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Cell Cycle-Regulated Association of E2F1 and Sp1 Is Related to Their Functional Interaction

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Because of the large number of growth-regulated genes containing binding sites for the transcription factors Sp1 and E2F and the reported ability of E2F to mediate cell cycle (growth) regulation, we studied interactions between E2F1 and Sp1. In transient transfection assays using *Drosophila melanogaster* SL2 cells, transfection with both Sp1 and E2F1 expression vectors resulted in greater than 85-fold activation of transcription from a hamster dihydrofolate reductase reporter construct, whereas cotransfection with either the Sp1 or E2F1 expression vector resulted in 30- or <2-fold activation, respectively. Therefore, these transcription factors act synergistically in activation of dihydrofolate reductase transcription. Transient transfection studies demonstrated that E2F1 could superactivate Sp1-dependent transcription in a promoter containing only Sp1 sites and that Sp1 could superactivate transcription of promoters through E2F sites, further demonstrating that these factors functionally interact with one another. Coimmunoprecipitation studies revealed that Sp1 and E2F1 are physically associated in *Drosophila* cells transfected with Sp1 and E2F1 expression vectors and in human cells, with maximal interaction detected in mid- to late G_1. Additionally, E2F1 and Sp1 interact in vitro through specific domains of each protein, and the physical interaction and functional synergism appear to require the same regions. Taken together, these data demonstrate that E2F1 and Sp1 both functionally and physically interact; therefore, through this interaction, Sp1 and E2F1 may regulate transcription of genes containing binding sites for either or both factors.

The transcription of many genes is activated at different times in the G_1 phase of the cell cycle. The so-called immediate-early genes (e.g., c-*fos*) are activated early in G_1 and immediately after release of quiescent cells into the cell cycle. Another subset of genes including c-*myc* is activated with delayed-early kinetics. A large group of genes are activated in mid- to late G_1; these include several genes whose expression is required for DNA synthesis (e.g., those encoding adenosine deaminase, thymidine kinase, dihydrofolate reductase [DHFR], and DNA polymerase α), as well as genes whose products control cell cycle progression (e.g., the retinoblastoma gene product [pRB] and cyclins A and E). Virtually all of these late G_1 genes lack a TATA box and have binding sites for both E2F and Sp1 transcription factors in their promoters.

E2F, first defined as a DNA-binding activity induced by adenovirus E1A, is now known to consist of a heterodimer composed of one of the E2F family members and one member of the DP family of proteins (of which three have been identified) (see reference 29 for a review). E2F1 was the first E2F family member to be cloned (18, 24), and subsequently four additional family members have been identified (see reference 1 for a review). pRB interacts with E2F1, -2, and -3 when it is hypophosphorylated and suppresses E2F-dependent transcription (8, 11, 16, 19). When pRB is phosphorylated in mid-G_1, E2F is released and apparently activates transcription. Because of this association with pRB and other cellular proteins, E2F is intimately involved with cell growth control and the regulation of genes involved in transformation, cell growth, and DNA replication (see references 7 and 37 for a review). Sp1 is a zinc finger DNA-binding protein that contains a glutamine-rich activation domain. It was the first cloned transcription factor and has largely been recognized as a basal factor for which there are binding sites in TATA-less promoters (22). We and others have shown that Sp1 sites often control initiation of transcription from downstream sites in TATA-less promoters (4, 27, 31).

The involvement of TATA-less genes containing E2F and Sp1 binding sites in DNA replication and cell growth control has led to much interest in their regulation. The role of E2F in regulation of several such genes, including the DHFR, DNA polymerase α, and thymidine kinase genes, c-*myc*, B-*myb*, and the E2F1 gene, has been carefully studied (3, 30, 33, 35, 38, 42). In keeping with its role in cell cycle control, E2F has been found to act as either a transcriptional repressor or activator on these promoters, depending on the growth state or cell cycle stage of the cells. Interestingly, these promoters differ in the timing of activation (e.g., c-*myc* is activated in early G_1, while the DNA polymerase α and DHFR genes are activated in late G_1), indicating that some additional factor(s) is involved in their regulation. These genes vary with respect to the position and number of Sp1 and E2F sites in their promoters, suggesting that Sp1-E2F interactions may be a factor in their differential regulation. This notion is consistent with the prevalence of Sp1 sites in late-G_1 genes (see reference 2 for a review) and the observation that pRB stimulates Sp1-dependent transcription (20, 26), both of which suggest that Sp1 plays a role in cell cycle regulation.

To further investigate the role of Sp1-E2F interactions in the control of these genes, we sought to determine whether these two factors cooperatively activate transcription and whether they physically interact. This report demonstrates that Sp1 and E2F1 interact functionally to synergistically activate DHFR transcription. In addition, they can superactivate (i.e., activate in the absence of a cognate binding site) transcription in cer-

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tain promoters containing a binding site for either Sp1 or E2F. Moreover, data which demonstrate that they interact physically both in vivo and in vitro are presented.

MATERIALS AND METHODS

Cell culture. *Drosophila melanogaster* SL2 cells (obtained from C. Benyajati) were cultured at 20°C in Schneider's *Drosophila* medium (GIBCO/BRL) supplemented with 10% fetal calf serum (Rehatuin; Invitrogen). Normal human diploid fibroblast cells, obtained at passage 5 from the American Type Culture Collection and used before passage 14, were grown at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM-H) supplemented with 10% fetal calf serum (GIBCO/BRL). These cells were synchronized by maintaining confluent cultures for 60 to 72 h in DMEM-H supplemented with 0.5% fetal calf serum. Cells were released into G_1 by addition of medium containing 10% fetal calf serum. To monitor the kinetics of entry of cells into S phase, medium containing 10% fetal bovine serum and 1 μCi of [^{3}H]thymidine per ml was added, and at various times thereafter, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and once with ice-cold trichloroacetic acid containing 1 mM thymidine. Following a further rinse with PBS, cells were dried. Trichloroacetic acid-precipitated material was extracted for 4 h with 1 M NaOH, and incorporated ^{3}H was quantified by scintillation counting.

Plasmid constructs. The DHFR reporter constructs used in transient transfection assays contain sequences derived from the hamster DHFR promoter driving expression of chloramphenicol acetyltransferase (CAT) or firefly luciferase. The wild-type DHFR promoter construct has nucleotides −210 to −23 (relative to the translational start site) of the DHFR promoter and contains all four Sp1 sites (GC boxes I to IV) and the dyad E2F sites of the DHFR promoter (4). The DHFR(ΔE2F) construct is identical to the wild-type construct except that the E2F dyad is inactivated by substitution of a TA at positions −57 and −56 (3).

Other constructs used in transient transfection assays were kindly provided as follows. The adenovirus E2a promoter-CAT constructs were obtained from P. Hearing. These included constructs containing the wild-type E2a promoter (E2WT), which contains an ATF site and two E2F sites (34), and the E2 promoter with both E2F sites mutated (E2aΔE2F). The Rous sarcoma virus–β-galactosidase plasmid was provided by Nenad Petrovic.

The Sp1 plasmid (pSP1-773C) used for in vitro translation was provided by J. Kadonaga. The Sp1 expression vector, pPAC-Sp1, which contains full-length Sp1 cDNA downstream of the *Drosophila* β-actin promoter, was provided by R. Tjian (13). Glutathione *S*-transferase (GST)–Sp1 constructs were provided by J. Horowitz (51). GST-E2F1 deletion constructs were provided by W. Kaelin, Jr., E. Harlow, and K. Helin. The point mutants and internal deletion mutants and the eukaryotic expression plasmid of E2F-1 were obtained from W. D. Cress and J. R. Nevins (14) and were subcloned into pGEX2T to produce GST-E2F1 fusion protein expression vectors (13). Full-length and E2F1 Δ113-120 cDNAs were subcloned in place of Sp1 in the pPAC vector for high expression in *Drosophila* cells.

Transient-transfection assays. SL2 cells were passaged the day before transfection into 75-cm^2 plastic tissue culture flasks (10^7 cells per flask). Cells were transfected with double-cesium-banded plasmid DNA by calcium phosphate coprecipitation (9). The total amount of transfected DNA was equalized to 20 μg with pUC18 DNA. Cells were harvested by mechanical detachment (vigorous tapping), centrifuged at 1,000 × *g*, resuspended in CAT harvest buffer (40 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA), recentrifuged, and suspended in 0.1 M Tris (pH 7.5). Cells were lysed by three freeze-thaw cycles, and clarified supernatants were assayed for protein content by the method of Bradford (5). CAT activity in 80 to 100 μg of supernatant protein was measured by a fluor diffusion assay using [^{3}H]acetyl coenzyme A (200 mCi/mmol; NEN) as described previously (36). CAT activity was calculated as picomoles of product acetylated per hour per microgram of protein. For the purposes of comparison, CAT activity in cells that were transfected with the reporter construct plus the transactivator was divided by CAT activity in cells transfected with the reporter construct alone. This quotient is referred to as fold activation. In some experiments, cotransfection of a second reporter not responsive to E2F1 and Sp1 was included as a control for transfection efficiency. Luciferase was measured on the extracts by using luciferase assay reagent from Promega as instructed by the manufacturer. β-Galactosidase was measured as described previously (43). In these experiments, the final effect (corrected for the cotransfected control) was expressed as fold activation. Transfections were performed a minimum of three times, results were averaged over the number of experiments, and analysis of variance was performed.

Interactions between Sp1 and E2F1 in cells. SL2 cells were harvested and rinsed with PBS (150 mM NaCl, 20 mM sodium phosphate [pH 7.4]) prior to extraction in radioimmunoprecipitation assay (RIPA) buffer (PBS containing 1% [wt/vol] Nonidet P-40 [NP-40], 0.5% [wt/vol] sodium deoxycholate, and 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) freshly supplemented with 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4, 5 μg of aprotinin per ml, and 5 mM NaF. Normal human diploid fibroblasts were rinsed with PBS and harvested by scraping, and cell pellets were lysed for 10 min on ice in a mixture containing 20 mM Tris (pH 7.2), 150 mM NaCl, 1% (wt/vol) NP-40, and phosphatase and protease inhibitors. Extracts were cleared by centrifugation and immunoprecipitated with rabbit polyclonal antibody (1 μg of immunoglobulin G [IgG]) specific for E2F1, Sp1 (Santa Cruz Biotechnology), or protein kinase Cζ (PKCζ) (GIBCO/BRL), using protein A-Sepharose as directed in the Santa Cruz product bulletin. Washed beads were boiled in SDS sample buffer, and released proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with the Sp1 antibody as directed by the supplier. Nuclear extracts used for Fig. 3 were prepared from log-phase HeLa cells as previously described (50). Prior to immunoprecipitation as described above, extracts were diluted in more than 10 volumes of RIPA buffer containing protease inhibitors.

Coupled transcription-translation reactions. cDNAs encoding Sp1 are in the pBluescript vectors, and full-length and mutated E2F1 cDNAs are in a cytomegalovirus expression vector with a T7 promoter (pRc-CMV; Clontech). These proteins were labeled with [^{35}S]methionine by coupled transcription-translation with a Promega TNT reticulocyte lysate kit. Reactions were performed with 1 μg of plasmid DNA in a total of 50 μl as recommended by the manufacturer. Control reactions were performed with either no added DNA or DNA encoding firefly luciferase.

GST fusion proteins. cDNA clones expressing GST fused to full-length Sp1 or E2F1 (amino acids 1 to 437) or various deletions were in the pGEX30X or the pGEX2T isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector (18). *Escherichia coli* DH5α cells transformed with these GST fusion plasmids were grown to an optical density at 595 nm of 0.5 in LB medium containing 50 μg of ampicillin per ml, and fusion protein was induced for 4 h with IPTG. Crude lysates were prepared at 4°C by mild sonication of the bacteria in binding buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 1 mM $MgCl_2$, 40 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. Extracts were cleared by centrifugation, and the supernatants were stored at −70°C in 10% glycerol. Thawed aliquots were added to glutathione-Sepharose beads equilibrated with binding buffer and rocked gently for 30 min at 4°C. After incubation, the beads were washed four times in binding buffer.

In vitro protein-protein interactions. Interactions between the [^{35}S]methionine-labeled Sp1 or E2F1 and GST-E2F1 or GST-Sp1 were examined by incubating 40 μl of a 50:50 slurry of the glutathione-Sepharose-bound GST fusion protein with reticulocyte lysate translation mix containing ^{35}S-labeled protein in 200 μl of binding buffer in the presence of 100 μg of ethidium bromide per ml. The mix was rocked gently at 4°C for 1 h. The Sepharose beads were washed four times with ice-cold binding buffer and boiled in SDS sample buffer. Released proteins were resolved on SDS–8 or 10% polyacrylamide gels and visualized by fluorography. Control incubations were performed with beads precoated with lysate from bacteria expressing GST protein alone or GST fused to the 72-kDa human cytomegalovirus immediate-early protein.

RESULTS

Synergistic activation of the DHFR promoter by Sp1 and E2F1. Since binding sites for Sp1 and E2F are found together in many promoters, we sought to determine whether they cooperatively activate transcription. The hamster DHFR promoter, which is relatively simple and well characterized, contains four Sp1 binding sites in the 140 bp 5′ to the major transcription start sites and two overlapping binding sites (in inverted orientation) for the transcription factor E2F that lie immediately 3′ to the major transcription start (3, 4). The hamster DHFR promoter (nucleotides −210 to −23 relative to ATG at +1) driving expression of CAT was transfected into *D. melanogaster* SL2 cells either alone or with E2F1 and/or Sp1 expression vectors. SL2 cells have no endogenous Sp1 and provide a very low background for DHFR transcription in the absence of exogenous Sp1. As shown in Fig. 1A, the DHFR promoter is very poorly expressed in *Drosophila* cells in the absence of a transactivator(s), and E2F1 alone caused at most a very slight stimulation (<2-fold) of transcription which was not significantly different from the basal level. This result confirms earlier observations that Sp1 is required for transcription of the DHFR gene (50). Sp1 alone stimulated DHFR transcription 30-fold; however, when it was cotransfected with E2F1, 85-fold activation was seen (Fig. 1A). This level of interaction between Sp1 and E2F1 is more than multiplicative, demonstrating that these two factors act synergistically in activation of DHFR transcription. To control for possible differences in transfection efficiency and possible effects of overexpression of these transcription factors on general transcription,

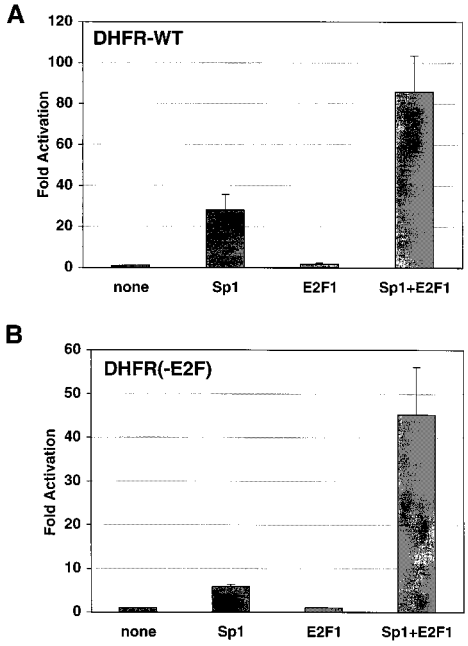

FIG. 1. Activation of various DHFR promoter constructs by E2F1 and/or Sp1. Various hamster DHFR promoter-CAT constructs (wild type [DHFR-WT] and mutated) were transfected into *D. melanogaster* SL2 cells with or without Sp1 (pPAC-Sp1) and/or E2F1 (pCMV-E2F1) expression vectors. (A) Five micrograms of wild-type DHFR-CAT (which contains four Sp1 sites and dyad E2F sites upstream of the CAT cDNA) was transfected into *D. melanogaster* SL2 cells alone or with 100 ng of pPAC-Sp1 (human Sp1 cDNA 3′ of the *Drosophila* actin promoter) and/or with 5 μg of pCMV-E2F1 (E2F1 cDNA 3′ of the cytomegalovirus immediate-early promoter). (B) Five micrograms of DHFR(ΔE2F) construct (DHFR promoter [−210 to −23] bearing a double point mutation to inactivate the E2F sites) was cotransfected as described for panel A. CAT activities were measured and normalized for total protein concentration. Data are presented as fold activation relative to the expression of the DHFR-CAT construct in the absence of mammalian transcription factors. Each histogram bar represents the mean of three separate transfection results ± the standard error of the mean.

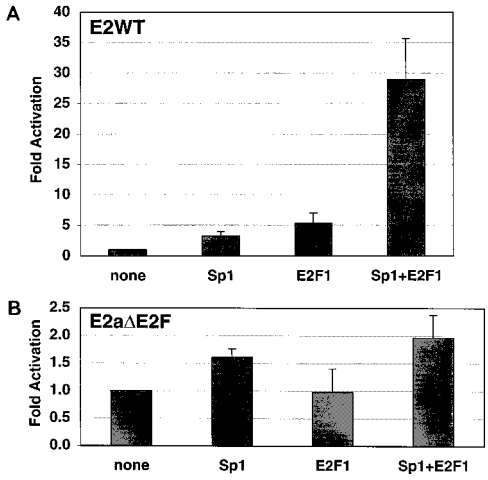

FIG. 2. Activation of the adenovirus E2a promoter by Sp1 and E2F. Fifteen-microgram aliquots of plasmids containing the CAT gene driven by the wild-type adenovirus E2a promoter (−115 to −35; E2WT) or by the E2a promoter in which both E2F binding sites have been mutated (E2aΔE2F) were cotransfected into *D. melanogaster* SL2 cells with 3 μg of plasmid containing the β-galactosidase gene driven by the Rous sarcoma virus promoter. Reporter plasmids were transfected alone or with plasmids harboring Sp1 and/or E2F genes driven by the *Drosophila* actin promoter (300 ng of pPAC-Sp1 and 50 ng of pPAC-E2F1 were used). The β-galactosidase plasmid served as a cotransfection control, and CAT activities were measured and normalized with respect to the β-galactosidase activity. Data are presented as fold activation relative to the expression of the E2a-CAT construct in the absence of mammalian transcription factors. Each histogram bar represents the mean of three separate transfection results ± the standard error of the mean.

this experiment was also performed by cotransfecting a plasmid containing the truncated adenovirus major late promoter (−174 to +33, relative to the transcription start site) driving expression of CAT along with a plasmid containing the DHFR promoter driving expression of the firefly luciferase gene. The results of this experiment showed the same pattern of activation as those shown in Fig. 1A; i.e., Sp1 and E2F1 synergistically activate the DHFR promoter (see Fig. 6, first four bars). The absolute differences are due to differences in the reporter and expression constructs used in the two experiments.

Superactivation of transcription by Sp1 and E2F1. To determine whether the synergy requires binding sites for both factors, a DHFR promoter with the E2F sites mutated was tested. This promoter has been shown to be unable to bind E2F as a result of a double point mutation changing the central two bases of the E2F sites (CG to TA) (3, 52). In SL2 cells, the activity of the DHFRΔE2F promoter is approximately twofold lower than that of the wild-type DHFR promoter. Sp1 transactivated this promoter sixfold, and E2F1 alone had no effect on transcription (Fig. 1B). Cotransfection of Sp1 and E2F1 activated transcription from the E2F site-mutated DHFR promoter 45-fold, indicating that E2F1, with Sp1, was able to superactivate transcription from a promoter possessing Sp1 sites but lacking an E2F site.

To determine whether Sp1 could superactivate E2F-dependent transcription, the effect of coexpression of E2F1 and Sp1 on the adenovirus E2a promoter (which has a TATA box, two upstream E2F sites, and an ATF site but no Sp1 sites) was tested. As shown in Fig. 2A, transfection with Sp1 or E2F1 alone stimulated transcription of the E2a promoter; moreover, cotransfection of E2F1 with Sp1 synergistically activated the E2a promoter. In contrast, an E2a promoter with both E2F sites mutated but containing an intact ATF site and still displaying significant unstimulated CAT activity (0.2 pmol/μg/h) did not respond to Sp1 or E2F1, either individually or in combination. Thus, the binding sites for E2F in the E2a promoter are necessary to confer superactivation by Sp1. These data also suggest that the activation of the E2a promoter observed following cotransfection with Sp1 expression vectors alone is mediated by endogenous *Drosophila* E2F (15, 39). The results with the E2a promoter also demonstrate that cooperative activation of transcription by Sp1 and E2F1 is not restricted to TATA-less promoters.

Association of E2F1 and Sp1 in cells. To further examine the functional interaction between E2F1 and Sp1, coimmunoprecipitation experiments were performed to determine whether E2F1 and Sp1 were associated in cells. Cellular extracts obtained from *Drosophila* cells transfected with Sp1 and/or E2F1 were immunoprecipitated with E2F1 antibody, and the immune complexes were analyzed by Western blotting (immunoblotting) for the presence of Sp1. In extracts from *Drosophila* cells that were cotransfected with E2F1 and Sp1, antibody to E2F1 was able to coimmunoprecipitate Sp1 (Fig. 3A, lane 2). The coimmunoprecipitation was specific, since it was dependent on the expression of both E2F1 and Sp1; antibody to E2F1 did not immunoprecipitate Sp1 from cells that were not cotransfected with Sp1 or from cells transfected with Sp1 but not E2F1. The expression of Sp1 in cells transfected with the Sp1 expression vector was confirmed by immunoprecipitation with anti-Sp1 antibody (Fig. 3A, lane 3). Immunoprecipitation

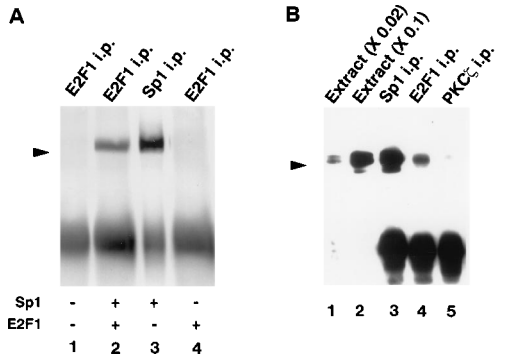

FIG. 3. Analysis of Sp1 content of E2F1 immunoprecipitates of extracts from SL2 cell transfectants and HeLa cells. (A) *D. melanogaster* SL2 cells were mock transfected or transfected with E2F1 (15 μg of pCMV-E2F1) and/or Sp1 (1 μg of pPAC-Sp1) expression vectors 48 h prior to extraction with RIPA buffer supplemented with protease inhibitors. Clarified extracts were immunoprecipitated (i.p.) with rabbit polyclonal IgG specific for E2F1 or Sp1 and protein A-Sepharose. Washed beads were boiled in SDS sample buffer, and precipitated proteins were resolved by SDS-PAGE (8% polyacrylamide gel) and analyzed for Sp1 by Western blot analysis using the anti-Sp1 antibody. Immunoreactive proteins were detected by using alkaline phosphatase-conjugated secondary antibody. The bands indicated by the arrowhead have a relative molecular weight of ~95,000 and represent precipitated Sp1. The band seen in all lanes at the bottom is due to immunoprecipitating antibody binding anti-rabbit IgG secondary antibody. (B) HeLa cell nuclear extracts (120 μg of protein) were immunoprecipitated in RIPA buffer, using anti-E2F1, anti-Sp1, or anti-PKCζ rabbit polyclonal IgG and protein A-Sepharose. Following electrophoresis on SDS–10% polyacrylamide gels, Sp1 which had been bound to beads was detected by Western blotting analysis of the transferred gel, using anti-Sp1 antibody and goat anti-rabbit secondary antibodies linked to peroxidase followed by enhanced chemiluminescence (Amersham). Lanes 1 and 2 show Sp1 from extract equivalent to 1/50 and 1/10 of that used for immunoprecipitation, respectively. The arrowhead indicates the bands due to precipitated Sp1.

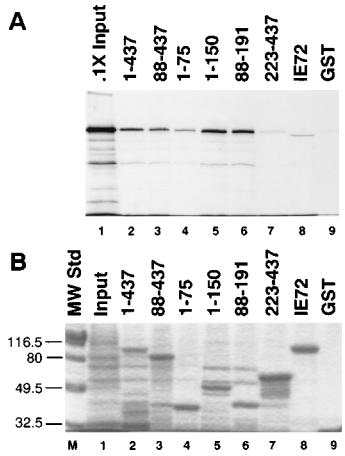

FIG. 4. In vitro binding assay for interaction of GST-E2F1 with Sp1. GST (lane 9) or GST-E2F1 (wild type or various deletion mutants; lanes 2 to 7) bound to glutathione-Sepharose was incubated with in vitro-translated ^{35}S-labeled Sp1 for 1 h at 4°C. Beads were washed, and bound proteins were solubilized in SDS-PAGE sample buffer and resolved by SDS-PAGE (10% polyacrylamide gel). Gels were dried, and ^{35}S-labeled proteins which had bound to the fusion proteins were visualized by autoradiography. Numbers above lanes signify the amino acid residues of E2F1 present in the various fusion proteins. Lane 1 shows 1/10 of the Sp1 protein used for the reactions, and lane 8 shows binding to a control IE72 protein. (B) Coomassie blue staining of the gel shown in panel A. MW Std, molecular weight standards (sizes are indicated in kilodaltons).

of E2F1 from cells transfected with E2F1 was also confirmed by the observation of an immunoreactive band migrating just above the IgG heavy chain only in immunoprecipitates from cells transfected with E2F1 (data not shown). Taken together, these data indicate that Sp1 and E2F1 can form a physical complex in *Drosophila* cells in which both proteins are present. The E2F1 antibody used here did not recognize *Drosophila* E2F (data not shown); therefore, these studies give no information about possible physical interaction of Sp1 with the endogenous E2F in SL2 cells.

To determine whether Sp1 and E2F1 are in a complex in cells in which they are normally expressed, HeLa nuclear extracts were tested. Three Sp1-immunoreactive bands were detected by Sp1 antibody on Western blots of cell extracts run directly or after anti-Sp1 immunoprecipitation (Fig. 3B, lanes 1 to 3). Results of other experiments indicate that the upper band of the doublet is the phosphorylated form of Sp1, as has also been shown by others (21); the fastest-migrating band also cross-reacts with an antibody specific to Sp3 (20a). In extracts from log-phase HeLa cells, a band corresponding to Sp1 was detected by Sp1 antibody in material immunoprecipitated by antibody to E2F1 (Fig. 3B, lane 4). The specificity of this immunoprecipitation was confirmed by the finding that nonspecific control antibody (anti-PKCζ) did not precipitate any Sp1-immunoreactive material (lane 5).

In vitro association of E2F1 and Sp1. The coimmunoprecipitation experiments demonstrated that E2F1 and Sp1 are present in a complex in *Drosophila* cells in which the two proteins are overexpressed or in mid-log-phase HeLa cells. To further characterize the interaction of Sp1 and E2F1, in vitro association assays were performed initially with GST-E2F1 and in vitro-translated Sp1. All of the in vitro association assays were performed in the presence of ethidium bromide (100 μg/ml) to eliminate any possible interference by or dependence on DNA in the protein-protein interactions (28). As shown in Fig. 4A, full-length GST-E2F1 (amino acids 1 to 437) was able to bind ^{35}S-labeled in vitro-translated Sp1. The specificity of the interaction was demonstrated by the very low background binding of Sp1 to glutathione-Sepharose beads coated with GST protein (lane 9) or a control GST fusion protein (GST-IE72; lane 8). Similarly, no interacting proteins were detected in control in vitro translation reactions (data not shown). Analysis of the interaction of Sp1 with E2F1 mutants fused to GST revealed that peptides composed of amino acid residues 88 to 191 or 1 to 150 of E2F1 contain the minimal regions required for interaction with Sp1 (Fig. 4A, lane 5 and 6), since deletion mutants containing only these amino acid residues were able to interact with Sp1. The specificity of the interaction within this region is further supported by the lack of interaction with mutants containing only amino acid residues 223 to 437. The peptide composed of amino acid residues 1 to 75 is significantly reduced in its ability to interact with Sp1 (Fig. 4A), and in some experiments, no interaction with this peptide was detected. The interaction detected in some experiments indicates that a weak site for Sp1 interaction may also lie in this region. The amounts of the GST-E2F fusion proteins used in the reactions were approximately equal, as shown by Coomassie blue staining of the gel (Fig. 4B). Identical regions of E2F1 were shown to be necessary and sufficient for interaction with Sp1 in nuclear extracts (data not shown).

A more precise analysis of the region of E2F1 that interacts with Sp1 was performed by using in vitro-translated E2F1 proteins bearing several specific mutations in the amino acid residues between positions 88 and 191 and assessing their abilities to bind GST-Sp1. Deletion of amino acid residues 113 to 120 greatly reduced the ability of E2F1 to interact with Sp1, whereas point mutations in amino acid residue 113, 120, 138, or 177 had little effect (Fig. 5A). This experiment also indicates that the D domain and zinc finger region of Sp1 are required

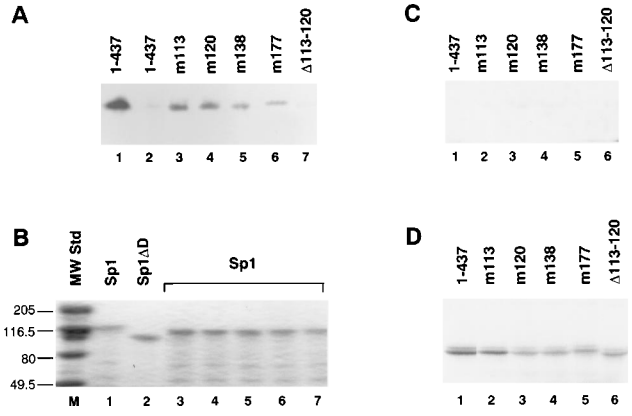

FIG. 5. In vitro binding assay for interaction of GST-Sp1 with E2F1. (A) GST–wild-type Sp1 (lanes 1 and 3 to 7) or mutant GST-Sp1 carrying a deletion of region D (expressing amino acid residues 1 to 603; lane 2) was tested for interaction with ^{35}S-labeled in vitro-translated E2F1 as described for Fig. 4A except that an 8% polyacrylamide gel was used. The samples in lanes 1 and 2 contain full-length E2F1 (amino acid residues 1 to 437). Numbers above the lanes signify the amino acid residues deleted (Δ113-120) or positions of amino acid residues where substitutions have been introduced in the in vitro-translated E2F1. (B) Coomassie blue staining of the gel shown in panel A. MW Std, molecular weight standards (sizes are indicated in kilodaltons). (C) Binding of different ^{35}S-labeled in vitro-translated E2F proteins used for panel A to a control GST protein. (D) Input proteins for the reactions shown in panels A and C. The different E2F1 proteins synthesized and ^{35}S labeled in rabbit reticulocyte lysate were subjected to fluorography after SDS-PAGE.

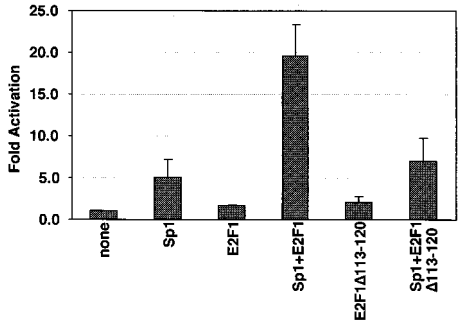

FIG. 6. Amino acid residues 113 to 120 are essential for full functional interaction of E2F with Sp1 on the DHFR promoter. Fifteen micrograms of the plasmid harboring the firefly luciferase gene driven by the DHFR promoter was cotransfected into *D. melanogaster* SL2 cells with 5 μg of the plasmid expressing the CAT gene under the control of a truncated adenovirus major late promoter. One hundred nanograms of the plasmid expressing Sp1 (pPAC-Sp1) and/or 20 ng of the plasmid expressing the wild-type or a mutated E2F gene (pPAC-E2F1 or pPAC-E2F1Δ113-120) were cotransfected with reporter plasmids as indicated. CAT expression served as the transfection efficiency control, and luciferase activity data were normalized with respect to CAT activities. Data are presented as fold activation relative to the expression of the DHFR-luciferase construct in the absence of mammalian transcription factors. Each datum point is the mean of the results with three independent transfections ± the standard error of the mean.

for interaction with E2F1; interaction of E2F1 (amino acids 1 to 437) with a GST-Sp1 fusion protein composed of amino acid residues 1 to 606 (i.e., domain D and zinc finger region deleted [12]) is greatly reduced in comparison with interaction with full-length Sp1 (Fig. 5A, lanes 1 and 2). The amounts of the GST-Sp1 fusion proteins used in the reactions were approximately equal, as indicated by Coomassie blue staining of the gel (Fig. 5B). The levels of input E2Fs are approximately equal (Fig. 5D). There was very little nonspecific interaction, as shown by the lack of interaction of the different radiolabeled E2Fs with GST-coated beads (Fig. 5C).

Activation of transcription by mutated E2F1 that cannot interact with Sp1. Although the E2F1Δ113-120 mutant activates E2F-dependent transcription to approximately the same extent as does the wild-type E2F1 (reference 14 and our unpublished data), it was unable to functionally interact with Sp1, in keeping with its impaired ability to interact with Sp1. Sp1 in combination with wild-type E2F1 was able to activate luciferase expression from the DHFR promoter 20-fold (Fig. 6); in contrast, E2F1Δ113-120 with Sp1 did not synergistically activate the DHFR promoter and gave the same level of activation as Sp1 alone (Fig. 6), suggesting that the interaction is likely responsible for the synergistic activation.

Cell cycle-specific association of E2F1 and Sp1. Since DHFR transcription and E2F1 activity are cell cycle regulated and maximal in mid- to late G_1, we sought to determine whether the interaction between E2F1 and Sp1 varied throughout the cell cycle. To address this issue, human diploid fibroblast cells were synchronized by contact inhibition and serum deprivation and stimulated to reenter the cell cycle by addition of serum. Cellular extracts were prepared at different time points after release from G_0 and subjected to immunoprecipitation with E2F1 antibody, after which Sp1 was detected by Western blot analysis of the immunoprecipitated material. As shown in Fig. 7A (top), the amount of Sp1 detected in extracts isolated throughout G_1 and in S phase did not vary. In contrast, the amount of Sp1 immunoprecipitated by E2F1 antibody in the same extracts was maximal 6 and 9 h after release into G_1 (middle), suggesting that the association between E2F1 and Sp1 is cell cycle specific. The transition from G_0 to S phase was monitored by measuring incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material; cells begin to synthe-

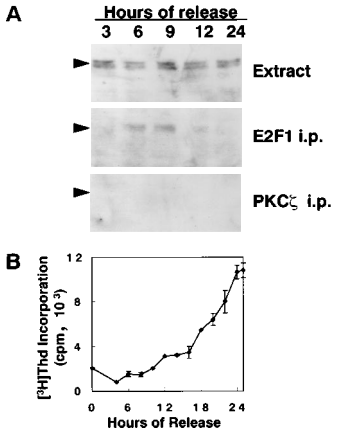

FIG. 7. Association of E2F1 and Sp1 at various stages of the cell cycle. (A) Normal human diploid fibroblasts were synchronized in G_0 by serum starvation and stimulated to reenter the cell cycle by addition of medium containing 10% fetal bovine serum. At various times thereafter, cells were harvested and frozen prior to extraction with 1% NP-40 buffer. A portion of the clarified extracts (equivalent to 10% of that used for immunoprecipitation [i.p.]) was solubilized in SDS sample buffer and subjected to SDS-PAGE (top), and the remainder was immunoprecipitated with anti-E2F1 or anti-PKCζ rabbit IgG and protein A-Sepharose. Proteins bound to the beads were released by boiling in SDS sample buffer followed by electrophoresis on an 8% polyacrylamide gel. Sp1 in the extract and in the immunoprecipitates was detected by Western blot analysis of the transferred gel using anti-Sp1 antibody and goat anti-rabbit secondary antibodies linked to peroxidase followed by enhanced chemiluminescence (Amersham). Only the section of the blots containing Sp1 is shown. (B) Normal human diploid fibroblasts were synchronized by serum starvation and stimulated to reenter the cell cycle by addition of medium containing 10% fetal bovine serum and [^{3}H]thymidine (1 μCi/ml). Cells were harvested at the indicated times, and trichloroacetic acid-precipitable ^{3}H was quantified by scintillation counting.

size DNA between 10 and 14 h after serum addition (Fig. 7B). Antibody to PKCζ used as a nonspecific antibody did not immunoprecipitate any Sp1-cross-reactive material at any of the time points (Fig. 7A, bottom). Thus, the association between E2F1 and Sp1 is specific to cells in mid- to late G1. This time period coincides with the period during which E2F is free, i.e., dissociated from pRB, and prior to association with cyclin A (6, 54).

DISCUSSION

Interaction of E2F1 and Sp1 has been established by the following observations: (i) synergistic activation of the DHFR promoter by coexpression of E2F1 and Sp1 in *Drosophila cells*, (ii) superactivation of a mutant DHFR promoter lacking E2F binding sites by E2F1, (iii) superactivation of the adenovirus E2a promoter by Sp1 through the E2F sites, (iv) coimmunoprecipitation of Sp1 from cell extracts with antibody against E2F1, and (v) in vitro association dependent on the presence of specific regions of both proteins. The interaction of Sp1 and E2F1 is likely responsible for synergistic activation, since an E2F1 mutant which can activate E2F-dependent transcription but is significantly impaired in its ability to interact with Sp1 cannot synergistically activate transcription from the DHFR promoter. Lastly, the association of Sp1 and E2F1 is maximal in mid- to late-G_1 cells, indicating that this association is cell cycle regulated.

E2F binds immediately 3′ to the major transcription start site in the hamster DHFR promoter (3) and thus may serve as an initiator. Sp1 sites enhance the level of transcription initiation at initiator elements in the terminal deoxytransferase promoter (40, 48, 49) and in the adenovirus-associated P5 promoter (46). The initiator sequence in the adenovirus P5 promoter is a YY1 site, and Sp1 has been shown to physically interact with YY1 (45). Thus, the synergistic activation of the DHFR promoter by E2F and Sp1 may be related to the ability of Sp1 to enhance E2F's function as an initiator, analogous to the situation seen with Sp1 and YY1. Activation of an Sp1-dependent promoter by YY1 has also been reported (45), providing a further similarity between the interaction of Sp1 with YY1 and that with E2F1.

The finding that E2F can stimulate initiation and elongation of transcription, whereas Sp1 stimulates only initiation of transcription (2a), suggests that the interaction between these factors is a potential mechanism of functional synergy in some promoters. In addition, the finding that E2F and Sp1 interact with TFIIH and TAF_{110}, respectively (17, 41a), would allow an additional potential mechanism of functional synergy through targeting different factors in the general transcription machinery, as has been observed with other transcription factors (10). While these possible mechanisms do not absolutely require physical interaction between the factors, they provide plausible explanations of how interacting factors could exert synergistic effects in the presence or absence of binding sites for both factors.

Our results with the DHFR promoter demonstrate that E2F1 and Sp1 can synergistically activate transcription from a promoter with Sp1 binding sites and/or E2F binding sites. However, not all promoters with binding sites for Sp1 and E2F give a synergistic response to coexpression of the factors. We have found that E2F and Sp1 synergistically activate the human DNA polymerase α promoter but have only a slightly greater than additive effect on the mouse thymidylate synthetase promoter (data not shown); both of these promoters are TATA-less and have single binding sites for Sp1 and E2F. The differences in the responses among these promoters and the DHFR promoter are not clear at present, but they highlight the complexity of regulation by these two factors and point to the potential importance of both the number and the position (relative to one another and to the transcription start) of the transcription factor binding sites.

On the basis of the ability of anti-E2F1 antibody to immunoprecipitate Sp1, we have concluded that E2F1 and Sp1 physically associate in cells. This coimmunoprecipitation is due to a specific interaction since (i) a control antibody was unable to immunoprecipitate Sp1, (ii) Sp1 could be immunoprecipitated by anti-E2F1 only in SL2 cells expressing both Sp1 and E2F1, and (iii) coimmunoprecipitation was not observed in early-G_1 cells (see below). The physical interaction between E2F1 and Sp1 was confirmed by in vitro experiments, which have also revealed the domains of both proteins involved in the interaction. GST-E2F1 binds in vitro-translated Sp1, and GST-Sp1 binds in vitro-translated E2F1. Sp1 from nuclear extract also interacts with GST-E2F1 fusion proteins, and this approach confirmed that the region of E2F1 interacting with Sp1 was that detected by using in vitro-translated Sp1 (25). Given that the interaction can be detected by these three different in vitro approaches and is not affected by addition of ethidium bromide, it is likely that the interaction between E2F1 and Sp1 is direct, although an indirect interaction cannot be excluded. Through the use of GST-E2F1 mutants, we have determined that the region of E2F1 from amino acid residues 88 to 151 is likely sufficient for this interaction. This region overlaps significantly with the DNA-binding domain. Analysis of E2F1 mutants revealed that the DNA-binding activity and the ability to interact with Sp1 can be separated. Deletion of amino acid residues 113 to 120, which has little effect on DNA binding or transactivation of E2F-dependent transcription (14), virtually abolished the interaction detected between GST-E2F1 and Sp1. E2F2 and E2F3 have also been shown to interact with Sp1, whereas E2F4 and E2F5 do not (25). We have made similar observations. The N-terminal regions of E2F1, E2F2, and E2F3 are very similar to one another but very different from those of E2F4 and E2F5 (44).

The region of Sp1 that appears to be necessary for interaction with E2F1 includes the zinc finger DNA-binding domain and the D domain; a GST fusion protein containing amino acid residues 1 to 606 does not interact with E2F1. The region of Sp1 required for interaction with E2F1 has not been as precisely mapped as the region of E2F1 that interacts with Sp1, but we have found that an Sp1 construct that expresses amino acid residues 1 to 699 can synergistically activate the DHFR promoter and can superactivate the adenovirus E2a promoter (data not shown). The region between amino acid residues 606 and 699 contains the zinc finger motifs required for DNA binding (23). Interactions between Sp1 and at least three other proteins (GATA-1 [32], YY1 [45], and E1a [53]) are mediated through its DNA-binding region. The region between amino acid residue 699 and the C terminus is required for Sp1 to synergistically activate Sp1-dependent transcription (41), suggesting that the region of Sp1 required for interaction with itself is different from the region involved in interaction with E2F1.

The ability of Sp1 and E2F1 to physically interact appears to be essential for their synergistic activation of DHFR transcription. A plasmid expressing E2F1 from which amino acid residues 113 to 120 have been deleted does not activate the DHFR promoter over the level of activation obtained with Sp1 alone. The DNA-binding and transactivation activities of this mutated E2F1 are not significantly affected (14), but the interaction detected between this mutated E2F1 and Sp1 was virtually abolished. In a preliminary experiment, cotransfection of

E2F4, which does not interact with Sp1 (reference 25 and our unpublished data), also does not synergistically activate transcription of the DHFR promoter. These data strongly indicate that the interaction between these factors mediates synergistic activation of DHFR transcription.

The physical interaction between Sp1 and E2F1 is cell cycle regulated, since the E2F1 antibody was able to coimmunoprecipitate a much larger amount of Sp1 from cells in mid- to late G_1 than from cells in early G_1 and S phase. This is particularly relevant in view of the cell cycle regulation of DHFR. E2F has been implicated in cell cycle control of the mouse DHFR gene (47). We have found that although E2F sites can confer late-G_1 expression on a heterologous promoter, mutation of the E2F site in the hamster DHFR promoter does not change the kinetics of its transcriptional activation during the G_0-to-S phase transition. The cell cycle-specific interaction between E2F and Sp1 may account for the unaltered cell cycle regulation of this mutant promoter. Moreover, Sp1 sites placed upstream of a TATA box in a truncated adenovirus major late promoter confer activation of transcription in late G_1 (21a). Thus, although E2F can mediate induction of expression in mid- to late G_1, binding sites for Sp1 can also mediate expression with similar kinetics; whether this effect is exerted through the cell cycle-specific association of E2F1 and Sp1 is being investigated. One possible explanation for the different levels of interaction of Sp1 and E2F in early G_1 is that E2F1 is bound by pRB in early-G_1 cells but is released in late G_1. By reducing levels of free E2F, pRB might interfere with E2F's ability to interact with Sp1 in early-G_1 cells. A further relationship between Sp1 and pRB is provided by the observation that pRB activates Sp1-dependent transcription (26, 51).

An emerging theme in transcriptional control is that regulation can result from physical interaction between transcription factors. The data showing physical interaction between E2F1 and Sp1, together with the observed functional interaction, indicate that this form of regulation may occur on promoters containing binding sites for either of these transcription factors. Such interactions would greatly broaden the possible regulation of these promoters. E2F is intimately involved in cell cycle-regulated transcription; its interaction with Sp1 may thereby extend this regulation to promoters containing Sp1 sites. The interaction between Sp1 and E2F1 is cell cycle regulated, and their interaction may be involved in the control of the many genes that contain binding sites for either or both of these factors, thus implicating Sp1 in cell cycle control of transcription.

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REFERENCES

1. **Adams, P. D., and W. G. Kaelin, Jr.** 1995. Transcriptional control by E2F. Semin. Cancer Biol. **6:**99–108.
2. **Azizkhan, J. C., D. E. Jensen, A. J. Pierce, and M. Wade.** 1993. Transcription from TATA-less promoters: dihydrofolate reductase as a model. Crit. Rev. Eukaryotic Gene Expression **3:**229–254.

2a. **Bentley, D.** Personal communication.

3. **Blake, M. C., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. **9:**4994–5002.
4. **Blake, M. C., R. C. Jambou, A. G. Swick, J. W. Kahn, and J. C. Azizkhan.** 1990. Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. Mol. Cell. Biol. **10:**6632–6641.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355:**176–179.
7. **Chellappan, S.** 1994. The E2F transcription factor: role in cell cycle regulation and differentiation. Mol. Cell. Differ. **2:**201–220.
8. **Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65:**1053–1061.
9. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
10. **Chen, J. L., L. D. Attardi, C. P. Verrijzer, K. Yokomori, and R. Tjian.** 1994. Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79:**93–105.
11. **Chittenden, T., D. M. Livingston, and W. G. Kaelin, Jr.** 1991. RB associates with an E2F-like, sequence-specific DNA-binding protein. Cold Spring Harbor Symp. Quant. Biol. **56:**187–195.
12. **Courey, A. J., D. A. Holtzman, S. P. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59:**827–836.
13. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55:**887–898.
14. **Cress, W. D., D. G. Johnson, and J. R. Nevins.** 1993. A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol. Cell. Biol. **13:**6314–6325.
15. **Dynlacht, B. D., A. Brook, M. Dembski, L. Yenush, and N. Dyson.** 1994. DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins. Proc. Natl. Acad. Sci. USA **91:**6359–6363.
16. **Flemington, E. K., S. H. Speck, and W. G. Kaelin, Jr.** 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA **90:**6914–6918.
17. **Gill, G., E. Pascal, Z. H. Tseng, and R. Tjian.** 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. USA **91:**192–196.
18. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70:**337–350.
19. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
20. **Horowitz, J. M.** 1993. Regulation of transcription by the retinoblastoma protein. Genes Chromosomes Cancer **6:**124–131.

20a. **Horowitz, J. M.** Personal communication.

21. **Jackson, S. P., J. J. MacDonald, S. Lees-Miller, and R. Tjian.** 1990. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell **63:**155–165.

21a. **Jensen, D. E., and J. C. Azizkhan.** Unpublished data.

22. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51:**1079–1090.
23. **Kadonaga, J. T., A. J. Courey, J. Ladika, and R. Tjian.** 1988. Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science **242:**1566–1570.
24. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma binding protein with E2F-like properties. Cell **70:**351–364.
25. **Karlseder, J., H. Rotheneder, and E. Wintersberger.** 1996. Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. Mol. Cell. Biol. **16:**1659–1667.
26. **Kim, S. J., U. S. Onwuta, Y. I. Lee, R. Li, M. R. Botchan, and P. D. Robbins.** 1992. The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol. **12:**2455–2463.
27. **Kollmar, R., K. A. Sukow, S. K. Sponagle, and P. J. Farnham.** 1994. Start site selection at the TATA-less carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase promoter. J. Biol. Chem. **269:**2252–2257.
28. **Lai, J.-S., and W. Herr.** 1992. Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. Proc. Nat. Acad. Sci. USA **89:**6958–6962.
29. **LaThangue, N. B.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **19:**108–114.

30. **Li, Y., J. E. Slansky, D. J. Myers, N. R. Drinkwater, W. G. Kaelin, and P. J. Farnham.** 1994. Cloning, chromosomal location, and characterization of mouse E2F1. Mol. Cell. Biol. **14:**1861–1869.
31. **Liao, W. C., J. Ash, and L. F. Johnson.** 1994. Bidirectional promoter of the mouse thymidylate synthase gene. Nucleic Acids Res. **22:**4044–4049.
32. **Merika, M., and S. H. Orkin.** 1995. Functional synergy and physical interaction of the erythroid transcription factor GATA-1 with the Krüppel family proteins Sp1 and EKLF. Mol. Cell. Biol. **15:**2437–2447.
33. **Moberg, K. H., T. J. Logan, W. A. Tyndall, and D. J. Hall.** 1992. Three distinct elements within the murine c-myc promoter are required for transcription. Oncogene **7:**411–421.
34. **Murthy, S. C., G. P. Bhat, and B. Thimmappaya.** 1985. Adenovirus EIIA early promoter: transcriptional control elements and induction by the viral pre-early EIA gene, which appears to be sequence independent. Proc. Natl. Acad. Sci. USA **82:**2230–2234.
35. **Neuman, E., E. K. Flemington, W. R. Sellers, and W. G. Kaelin, Jr.** 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. **14:**6607–6615.
36. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A rapid fluor diffusion assay for detection of chloramphenicol acetyl transferase activity. BioTechniques **5:**444–448.
37. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
38. **Ogris, E., H. Rotheneder, I. Mudrak, A. Pichler, and E. Wintersberger.** 1993. A binding site for transcription factor E2F is a target for *trans* activation of murine thymidine kinase by polyomavirus large T antigen and plays an important role in growth regulation of the gene. J. Virol. **67:**1765–1771.
39. **Ohtani, K., and J. R. Nevins.** 1994. Functional properties of a *Drosophila* homolog of the E2F1 gene. Mol. Cell. Biol. **14:**1603–1612.
40. **O'Shea-Greenfield, A., and S. T. Smale.** 1992. Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription. J. Biol. Chem. **267:**6450–6455.
41. **Pascal, E., and R. Tjian.** 1991. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. **5:**1646–1656.
41a. **Pearson, A., and J. Greenblatt.** Personal communication.
42. **Pearson, B. E., H.-P. Nasheuer, and T. S.-F. Wang.** 1991. Human DNA polymerase α gene: sequences controlling expression in cycling and serum-stimulated cells. Mol. Cell. Biol. **11:**2081–2095.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R. A. Weinberg.** 1995. E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA **92:**2403–2407.
45. **Seto, E., B. Lewis, and T. Shenk.** 1993. Interaction between transcription factors Sp1 and YY1. Nature (London) **365:**462–464.
46. **Seto, E., Y. Shi, and T. Shenk.** 1991. YY1 is an initiator sequence-binding protein that directs and activates transcription *in vitro*. Nature (London) **354:**241–245.
47. **Slansky, J. E., Y. Li, W. G. Kaelin, and P. J. Farnham.** 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. Mol. Cell. Biol. **13:**1610–1618.
48. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57:**103–113.
49. **Smale, S. T., M. C. Schmidt, A. J. Berk, and D. Baltimore.** 1990. Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. Proc. Natl. Acad. Sci. USA **87:**4509–4513.
50. **Swick, A. G., M. C. Blake, J. W. Kahn, and J. C. Azizkhan.** 1989. Functional analysis of GC element binding and transcription in the hamster dihydrofolate reductase gene promoter. Nucleic Acids Res. **17:**9291–9304.
51. **Udvadia, A. J., K. T. Rogers, P. D. Higgins, Y. Murata, K. H. Martin, P. A. Humphrey, and J. M. Horowitz.** 1993. Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression. Proc. Natl. Acad. Sci. USA **90:**3265–3269.
52. **Wade, M., M. C. Blake, R. C. Jambou, K. Helin, E. Harlow, and J. C. Azizkhan.** 1995. An inverted repeat motif stabilizes binding of E2F and enhances transcription of the dihydrofolate reductase gene. J. Biol. Chem. **270:**9783–9791.
53. **Wagner, S., and M. R. Green.** 1994. DNA-binding domains: targets for viral and cellular regulators. Curr. Opin. Cell Biol. **6:**410–414.
54. **Xu, M., K.-A. Sheppard, C.-Y. Peng, A. S. Yee, and H. Piwnica-Worms.** 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol. **14:**8420–8431.