important to determine which of these interact with the p18 promoter. For example, two human E2F3 proteins, designated E2F3a and E2F3b, have been described in HL-60 cells (63). The antibody we used to detect E2F3 in supershift experiments detects a predominant polypeptide of ~55 kDa (presumably E2F3a) in Western blots of HeLa extracts. Similarly, the SP3 transcript gives rise by internal translation initiation to at least three proteins of different sizes. The shorter forms are truncated in their transactivation domain, a fact that could in part explain why Sp3 can also act as a transcriptional repressor (60). Notwithstanding these limitations, which will be resolved in part when appropriate immunologic reagents are available, the results of the EMSA experiments provide a good indication of the type of proteins that interact with the E2F-responsive region of the human p18 promoter.

The functional relevance of the aforementioned regulatory elements of the p18 promoter was tested in human MCF7 breast cancer cells and D. melanogaster SL2 cells, as well as in other cell lines (data not shown), in transient expression experiments using p18 promoter constructs harboring mutations in one or more of these elements. These experiments showed that the Sp1-binding sites in the −56 to −28 cluster play a particularly important role in controlling basal p18 promoter activity. The protein-DNA interaction data suggest that the Sp1 and/or Sp3 transcription factors are responsible for the basal level of p18 gene expression. These factors belong to a large family of zinc finger transcription factors that are not, as was once believed, only implicated in the transcription of housekeeping genes. The ubiquitous Sp1 and Sp3 proteins are also important for the control of the expression of several genes in response to a variety of stimuli such as mitogenic signaling and differentiation. Moreover, they have the ability to interact with different transcriptional modulators, including E2F1 and pRb (64).

In terms of the response to E2F1, we found that either the −23 E2F site alone or the Sp1 cluster could mediate a robust (~10-fold) stimulation by E2F. On the other hand, both basal promoter activity as well as the magnitude of the response to E2F were severely impaired when both the Sp1 cluster and the −23 E2F site were mutated in combination with mutations of the −121 E2F/Sp1 and/or −98 Sp1 sites. These results indicate that these elements are functionally redundant, at least in terms of their role in mediating activation by E2F1. Interestingly, we observed a strong cooperative effect between E2F1 and Sp1 on p18 promoter activity. The promoter of the dihydrofolate reductase gene, which has been well characterized in hamster, mouse, and human, also contains Sp1 and E2F response elements in the vicinity of its transcription initiation region (42). The basal transcription of this gene relies on the presence of these sites, because the position of the E2F site influences the transcription initiation position, and because the absence of functional Sp1 protein or Sp1-binding sites in the promoter abolishes transcription. Moreover, it was found that the E2F site of the dihydrofolate reductase promoter is involved in promoter silencing in serum-starved conditions, whereas serum responsiveness relies on the Sp1 sites (65). Others have suggested that E2F2 and Sp1 could act in a synergistic fashion on the promoter of different genes, including dihydrofolate reductase, to recruit the transcriptional apparatus and to enhance transcriptional elongation (55, 66–68). There is also mounting evidence that the E2F-Sp1 cooperation may form, along with pRb and HDAC1, the core of a “transcriptional switch,” whose function is to couple transcriptional regulation with cell cycle phase transition (69, 70). These topics are beyond the scope of the work reported here but clearly deserve to be investigated in the future, because they could...
Help to better understand the coupling of transcriptional regulation and cell cycle progression.

Previous studies that identified E2F as an inducer of p18 expression relied solely on overexpression of E2F proteins (22, 53, 71). We have also used adenoviral vectors and transient expression assays to assess the role of E2F on p18 expression and promoter activity. However, these models are not without flaws because overexpression of E2F may cause major perturbations in a cell and thereby elicit nonspecific responses. By using a more physiological approach, i.e. chromatin immunoprecipitation assays, we showed here that E2F1 and E2F4 can associate with the promoter of the p18 gene in unperturbed WI-38 cells. These results constitute additional and compelling evidence that E2F proteins are involved in the regulation of p18 gene expression.

Of the seven cyclin-dependent kinase inhibitor genes characterized to date, three (p18, p19, and p21) have been shown to be transcriptionally induced by E2F. The induction of p21 expression by E2F is not at odds with the properties of E2F when one considers that p21 can act as an assembly factor for cyclin-DK complexes (72, 73). On the other hand, the observation that E2F induces p18 and p19 is not so easily rationalized. Hirai et al. (20) hypothesized that the maximum levels of p19\textsuperscript{INK4c} (and of p18\textsuperscript{INK4c}) in S phase could serve to inhibit CDK4 and CDK6 activity once the G\textsubscript{1} phase is over. This concept was reiterated by DeGregori et al. (22) who proposed that the E2F-p18 link could constitute a retro-feedback loop that would prevent E2F activity beyond the restriction point.

Others have proposed that p18 mRNAs derived from alternate promoters of the p18 gene may not be translated with equal efficiency such that differential expression of said transcripts could influence p18 protein levels. This hypothesis is based on the work of Phelps et al. (29) who observed that a translationally inefficient long form of the p18 mRNA was down-regulated during myogenic differentiation of murine C2C12 myoblasts, whereas the short form of p18 mRNA, which is translated efficiently, was up-regulated during this process. The translational efficiency of the short and long forms of human p18 mRNA has not yet been determined, but the fact that p18 protein levels correlated to p18 mRNA levels in WI-38 cells suggests that E2F-expressing adenovirus would argue against such a mechanism in human cells.

The present work provides a solid basis to investigate further the regulation of p18 expression by E2F, particularly during the different phases of the cell cycle. In fact, whereas the data presented herein clearly indicate that E2F1 stimulates p18 promoter activity and up-regulates p18 mRNA levels, the role of the other p18 E2F proteins is less clear. For example, E2F4 associates with the p18 promoter in EMSA and chromatin immunoprecipitation experiments, but transient overexpression of E2F4 in MCF7 cells caused only minor increases in p18 promoter activity (data not shown). Because both EMSA and chromatin immunoprecipitation assays were performed with asynchronously growing cells, it is possible that E2F4, as well as other members of the E2F family, acts in complex with a pocket protein (and possibly other co-repressors) as a repressor of p18 promoter activity in early G\textsubscript{1} phase, as is the case for numerous cell cycle-regulated genes (52, 75). This observation is in accordance with previously published work (22) and with the fact that E2F4, in contrast to E2F1, localizes preferentially to the cell cytoplasm in proliferating cells and associates with pocket proteins in the nucleus only in G\textsubscript{1} and early G\textsubscript{2} phases (76–78). In the future it will be possible to determine which E2F proteins associate with the p18 promoter in various phases of the cell cycle by performing chromatin immunoprecipitation using extracts from synchronized cells. Combining this approach with stable or transient expression of wild-type and mutated p18 promoter constructs could provide additional information on the respective role of each response element.

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