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E2F Activity Is Regulated by Cell Cycle-Dependent Changes in Subcellular Localization

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E2F directs the cell cycle-dependent expression of genes that induce or regulate the cell division process. In mammalian cells, this transcriptional activity arises from the combined properties of multiple E2F-DP heterodimers. In this study, we show that the transcriptional potential of individual E2F species is dependent upon their nuclear localization. This is a constitutive property of E2F-1, -2, and -3, whereas the nuclear localization of E2F-4 is dependent upon its association with other nuclear factors. We previously showed that E2F-4 accounts for the majority of endogenous E2F species. We now show that the subcellular localization of E2F-4 is regulated in a cell cycle-dependent manner that results in the differential compartmentalization of the various E2F complexes. Consequently, in cycling cells, the majority of the p107-E2F, p130-E2F, and free E2F complexes remain in the cytoplasm. In contrast, almost all of the nuclear E2F activity is generated by pRB-E2F. This complex is present at high levels during G_1 but disappears once the cells have passed the restriction point. Surprisingly, dissociation of this complex causes little increase in the levels of nuclear free E2F activity. This observation suggests that the repressive properties of the pRB-E2F complex play a critical role in establishing the temporal regulation of E2F-responsive genes. How the differential subcellular localization of pRB, p107, and p130 contributes to their different biological properties is also discussed.

E2F is a transcriptional regulator that plays a pivotal role in the regulation of cellular proliferation (reviewed in reference 59; 8). Many E2F-responsive genes have been identified, and their products are components of either cell cycle control (e.g., cyclin E, cyclin A, and cdc2) or DNA synthesis (e.g., dihydrofolate reductase, thymidine kinase, or DNA polymerase α) machinery. In each case, E2F is thought to restrict the expression of these genes to the point of the cell cycle at which their products act (38).

E2F is regulated by the retinoblastoma protein (pRB) (3, 5, 12), a tumor suppressor that is functionally inactivated in a large proportion of all human tumors (reviewed in reference 67). Consistent with its antiproliferative role, pRB blocks the ability of E2F to activate transcription (32, 33). In addition, overexpression studies have indicated that the resultant pRB-E2F complex can act as a transcriptional repressor, in which E2F provides the sequence-specific DNA binding activity and pRB inhibits transcription by sequestering adjacent transcription factors (2, 10, 62, 69, 70). This idea suggests that E2F participates in both the activation and the inhibition of cellular proliferation. Consistent with this hypothesis, homozygous deletion of the murine E2F-1 gene causes atrophy in some tissues and tumors in others (26, 73).

The growth-inhibitory properties of pRB are regulated by its cell cycle-dependent phosphorylation (reviewed in reference 7). Phosphorylation is catalyzed by one or more of the cell cycle-dependent kinases (24, 35, 39, 48, 53, 55), and overexpression studies have indicated that this modification is essential for S-phase entry (35). In vivo studies confirm that the phosphorylation of pRB is sufficient to induce the release of free, presumably transcriptionally active E2F (12). Because of the dual role of the E2F complex, phosphorylation of pRB provides a simple mechanism for switching E2F-responsive genes from the fully repressed to the fully induced state. Consistent with this model, the timing of transcriptional activation of E2F-responsive genes correlates closely with the induction of pRB phosphorylation at the G_1-S transition.

Our understanding of E2F is complicated by the finding that its activity is regulated by two other proteins, p107 and p130 (11, 16, 19, 63). These two proteins share significant sequence similarity with pRB (25, 29, 50, 54), and overexpression studies have confirmed that they can regulate E2F in a similar manner (65, 74). Despite these similarities, pRB, p107, and p130 interact with E2F at different stages of the cell cycle (11, 13, 16, 47, 58, 63). Moreover, unlike that of the pRB-E2F complex, the timing of the appearance or disappearance of the p130-E2F and p107-E2F species does not correlate with the timing of the repression or activation of known E2F-responsive genes. These findings suggest that pRB, p107, and p130 do not regulate E2F in the same way in vivo, and genetic analyses have confirmed that these proteins have different biological consequences. While pRB is mutated in 30% of all human tumors, neither p107 nor p130 is a tumor suppressor (68). Similarly, the mutation of pRB, p107, or p130 within otherwise isogenic mouse strains gives rise to very different phenotypes (14, 15, 41, 45, 46). Clearly, the different biological consequences of pRB, p107, and p130 action could reflect differences in their regulation of E2F or of non-E2F targets.

To date, at least seven human genes that encode components of E2F transcriptional activity have been identified (reviewed in reference 8). These can be divided into two distinct groups, termed E2F-1 through E2F-5 and DP-1 and DP-2, that share little sequence similarity. E2F and DP proteins heterodimerize, and this association is essential for high-affinity DNA binding, transcriptional activity, and interaction with pRB, p107, or p130 (6, 31, 42, 61, 71). In vivo studies have confirmed that endogenous E2F activity arises from the con-

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certed action of multiple E2F-DP complexes (57, 71). These individual E2F-DP complexes have different pRB, p107, and p130 binding properties. Complexes containing E2F-1, -2, or -3 associate with pRB but not p107 or p130 in vivo (23, 49). In contrast, complexes containing E2F-4 or -5 have been reported to bind preferentially to p107 and p130 (9, 27, 34, 66). Consistent with these findings, sequence comparisons have indicated that E2F-1, -2, and -3 are more closely related to each other than they are to E2F-4 and -5. Taken together, these observations suggest that the two E2F subsets (E2F-1, -2, and -3 versus E2F-4 and -5) play distinct roles in vivo that at least partially account for the different biological consequences of pRB, p107, and p130 action. Overexpression assays have revealed some differences in the properties of the individual E2F-DP complexes (18, 36, 52), but the mechanistic distinction(s) between these species remains unclear.

We previously characterized the cell cycle regulation of the individual E2F-DP complexes (57). That study revealed clear differences in the relative contributions and potential activities of these species. E2F-1, -2, -3, and -5 exist at low levels in vivo and together comprise less than one-third of the endogenous E2F species. In contrast, E2F-4 accounts for the majority of E2F complexes at every stage of the cell cycle. Moreover, in addition to binding p107 and p130, E2F-4 was found to be the major component of pRB-associated E2F activity. These findings suggest that E2F-4 plays a pivotal role in establishing the biological properties of cellular E2F activity. Nevertheless, we also found that the appearance of free E2F-4–DP, which occurs early in G_1, is insufficient to induce the activation of known E2F-responsive genes. This observation can be explained in two distinct ways: the transcriptional activity of free E2F-4 is regulated by an unknown mechanism and/or it is directed at an unknown set of target genes. Since E2F-4 accounts for most of the endogenous E2F species, either mechanism will have a profound effect upon the biological consequences of E2F action.

In this study, we used a combination of in vitro and in vivo assays to investigate these two hypotheses. These experiments did not allow us to examine whether E2F-4 has a different target specificity than the other E2F species. However, our data indicate that, unlike that of E2F-1, -2, and -3, the transcriptional activity of E2F-4 is regulated at the level of subcellular localization. In vivo, the nuclear localization of E2F-4 is restricted to certain stages of the cell cycle and is limited to a specific subset of the E2F-4 complexes. This novel mode of E2F-4 regulation provides new insight into the molecular mechanism(s) that establishes the different biological properties of the individual E2F family members and may offer important insight into the in vivo roles of the E2F regulators pRB, p107, and p130.

MATERIALS AND METHODS

Cell cultures. Human cell lines ML-1 (premyeloid leukemia), C33-A (cervical carcinoma), WI-38 (normal diploid lung fibroblast), 293 (renal adenocarcinoma), T98G (glioblastoma), R12 (Rat1A derivative cell line), and U2OS and SAOS-2 (osteosarcomas) were grown under standard conditions of 5% CO_2 in Dulbecco modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Murine 3T3 fibroblasts were grown in DMEM supplemented with 10% calf serum. The HL60 (human lymphoma) cell line was cultured in RPMI medium supplemented with 20% fetal bovine serum. Transient transfections were carried out exactly as described previously (57).

Cell synchronization. U2OS cells were arrested at metaphase by sequential culturing in media containing 2 mM thymidine (12 h) and then 1.7 μM nocodazole (24 h), washed several times, and replated in fresh media on coverslips. Cells were collected every 6 h and processed for either immunofluorescence or fluorescence-activated cell sorting (FACS) analysis. HL60 cells were separated by centrifugal elutriation with standard procedures, and a small proportion of each fraction was subjected to FACS analysis to determine cell cycle staging. Synchronized G_0-G_1 WI-38 human diploid fibroblasts were obtained by starvation of early-passage cells in 0.1% serum for 48 h; enriched S-phase populations were generated by replating arrested cells in 20% fetal bovine serum for 14 h (56).

Plasmids. The following plasmids were described previously: pCMV-E2F-1, -2, -3, and -4 (57) and pCMV-DP-1 and -2 and pCMV-pRB (71). The E2F-responsive reporter plasmid $E2F_4$-CAT and pRSV-luciferase were described by Helin et al. (31). Chimeric E2F cDNAs were constructed in a derivative of the pBKSII$^+$ vector (Stratagene) in which the *Xba*I site had been deleted. Briefly, domains of either E2F-2 or E2F-4 were amplified by PCR with primers containing silent restriction sites which did not alter codon usage within the relevant open reading frame. Hybrid cDNAs were created by ligation of these individual domains together in the pBKSII$^+$ΔXba vector with the engineered restriction sites. Full-length chimeric cDNAs were then subcloned as *Bam*HI fragments into the *Bam*HI site of the pCMV-Neo-Bam vector (4). The E2F-2- and E2F-4-specific primer pairs are as follows.

(i) E2F-2. For E2F-2, the primer pairs were 10.18 (5′-GGATCCATGCTGC AAGGGCCCCGGGCCTTG-3′) and 10.24Xba (5′-CCTAAGCTTCTAGAAC GTTGGTGATGTCATAG-3′), 10.23Xba (5′-CGTTCTAGAAGGCACTCAG CTCATC-3′) and 10.22HIII (5′-GAGAAGCTTATCAGAGGGGAG-3′), and 10.21HIII (5′-GATAAGCTTCTCCCCATCCTTG-3′) and 10.20 (5′-GGTGGT ACCGGATCCTCAATTAATCAACAGGTCC-3′).

(ii) E2F-4. For E2F-4, the primer pairs were 4.13 (5′-GGATCCATGGCGG AGGCCGGGCCACAG-3′) and 4.19Xba (5′-CCTAAGCTTCTAGAACATTG GTAATGTCGTA-3′), 4.18Xba (5′-TGTTCTAGAAGGTATCGGGCTAATC-3′) and 4.17HIII (5′-GAAAAGCTTAGCAGAGGGGCAAACACT-3′), and 4.16HIII (5′-GCTAAGCTTATCTCCACCCCCGGGAGAC-3′) and 4.15 (5′-G GTGGTACCGGATCCTCAGAGGTTGAGAACAGG-3′).

For pCMV-2444, the N terminus of E2F-2 was amplified by PCR with primers 10.18 (see above) and 10.2N (5′-GATGGATCCGAGGCCATCCACTCTGAT-3′). The N-terminal deletions of E2F-2, Δ83, Δ88, and Δ117, were constructed by PCR amplification of a wild-type E2F-2 cDNA template by use of the following primers together with primer 10.20 (see above): 10.83 (5′-GGTGGATCCATG GCCAAAAGGAAGCTGG-3′), 10.88 (5′-GGTGGATCCATGCTGGATCTG GAGGGGATTG-3′), and 10.117 (5′-GGTGGATCCATGGGCCCCAAAACC CCCAAATC-3′). The F2ΔNLS construct was generated by PCR amplification of E2F-2 cDNA with the primer pair 10.18 (see above) and 10.ΔNLS2 (5′-CTTC AAGCTTCTACAGGCACTCAGCCGTCCTGCCGGCAG-3′) and the primer pair 10.20 (see above) and 10ΔNLS1 (5′-GTTGAAGCTTGTTTGTGGCGGG GATTGGGAGGCC-3′). The two fragments were then ligated together to yield an E2F-2 cDNA containing a novel *Hin*dIII site within the altered nuclear localization signal (NLS) sequences.

E2F transactivation assays. Chloramphenicol acetyltransferase (CAT) and luciferase assays were performed as described by Helin et al. (31). Briefly, cells were harvested 36 h posttransfection and lysed in 0.025 M Tris-HCl (pH 8.0) by three rapid freeze-thaw cycles. Extracts were clarified by a 15,000 × *g* spin for 10 min, and the supernatants were assayed for CAT and luciferase activities.

E2F-inducible cell lines. E2F-2- or E2F-4-inducible cell lines were generated by the technique of Gossen and Bujard (28). U2OS cells were cotransfected with pUHD15-1 and pCMVneo by the calcium phosphate method. After selection in G418 (250 μg/ml), stable cell lines were assayed by transient transfection for tetracycline-regulated activity of a luciferase reporter (pUHC13-3). One line, U2tTA10, which showed a 2×10^3 increase in luciferase activity upon tetracycline withdrawal, was transfected with pTK-HYG and the pUHD10-3 expression vector containing either the E2F-2 or the E2F-4 cDNA. After selection in the presence of hygromycin (100 μg/ml) and tetracycline (0.1 μg/ml), extracts from stable cell lines were screened by Western blotting for the induction of the relevant E2F species.

Microinjection and immunofluorescence. 3T3 and R12 cells were plated on glass coverslips and grown to 70% confluency. E2F expression plasmids (25 μg/ml) were coinjected with a plasmid encoding β-galactosidase (5 μg/ml) to mark injected cells. After injection, the cells were grown in DMEM-HEPES supplemented with 10% serum for 3 to 4 h. Following fixation and permeabilization, cells were incubated for 30 min with rabbit anti-β-galactosidase antibodies (1:50 dilution; 5 Prime-3 Prime, Inc.) and a cocktail of mouse anti-E2F monoclonal antibodies (1:25 dilution). The E2F antibodies used were KH20 (anti-E2F-1), LLF2-1 (anti-E2F-2), LLF3-1 (anti-E2F-3), and LLF4-1 (anti-E2F-4). KH20, LLF2-1, and LLF3-1 were described previously (57). The LLF4-1 monoclonal antibody hybridoma cell line was isolated from BALB/c mice immunized with six-His-tagged E2F-4 (amino acids 147 to 413) exactly as described by Moberg et al. (57). Following incubation in primary antibody, cells were washed with phosphate-buffered saline (PBS) and incubated for 30 min in secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse antibody at a 1:1,000 dilution [Cappel] and rhodamine-conjugated goat anti-rabbit antibody at a 1:1,000 dilution [Cappel]). The cells were then washed four times with PBS, incubated with 4′,6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml) for 5 min, washed again, and mounted on glass slides with Mowiol.

For detection of transfected proteins, 50% confluent U2OS cells, plated on coverslips 48 h earlier, were transfected with expression constructs together with cytomegalovirus (CMV)–β-galactosidase to mark transfected cells and processed 24 h later for immunofluorescence exactly as described above. The murine anti-influenza virus hemagglutinin (HA) antibody 12CA5 was used to detect HA-tagged proteins.

For detection of endogenous E2F-4, synchronous or asynchronous U2OS cells

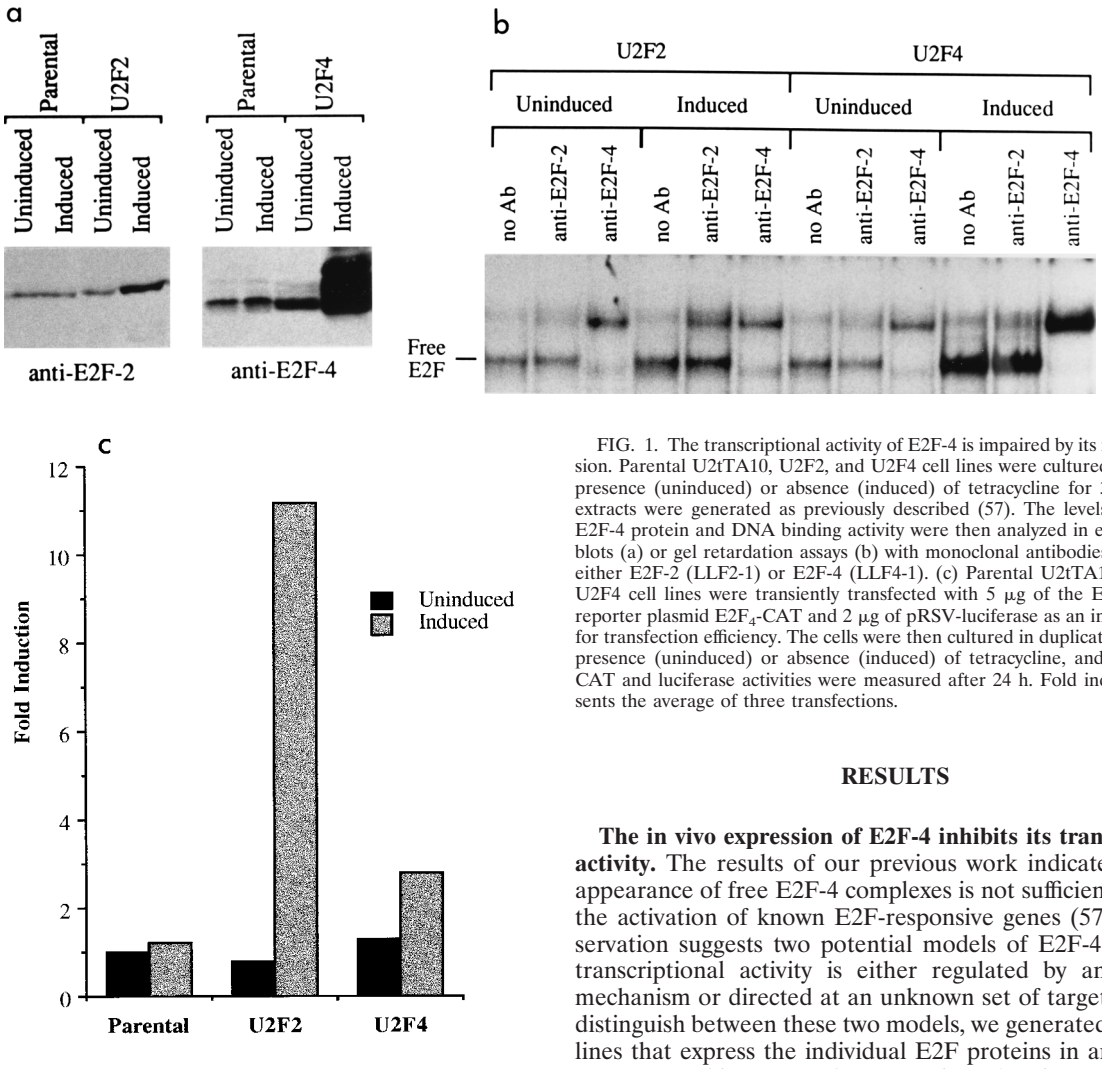


FIG. 1. The transcriptional activity of E2F-4 is impaired by its in vivo expression. Parental U2tTA10, U2F2, and U2F4 cell lines were cultured in either the presence (uninduced) or absence (induced) of tetracycline for 36 h, and cell extracts were generated as previously described (57). The levels of E2F-2 or E2F-4 protein and DNA binding activity were then analyzed in either Western blots (a) or gel retardation assays (b) with monoclonal antibodies (Ab) against either E2F-2 (LLF2-1) or E2F-4 (LLF4-1). (c) Parental U2tTA10, U2F2, and U2F4 cell lines were transiently transfected with 5 μg of the E2F-responsive reporter plasmid $E2F_4$-CAT and 2 μg of pRSV-luciferase as an internal control for transfection efficiency. The cells were then cultured in duplicate in either the presence (uninduced) or absence (induced) of tetracycline, and the levels of CAT and luciferase activities were measured after 24 h. Fold induction represents the average of three transfections.

or WI-38 cells were plated on glass coverslips, and immunofluorescence was detected as described above but with the following modifications. After cells were fixed and permeabilized, blocking solution (5% goat serum, 0.2% fish skin gelatin [Sigma], 0.2% Tween 20) was added for 60 min. Cells were then incubated with mouse anti-E2F-4 monoclonal antibody (diluted 1:25 in blocking solution) for 60 min, washed twice with PBS–0.2% Tween 20, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 30 min. After several washes with PBS–Tween-20 and DAPI staining for 5 min, the cells were mounted on glass slides with VectaShield (Vector).

Subcellular fractionation, Western blotting, and gel shift assays. Fractionation of cultured cells was performed as follows. Cell pellets were resuspended in two packed-cell volumes (PCV) of hypotonic buffer (10 mM HEPES [pH 7.5], 10 mM KCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EDTA [pH 8.0], 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mg of aprotinin per ml, 1 mg of leupeptin per ml) and incubated for 5 min on ice. Nuclei were separated by a 500 × *g* spin for 5 min and washed twice in hypotonic buffer. Nuclei were then lysed in three PCV of lysis buffer (57) and clarified by a 45-min spin at 100,000 × *g* as described previously (57). The cytoplasmic supernatant from the original 500 × *g* spin was supplemented with glycerol to 35% (final volume, three PCV) and clarified by a 20,000 × *g* spin for 10 min. Protein concentrations were determined with protein dye reagent (Bio-Rad). As the final volumes of cytoplasmic and nuclear extracts from a given cell population were equivalent, the ratio of protein concentrations was taken as a measure of the per-cell ratio of cytoplasmic and nuclear proteins.

Western blotting and gel shift assays were performed exactly as described previously (57). Briefly, equal volumes of cytoplasmic and nuclear extracts were assayed for E2F protein (Western blotting) or E2F DNA binding activity (gel shift assays) in the presence of antibodies. E2F-4 was detected in both assays with the monoclonal antibody LLF4-1 supernatant described above. Additional antibodies used were KH20 (anti-E2F-1), XZ55 (anti-pRB), SD15 (anti-p107), and sc-317X (anti-p130), from Santa Cruz Biotech., Inc.

RESULTS

The in vivo expression of E2F-4 inhibits its transcriptional activity. The results of our previous work indicated that the appearance of free E2F-4 complexes is not sufficient to trigger the activation of known E2F-responsive genes (57). This observation suggests two potential models of E2F-4 action: its transcriptional activity is either regulated by an unknown mechanism or directed at an unknown set of target genes. To distinguish between these two models, we generated stable cell lines that express the individual E2F proteins in an inducible manner. In this system, the expression of a given transgene is controlled by a transcriptional regulator, the hybrid VP16-tetracycline repressor, whose activity is inhibited in the presence of tetracycline (28). Using this approach, we selected two cell lines (called U2F2 and U2F4) that expressed either E2F-2 or E2F-4 in a strictly regulated manner (Fig. 1a). When cells were cultured in the presence of tetracycline, the levels of E2F-2 or E2F-4 in the U2F2 or U2F4 cell lines were similar to those detected in the parental cell line. In contrast, tetracycline withdrawal produced a modest increase in E2F-2 levels (between 3- and 8-fold) in the U2F2 cells and a dramatic increase in E2F-4 levels (greater than 40-fold) in the U2F4 cells.

To establish the biological properties of the induced E2F proteins, we compared the levels of E2F DNA binding activity and transcriptional activity present in parental U2tTA10, U2F2, or U2F4 cells after culturing in either the absence or the presence of tetracycline. DNA binding was assessed by screening whole-cell lysates for their ability to bind to the consensus E2F site in a gel retardation assay (Fig. 1b). Consistent with our expression data, the uninduced cells contained similar levels of E2F DNA binding activity. In contrast, induction of the U2F2 and U2F4 cell lines increased the levels of a single E2F complex, the fastest migrating, free E2F species. Supershift experiments confirmed that this increase was caused by a direct increase in the levels of either free E2F-2–DP (U2F2) or E2F-4–DP (U2F4) (Fig. 1b). These data indicate that the induction

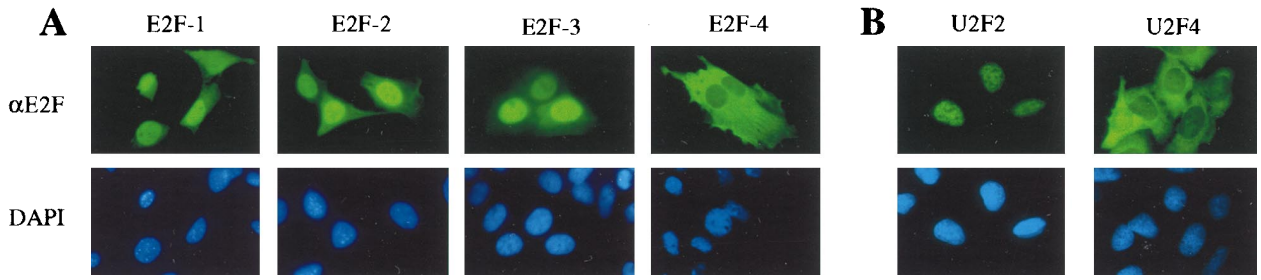


FIG. 2. Exogenously expressed E2F-4 localizes to the cytoplasm. (A) R12 cells were microinjected with CMV expression constructs encoding the indicated E2F proteins along with CMV–β-galactosidase to mark injected cells (data not shown). Immunofluorescence was detected with either control (data not shown) or anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), anti-E2F-3 (LLF3-1), or anti-E2F-4 (LLF4-1) antibodies (αE2F) and with DAPI. (B) U2F2 and U2F4 cell lines were plated on coverslips and then cultured in the absence (induced) of tetracycline for 36 h. Immunofluorescence was detected with control (data not shown) or anti-E2F-2 (LLF2-1) or anti-E2F-4 (LLF4-1) antibodies (αE2F) and with DAPI.

of E2F-2 or E2F-4 resulted in an increase in the levels of free E2F-2 or E2F-4 complex without altering the levels of the other, endogenous E2F species. The transcriptional activity of these induced E2F complexes was tested by transiently transfecting these cells with an artificial E2F-responsive reporter plasmid, called $E2F_4$-CAT, in which the expression of the CAT gene is controlled by a minimal promoter containing the E1B TATA box and four consensus E2F sites (31). This reporter was selected because it has been shown to respond with similar efficiencies to free E2F-2–DP and E2F-4–DP complexes in in vitro transcription assays (22). Although induction of the U2F2 cell line produced a relatively small increase in the levels of the E2F-2–DP complex (Fig. 1b), E2F transcriptional activity increased by more than 11-fold (Fig. 1c). In contrast, tetracycline withdrawal caused little change in the transcriptional activity in the U2F4 cell line, despite the much greater increase in the levels of the free E2F-4–DP complex (Fig. 1b and c). Since this complex can efficiently activate this reporter in vitro, we conclude that there are additional factors in vivo that prevent the activation of transcription by free E2F-4. Although this finding does not rule out the possibility that the individual E2F proteins activate different target genes in vivo, it suggests the existence of an unidentified regulatory mechanism that controls the activity of E2F-4 differently from that of E2F-2.

The transcriptional activity of E2F-4 is inhibited by its cytoplasmic localization. To determine the mechanism responsible for the inhibition of E2F-4 transcriptional activity, we investigated whether there were any obvious differences in the regulation of the individual E2F proteins. As part of this study, we examined the localization properties of E2F proteins expressed in microinjection assays (Fig. 2A). Consistent with their role as transcriptional regulators, the three pRB-specific E2F proteins, E2F-1, -2, and -3, were all detected in the nucleus. In contrast, the vast majority of the E2F-4 protein localized to the cytoplasm. Although we could not rule out the possibility that the localization of this protein was an artifact of its overexpression and presumably monomeric state, this finding strongly suggested that the differential localization of E2F-2 and E2F-4 might account for the differences in their transcriptional activity revealed in the inducible cell lines. To test this hypothesis, we used indirect immunofluorescence to examine the localization of E2F-2 or E2F-4 that had been induced in U2F2 or U2F4 cells (Fig. 2B). These proteins were expressed at considerably lower levels than in the microinjection assays; therefore, a much greater proportion formed productive E2F-DP heterodimers. However, their localization was identical to that observed in microinjection assays; E2F-2 was detected in the nucleus, while E2F-4 was predominantly cytoplasmic (Fig. 2B). These findings suggest that the transcriptional activity of exogenously expressed E2F-4 is inhibited in vivo by its cytoplasmic localization.

The localization of the endogenous E2F-4 protein is regulated in a cell cycle-dependent manner. We previously showed that the appearance of endogenous free E2F-4 complexes does not induce the transcription of known E2F-responsive genes (57). Our data now suggest that the transcriptional activity of these complexes could be blocked by their sequestration in the cytoplasmic compartment. To test this hypothesis, we compared the subcellular localizations of the endogenous E2F proteins. Initially, we used standard methods to prepare nuclear and cytoplasmic extracts for a wide variety of human cell lines. These fractions were then screened by Western blotting for the presence of either E2F-1 or E2F-4 (Fig. 3A). Regardless of the cell line, E2F-1 was consistently detected in the nuclear fraction. Similar results were also observed with E2F-2 and E2F-3 (data not shown). In contrast, the majority of the endogenous E2F-4 protein was contained within the cytoplasm. These findings confirm that the endogenous E2F proteins localize to different subcellular compartments in a manner similar to that of overexpressed E2F proteins. To reinforce these data, we also examined the localization of endogenous E2F-4 by indirect immunofluorescence. Initially, we screened for E2F-4 in an asynchronous population of U2OS cells (Fig. 3B). Within this population, the individual cells had one of two distinct staining patterns: E2F-4 was either predominantly cytoplasmic or present in both the cytoplasm and the nucleus. This dual staining pattern was observed with multiple E2F-4 monoclonal antibodies in several different cell types (data not shown).

The presence of two distinct E2F-4 staining patterns within asynchronous cells suggested that the localization of E2F-4 might change through the cell cycle. To test this idea, we compared the patterns of E2F-4 staining at different cell cycle stages. Initially, U2OS cells were released from a drug-induced G_2-M arrest and harvested at 6-h intervals for both FACS analysis and E2F immunofluorescence (Fig. 3B). Consistent with our hypothesis, E2F-4 was detected in both the nucleus and the cytoplasm in the enriched G_1 population but was predominantly cytoplasmic in cells that had entered S phase. Since the pRB pathway is known to be disrupted in most if not all tissue culture cell lines, we also examined the localization of E2F-4 in a primary human diploid fibroblast cell line, WI-38. In this case, the cells were arrested in G_0-G_1 by contact inhibition and serum starvation and then stimulated to reenter the cell cycle. The localization of E2F-4 was then examined in the peak G_0-G_1- and S-phase fractions (as judged by FACS anal-

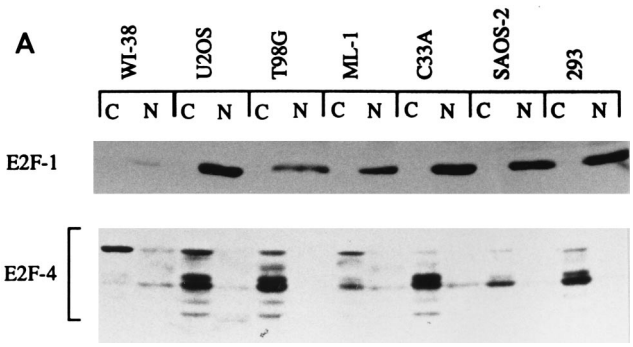
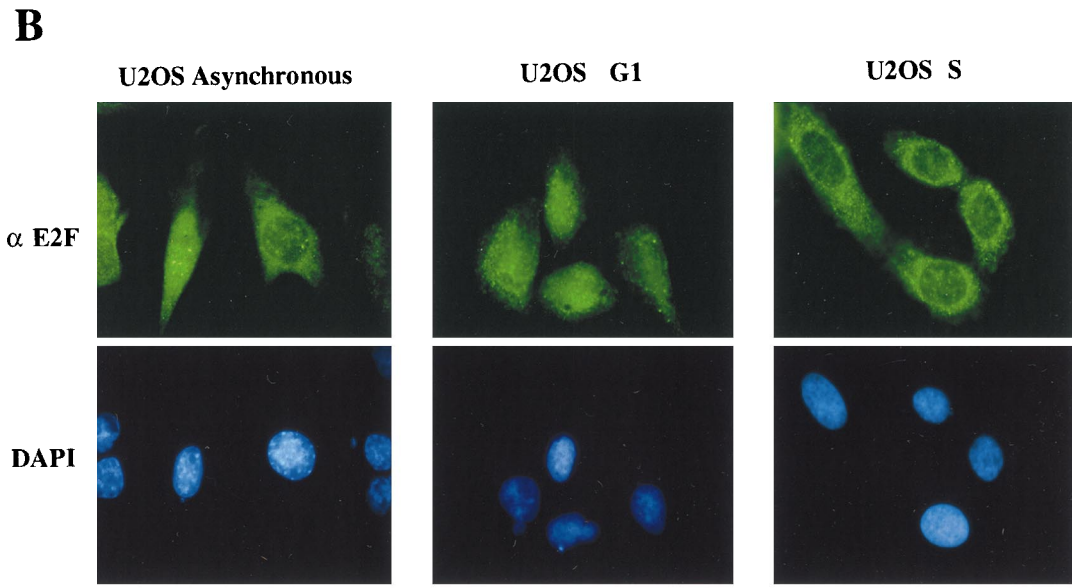
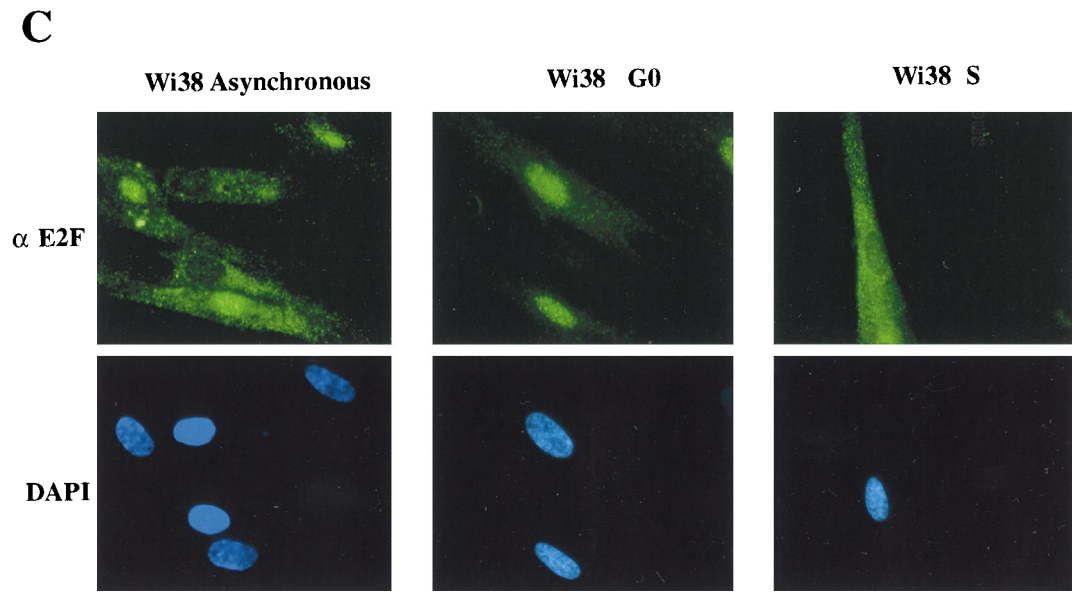


FIG. 3. Localization of the endogenous E2F-4 protein is regulated in a cell cycle-dependent manner. (A) Asynchronous WI-38, U2OS, T98G, ML-1, C33-A, SAOS-2, and 293 cells were fractionated as described in Materials and Methods. Equivalent volumes of cytoplasmic or nuclear extracts were resolved by SDS–10% PAGE and transferred to nitrocellulose. The blots were probed with antibodies that specifically recognize either E2F-1 (KH20) or E2F-4 (LLF4-1). (B) U2OS cells were cultured in the presence of thymidine (for 12 h) and then nocodazole (for 24 h) to generate a synchronized cell population. The cells were plated on coverslips and cultured in normal media. Fractions were removed every 6 h, and cell cycle staging was assessed by FACS analysis. Immunofluorescence was detected in asynchronous or peak G_1- or S-phase populations with either control (data not shown) or anti-E2F-4 (LLF4-1) antibodies (αE2F-4) and with DAPI. (C) WI-38 cells synchronized in G_0-G_1 by starvation or released into S phase by readdition of serum were subjected to indirect immunofluorescence localization of E2F-4 protein as described above.





ysis) by immunofluorescence (Fig. 3C). In this primary cell line, the cell cycle-dependent change in the subcellular localization of E2F-4 was even more pronounced. Almost all of the E2F-4 protein was retained in the nucleus in the G_0-G_1 population. However, by the time that the cells had entered S phase, the vast majority of E2F-4 was detected in the cytoplasm.

These experiments yield several important findings. First, our data suggest that E2F-4 but not E2F-1, -2, or -3 is regulated at the level of subcellular localization. Second, these changes appear to be linked to the state of cell cycle progression. In either G_0 or G_1 cells, a significant proportion of endogenous E2F-4 is retained in the nucleus, but this protein is almost entirely cytoplasmic by S phase. The dramatic alteration in the relative levels of nuclear and cytoplasmic E2F-4 could be caused by either the translocation of preexisting E2F-4 or the combined effect of the degradation of nuclear E2F-4 and the appearance of newly synthesized cytoplasmic protein. Finally, our analysis of the E2F-inducible cell lines suggests that the cytoplasmic form(s) of E2F-4 is unable to activate transcription. By extension of this logic, our data suggest that the transcriptional effects of endogenous E2F-4 are primarily exerted during G_0 and G_1.

The subcellular localization of the endogenous E2F complexes changes through the cell cycle. The identity and regulation of the endogenous E2F complexes have been analyzed in a wide variety of cell types and growth conditions (11, 13, 19, 57, 63). However, all of these studies have been conducted with whole-cell rather than nuclear extracts. We previously showed that E2F-4 comprises more than 80% of the endogenous E2F species and makes a major contribution to each of the pRB-E2F, p107-E2F, and p130-E2F complexes (57). The localization properties of this protein suggested that significant proportions of the endogenous E2F complexes exist in the cytoplasm at certain stages of the cell cycle. To address this issue, we used counterflow centrifugal elutriation to generate populations of a human lymphoma cell line, HL60, that were highly enriched in G_1 (93%), S (78%), or G_2-M (81%) cells. These cells were then fractionated to yield nuclear and cytoplasmic extracts. Initially, we used Western blotting to compare the subcellular localizations of the E2F-1 and E2F-4 proteins (Fig. 4a). Regardless of the cell cycle staging, the vast majority of the endogenous E2F-1 protein was detected in the nuclear fraction. In contrast, this experiment confirmed that the localization of E2F-4 changed through the cell cycle; this protein was present in both the nucleus and the cytoplasm during G_1 but became increasingly cytoplasmic as the proportion of G_1 cells declined. These changes strictly mirrored those detected in our immunofluorescence studies.

To determine the localization of the E2F-DP complexes, we used gel retardation assays to compare the levels of E2F DNA binding activity in the cytoplasmic and nuclear fractions of each cell cycle population (Fig. 4b). These experiments demonstrated that localization has a profound effect upon the profile of E2F complexes. In the first cell cycle fraction, comprised of 93% G_1 cells, the nucleus contained considerably more E2F activity than the cytoplasm. However, the ratio of cytoplasmic to nuclear E2F activity increased dramatically as the HL60 cells progressed through the cell cycle. This change arose from two distinct effects: a steady increase in the levels of cytoplasmic E2F activity and a dramatic reduction in the levels of nuclear E2F complexes. By the last three elutriation fractions (which were almost free of contaminating G_1 cells), less than 10% of the E2F DNA binding activity was retained in the nucleus. Consistent with our finding that E2F-4 accounts for the majority of endogenous E2F activity (57), the changes in the relative levels of nuclear and cytoplasmic E2F complexes closely mirrored the changes in the localization of the E2F-4 protein detected by either Western blotting or immunofluorescence. This experiment yielded one other critical finding: the cytoplasmic and nuclear E2F complexes migrated with different mobilities. Of the three major E2F complexes, two (labeled A and C in Fig. 4b) were predominantly cytoplasmic, while the third (labeled B) accounted for almost all of the nuclear E2F activity. This result strongly suggested that the cytoplasmic and nuclear E2F activities were generated by different E2F species.

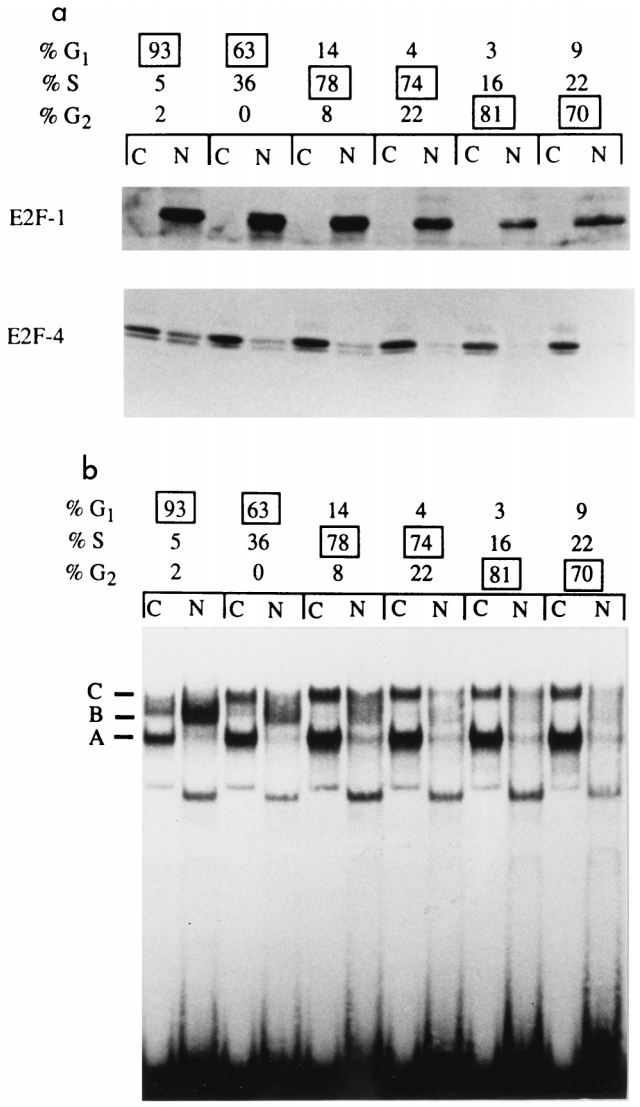


FIG. 4. Localization of the endogenous E2F-4 complexes is regulated in a cell cycle-dependent manner. Human HL60 cells were separated by centrifugal elutriation, and the cell cycle distribution was determined by FACS analysis of a proportion of the resultant populations. The remaining cells were then fractionated to yield nuclear and cytoplasmic extracts. (a) Equivalent volumes of cytoplasmic (C) and nuclear (N) extracts were subjected to Western blot analysis of E2F-1 (KH20 monoclonal antibody) and E2F-4 (LLF4-1 monoclonal antibody). (b) Cytoplasmic (C) and nuclear (N) extracts from the elutriation fractions were screened for the presence of E2F DNA binding activity in gel retardation assays.

The pRB-E2F, p107-E2F, and p130-E2F complexes localize preferentially to either the cytoplasm or the nucleus. pRB, a known tumor suppressor, and its related proteins, p107 and

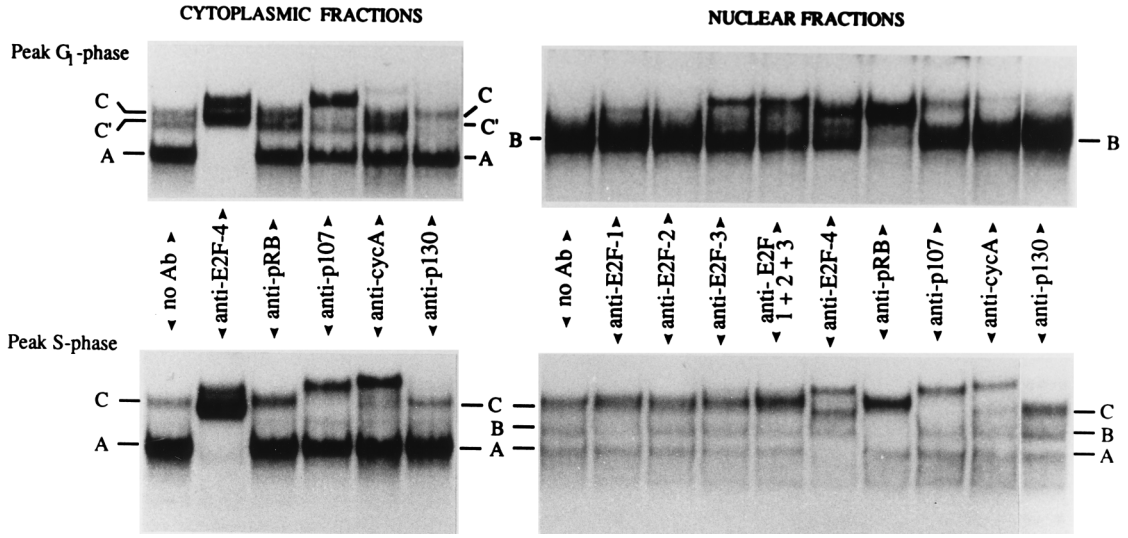


FIG. 5. The pRB-E2F, p107-E2F, and p130-E2F complexes localize preferentially to either the cytoplasm or the nucleus in actively dividing cells. To identify the constituent components, the cytoplasmic and nuclear fractions of the peak G_1-phase (93% G_1, 5% S, and 2% G_2-M) and S-phase (14% G_1, 78% S, and 8% G_2-M) populations were analyzed in gel retardation assays in the absence or presence of monoclonal antibodies (Ab) specific for the individual E2F or regulatory proteins. The anti-E2F antibodies all supershifted pocket protein-containing E2F complexes as efficiently as their free E2F-DP counterparts (57). Similar results were obtained with several other anti-E2F-1, -2, -3, or -4 monoclonal or polyclonal antisera (data not shown). cycA, cyclin A.

p130, are thought to play a pivotal role in determining the biological properties of the endogenous E2F complexes (reviewed in reference 8). Although it is widely accepted that these three proteins must regulate E2F in different ways in vivo, overexpression assays have failed to reveal any obvious differences in the properties of the pRB-E2F, p107-E2F, and p130-E2F complexes. The experiments described above raised the possibility that these complexes are preferentially sequestered in different subcellular compartments in vivo. To address this hypothesis, we used specific monoclonal antibodies to identify the components of the nuclear and cytoplasmic complexes in each cell cycle fraction. Our analysis of the peak G_1- and S-phase fractions is shown in Fig. 5.

Initially, we focused our attention on identifying the E2F species that localize to the nucleus and therefore likely participate in the transcriptional regulation of E2F-responsive genes. In G_1 cells, most if not all of the nuclear activity arose from a single complex, complex B (Fig. 4b and 5). Supershift experiments revealed that this band comprised a mixture of E2F species, of which E2F-4 was the most prevalent (Fig. 5, nuclear G_1 phase). These G_1-phase nuclear complexes also contained an associated regulatory protein. Although p107 accounted for a small subset of the nuclear E2F species, the vast majority (>90%) contained pRB. As described above, the level of nuclear E2F DNA binding activity dropped dramatically as cells entered the S phase. Even though present at low levels, it was important that we identify the nuclear species. To achieve this goal, we used fivefold-higher levels of the S-phase nuclear extract in gel retardation assays (Fig. 5, nuclear S phase). Under these conditions, we were able to demonstrate that the constituent E2F activity was generated by three distinct E2F complexes. First, the S-phase cells contained a low level of the pRB-E2F complex (complex B). The reduction in the level of this species (relative to that in G_1-phase cells) was entirely consistent with the known dissociation of the pRB-E2F complex at the G_1-S transition (reviewed in reference 8). Whether the remaining pRB-E2F complexes are a true component of S-phase cells or whether they are derived from the low level of contaminating G_1-phase cells is unclear. The other two species, complexes A and C, were both identified as E2F-4 complexes. This finding is consistent with our previous finding that the nucleus contained low levels of E2F-4 protein at this stage of the cell cycle (Fig. 4a). Further analysis identified these species as free E2F-4 (complex A) and p107–cyclin A–E2F-4 (complex C) (Fig. 5).

Having characterized the nuclear E2F activity, we turned our attention to the cytoplasmic complexes. Regardless of the cell cycle stage, we found that E2F-4 accounted for all of the cytoplasmic activity (Fig. 5). G_1 cells contained high levels of three different E2F-4 complexes, A, C, and C′. Consistent with our analysis of nuclear E2F activity, the A and C species were identified as free E2F-4 and p107–E2F-4, respectively (Fig. 5, cytoplasmic G_1 phase). The remaining complex, C′, was specifically recognized by antibodies against p130. Although pRB-E2F was present at high levels in the nuclei of G_1 cells, this complex was absent from the cytoplasmic fraction. Upon S-phase entry, we detected changes in the cytoplasmic complexes that were consistent with the known cell cycle regulation of p130-E2F and p107-E2F. Specifically, p130–E2F-4 disappeared, while the level of p107–E2F-4 increased steadily. Notably, this p107 complex was now bound to the S-phase kinase, cyclin A-CDK2, despite being located in the cytoplasm (60). Finally, the levels of cytoplasmic free E2F-4 also were increased considerably in cells that had entered S phase.

In summary, our analysis of the cell cycle fractions confirms that the endogenous E2F complexes are regulated at the level of subcellular localization. Most importantly, the individual E2F species were found to be preferentially located in either the cytoplasm or the nucleus. The vast majority of the nuclear E2F activity is generated by a single species, the pRB-E2F complex, that is present at high levels in the G_1 population. In contrast, the vast majority of the remaining E2F complexes, p130-E2F, p107-E2F, and free E2F, is predominantly located in the cytoplasm. Consistent with previous studies, the p130-E2F complex is detected only during the early stages of the cell cycle. In contrast, the levels of both the p107-E2F and the free E2F species increase as cells progress through the cell cycle, resulting in a steady increase in the level of cytoplasmic E2F

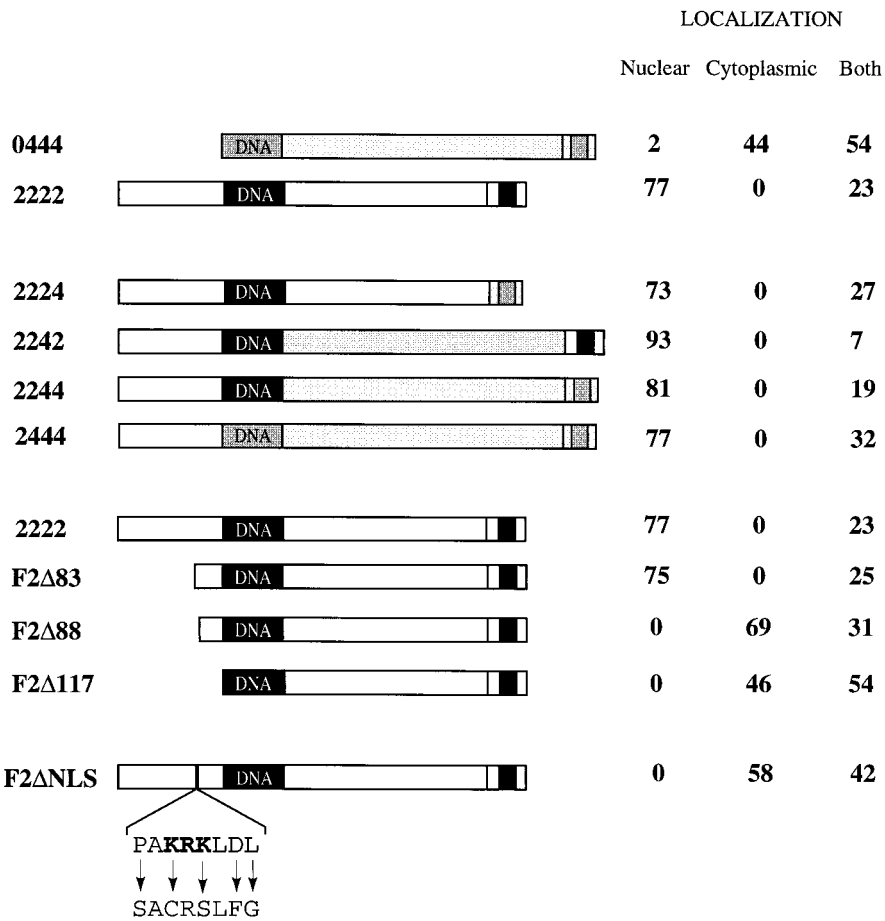


FIG. 6. Localization properties of chimeric E2F proteins. U2OS cells were transiently transfected with 1 μg of the indicated CMV-E2F constructs along with CMV–β-galactosidase to mark transfected cells. Immunofluorescence was detected as described in Materials and Methods with anti-E2F-2, anti-E2F-4, and anti-β-galactosidase antibodies (data not shown). Two hundred β-galactosidase-positive cells were scored for E2F protein localization, and the percentage of cells displaying exclusively nuclear, exclusively cytoplasmic, or both nuclear and cytoplasmic staining was determined.

activity. In each case, the cytoplasmic localization of these three E2F species correlates with the presence of E2F-4. This finding directly supports our conclusion that cytoplasmic localization is a particular property of E2F-4 and not of E2F-1, -2, or -3. However, our data also indicate that E2F-4 is not sufficient to ensure cytoplasmic localization, since this E2F family member is also the major E2F component of the nuclear pRB-E2F complex.

E2F-1, -2, and -3 contain an NLS that is absent in E2F-4. Our data indicate that a significant proportion of the endogenous E2F species is localized in the cytoplasm and not the nucleus. This observation suggests that subcellular localization could have a profound influence upon the biological properties of the individual E2F complexes. To address this issue, we need to identify the molecular mechanism(s) that controls this process. Our previous experiments suggested that this process may be determined by two distinct factors. First, localization of the E2F complexes appears to be partially dependent upon the localization properties of the constituent E2F proteins. Complexes containing E2F-1, -2, or -3 are exclusively nuclear, while the cytoplasmic localization of the endogenous E2F complexes seems to be dependent upon the presence of E2F-4. Second, although monomeric E2F-4 is predominantly cytoplasmic, this E2F family member participates in both cytoplasmic and nuclear E2F complexes in vivo. This fact suggests that the localization of the E2F-4 may be altered by the presence of one or more of its associated proteins.

Our first goal was to identify the signal(s) that establishes the localization of the monomeric (i.e., non-DP-associated) E2F proteins. To address this issue, we generated a series of chimeras in which one or more domains (the N terminal, DNA binding, dimerization-transactivation, or pRB, p107, or p130 binding) were exchanged between these proteins (Fig. 6). The resultant mutants were named to indicate the origin of each domain; for example, 2224 contains the N-terminal, DNA binding, and dimerization-transactivation domains of E2F-2 and the pRB, p107, and p130 binding domain of E2F-4. The localization properties of each chimera were determined by indirect immunofluorescence after transient transfection into U2OS cells (Fig. 6). Consistent with the results of our microinjection studies, transiently transfected E2F-2 was predominantly nuclear, while exogenously expressed E2F-4 was preferentially localized to the cytoplasm. We next examined the localization of mutants in which various functional domains of E2F-2 had been replaced with the corresponding region of E2F-4 (2224, 2242, 2244, and 2444). In each case, these mutants localized to the nucleus with an efficiency similar to or greater than that of the parental E2F-2 protein (Fig. 6). In fact, it was possible to exchange all of the E2F-2 sequences from the start of the DNA binding domain to the end of the protein

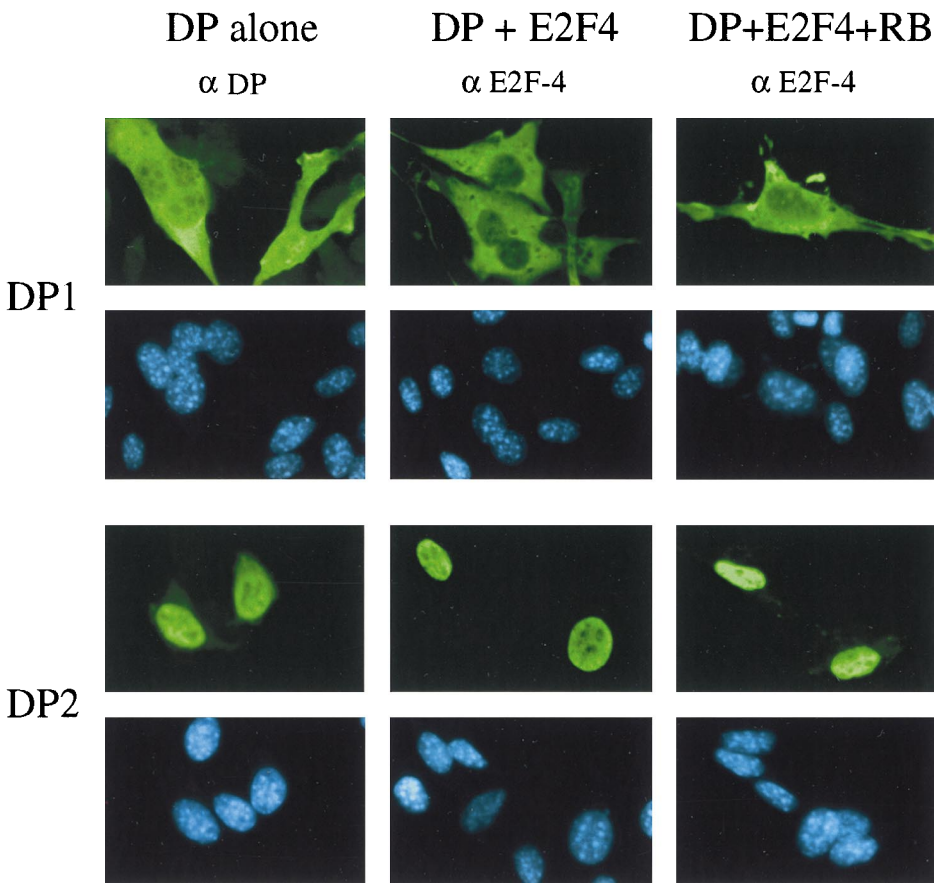


FIG. 7. Associated proteins modulate the localization of E2F-4. Murine 3T3 fibroblasts were microinjected with CMV expression constructs encoding HA-tagged human DP-1 or DP-2 either alone or in combination with the indicated plasmids. CMV–β-galactosidase was included to mark injected cells. Immunofluorescence was detected as described in Materials and Methods with anti-E2F-4, anti-HA, and anti-β-galactosidase antibodies.

(amino acids 118 to 437) with the corresponding region of E2F-4 (amino acids 2 to 416) without impairing nuclear import. We therefore conclude that E2F-2 contains within its N-terminal domain an NLS that, when fused to E2F-4, can induce this normally cytoplasmic protein to enter the nucleus.

To precisely map the E2F-2 NLS, we examined the localization properties of N-terminal deletion mutants (Fig. 6). Deletion of the first 83 amino acids of E2F-2 did not affect its nuclear localization. However, deletion of an additional 5 amino acids (to generate F2Δ88) caused the protein to shift from being predominantly nuclear (75% of cells) to being predominantly cytoplasmic (69% of cells) in a manner similar to that of E2F-4. Deletion of additional N-terminal sequences (F2Δ117) did not further increase cytoplasmic localization. We therefore conclude that E2F-2 contains a single NLS that encompasses residues 83 and 88. This region encompasses a short motif, PAKRKLDL (residues 84 to 91), that is closely related to the NLS of the c-myc protein (17). Moreover, this sequence is highly conserved in the other nuclear E2F proteins, E2F-1 (PVKRRLDL) and E2F-3 (PAKRRLEL), and represents the only region of homology in the N-terminal domain of these three E2F family members. To directly demonstrate the importance of this domain, we used site-directed mutagenesis to alter the basic residues within this motif. When tested in the transient transfection assay, the resulting mutant (named F2ΔNLS) localized to the cytoplasm in a manner similar to that of either F2Δ88 or E2F-4 (Fig. 6). We therefore conclude that the nuclear localization of E2F-1, -2, and -3 is mediated by the P(A/V)KR(K/R)L(D/E)L motif.

In addition to the mutants shown in Fig. 6, we tested the localization properties of several other chimeric and deletion mutants (data not shown). The localization properties of these mutants did not yield any evidence for the existence of a nuclear export signal (NES) within E2F-4. Although we cannot rule out the possibility that E2F-4 contains a weak NES, our data argue that the predominant cytoplasmic localization of this protein results from the lack of an NLS. This conclusion is supported by the finding that E2F-2 mutants that lack the NLS (F2Δ88, F2Δ117, and F2ΔNLS) localize to the cytoplasm with an efficiency similar to that of E2F-4.

Associated proteins can mediate the nuclear localization of E2F-4. Our data suggest that monomeric E2F-4 is unable to enter the nucleus because it lacks an NLS. However, our analysis of the cellular E2F complexes indicates that E2F-4 participates in both cytoplasmic and nuclear E2F complexes in vivo (Fig. 5). What mediates the localization of these nuclear E2F-4 complexes? One likely possibility is associated proteins. Indeed, our analysis of endogenous E2F complexes revealed a clear difference in the localization of the E2F-4 complexes that were associated with p107 or p130 (predominantly cytoplasmic) rather than pRB (exclusively nuclear) (Fig. 5). To determine whether any of the known associated proteins could influence E2F-4 localization, we used microinjection assays to compare the localizations of E2F-4 that had been expressed in

either the absence or the presence of these proteins (Fig. 7). Since functional E2F activity requires the formation of an E2F-DP heterodimer, we initiated this study by examining the effect of the known E2F heterodimeric partners, DP-1 and DP-2. The monomeric DP-1 protein was found to be predominantly cytoplasmic, and this protein caused little or no change in the nuclear uptake of the coexpressed E2F-4 protein. In contrast, E2F-4 became almost exclusively nuclear when coexpressed with DP-2, and this localization clearly reflected the nuclear localization properties of the monomeric DP-2 protein. We therefore conclude that the association of DP-2 but not of DP-1 is sufficient to trigger the nuclear localization of E2F-4.

Since our in vivo studies indicated that the association of pRB but not of p107 or p130 correlates with the nuclear localization of E2F-4, we also investigated whether pRB is able to induce nuclear uptake of the cytoplasmic E2F-4–DP-1 complex (Fig. 7). When coexpressed in microinjection assays, pRB had no effect upon the localization of either the E2F-4–DP-1 (predominantly cytoplasmic) or the E2F-4–DP-2 (nuclear) species. Although we cannot rule out the possibility that pRB plays some role in determining the localization of E2F-4 in vivo, these experiments indicate that it is not sufficient to induce the nuclear uptake of this E2F family member. In contrast, our data suggest that the differential localization properties of the two DP proteins could provide the underlying basis for the differential localization of the individual E2F-4 complexes.

The nuclear localization of the individual E2F proteins correlates with their ability to activate transcription. The transcriptional role of the individual E2F species has been the focus of extensive study. Overexpression experiments have suggested that each of the free E2F-DP complexes induces the activation of E2F-responsive genes, while complexes containing pRB, p107, or p130 repress their transcription. In this study, we have shown that a significant proportion of these complexes is localized in the cytoplasm and not in the nucleus in vivo. This observation raises clear questions about how the subcellular localization of these species affects their ability to regulate transcription. To directly address this issue, we examined the functional properties of various nuclear or cytoplasmic versions of the E2F-2 and E2F-4 proteins. Initially, we wanted to verify that the construction of these mutants did not disrupt their ability to dimerize with DP and/or bind to DNA. To this end, the relevant chimeric and deletion mutants were transiently transfected into C33-A cells along with pCMV-DP-1, and whole-cell extracts were screened in gel retardation assays (Fig. 8a). In each case, we recovered a significant proportion of E2F DNA binding activity, confirming the structural integrity of these proteins. We then examined the transcriptional activity of these mutants by transiently transfecting their eukaryotic expression vectors into C33-A cells along with the E2F-responsive reporter plasmid $E2F_4$-CAT and an internal control for transfection efficiency, pRSV-luciferase (Fig. 8b). That these proteins were expressed at similar levels was confirmed by Western blotting (data not shown). Consistent with our analysis of the E2F-inducible cell lines (Fig. 1), E2F-2 activated the transcription of the reporter with a much greater efficiency than E2F-4. However, deletion or mutation of the E2F-2 NLS (mutants F2Δ88 and F2ΔNLS) significantly reduced its transcriptional activity. In contrast, the transcriptional activity of E2F-4 was significantly increased when this protein was fused in frame to either the N-terminal domain of E2F-2 (mutant 2444) or the NLS of the simian virus 40 large T antigen (mutant F4+NLS). In fact, the transcriptional activity of the latter mutant significantly exceeded that of the wild-type E2F-2 protein.

In every case, the transcriptional activity of the deletion and chimeric proteins correlated with their localization rather than with the origin (either E2F-2 or E2F-4) of their transactivation domain. This result suggests that the mutants have similar capacities to activate transcription but that these capacities are restricted by their ability to localize to the nucleus. If this hypothesis is true, we would predict that the association of DP-2, which was sufficient to induce the nuclear uptake of E2F-4 (Fig. 7), should active the transcriptional potential of the cytoplasmic mutants. To test this idea, we compared the transcriptional activities of the mutants after cotransfection with DP-2. The presence of DP-2 was sufficient to mediate the nuclear localization of all of the mutants (data not shown). Under these conditions, the chimeric and deletion mutants activated transcription with similar efficiencies (Fig. 8b). We therefore conclude that E2F-2 and E2F-4 have similar potentials to activate transcription but that these activities are dependent upon their subcellular localization.

DISCUSSION

The cellular transcription factor E2F plays a critical role in directing the cell cycle-dependent transcription of the genes that control cellular proliferation. It is well established that E2F activity arises from the combined properties of multiple E2F-DP heterodimers. However, despite extensive study, the precise role of the individual E2F-DP species is not well understood. We have focused our attention on one member of the E2F family, E2F-4, for the following reasons. First, this E2F protein accounts for the vast majority of the endogenous E2F complexes, including most of pRB-, p107-, or p130-associated E2F activity (40, 57). This observation suggests that E2F-4 plays a pivotal role in establishing the properties of endogenous E2F activity. Second, free E2F-4–DP complexes accumulate early in the cell cycle but are unable to induce the transcription of known E2F-responsive genes (57). This finding led us to propose two possible, although not mutually exclusive, models of E2F-4 action: the transcriptional activity of E2F-4 either is directed at an unknown set of target genes or is regulated by a previously unknown mechanism.

To distinguish between these two models, we generated a stable cell line that showed inducible E2F-4 DNA binding activity but, surprisingly, did not show inducible E2F transcriptional activity. Although this result prevented us from addressing whether E2F-4 activates a novel set of target genes in vivo, this observation led us to the finding that monomeric E2F-4 is sequestered in the cytoplasm. The transcriptional activity of this protein was rescued by the induction of its nuclear uptake. Moreover, under these conditions, E2F-4 could activate transcription with an efficiency similar to that of the other E2F family members. Taken together, these data led us to conclude that E2F-4 may activate transcription in vivo but, in contrast to E2F-1, -2, and -3, activation by E2F-4 is dependent upon an additional step that mediates the nuclear localization of this protein.

These findings suggest the need to reevaluate previous studies concerning the biological properties of the individual E2F proteins. Overexpression studies have revealed several differences in both the transcriptional and the cell cycle effects of E2F-4 and of E2F-1, -2, and -3 (20, 36, 52). For example, Lukas et al. (52) have shown that microinjection of E2F-1, -2, or -3 is sufficient to induce quiescent cells to initiate DNA synthesis, whereas that of E2F-4 is unable to trigger this event. That study and another study (20, 52) further showed that overex-

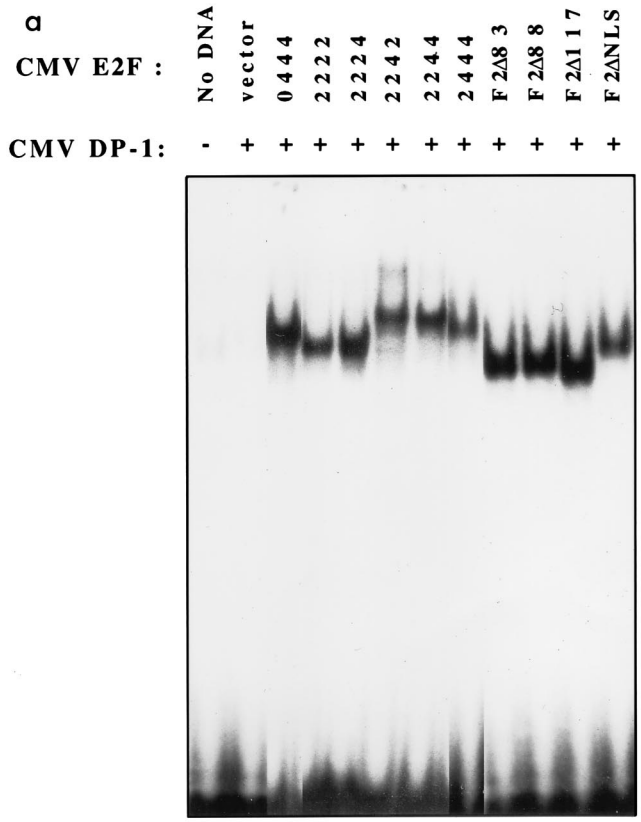
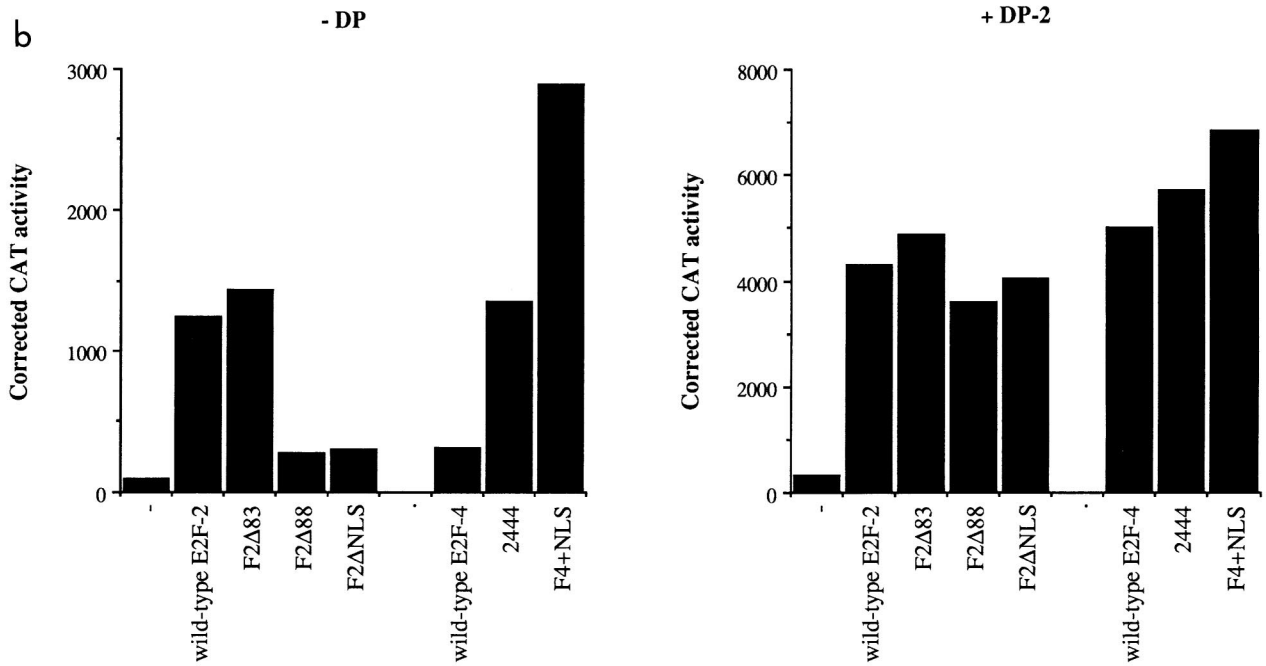


FIG. 8. Localization of E2F proteins determines their ability to activate transcription in vivo. The properties of the chimeric E2F molecules were assayed by transfection in human C33-A cells. (a) DNA binding activity was determined by transfection of 10 μg of the relevant CMV-E2F expression constructs together with 10 μg of CMV–HA–DP-1. Whole-cell extracts were generated as described previously (57) and assayed for E2F DNA binding activity in gel shift assays (see Materials and Methods). (b) The transcriptional activity of the chimeric E2F molecules in vivo was tested by cotransfection of 200 ng of the relevant CMV-E2F construct, 4 μg of $E2F_4$-CAT, or 2 μg of pRSV-luciferase in the presence or absence of 1 μg of CMV–HA–DP-2. Normalized CAT values were used as a measure of transcriptional induction.



pression of E2F-1 but not of E2F-4 can override the G_1 arrest induced by either p16 or p21. Our data suggest that these biological differences arise as a direct consequence of the differential localization of these overexpressed proteins. In addition, our data also affect the interpretation of E2F-1 mutants. Previous studies identified a short motif within the N terminus of E2F-1, -2, and -3 that mediates their cyclin A-CDK2 binding properties (1, 43). This kinase is thought to ensure the S-phase-specific inactivation of the E2F-1–DP, E2F-2–DP, and E2F-3–DP species through its interaction with the E2F subunit and phosphorylation of the associated DP protein (21, 22, 44, 72). Interestingly, our mapping studies indicated that this motif is

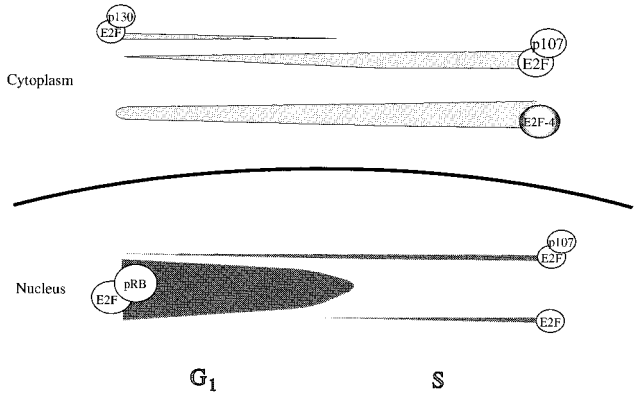


FIG. 9. Summary of the nuclear and cytoplasmic E2F complexes throughout the cell cycle.

also responsible for mediating the nuclear localization of these three E2F proteins. At this point, the biological consequences of the colocalization of the cyclin A binding and nuclear localization functions of E2F-1, -2, and -3 are unclear. It will be important to reevaluate studies that have used deletion mutagenesis to assess the role of cyclin A binding in E2F regulation (for example, see reference 44), given our finding that these mutations also disrupt the nuclear localization of the E2F proteins.

Cell cycle regulation of endogenous E2F-4 localization. The localization properties of E2F-4 are only relevant if they extend to the endogenous protein. Both subcellular fractionation and immunofluorescence confirmed that a significant proportion of the endogenous E2F-4 protein is localized in the cytoplasm. Moreover, the relative levels of nuclear and cytoplasmic E2F-4 proteins alter dramatically as cells progress through the cell cycle. While cytoplasmic E2F-4 protein exists throughout the cell cycle, nuclear E2F-4 protein is primarily detected during G_0 and G_1. These observations strongly suggest that the regulation of E2F-4 subcellular localization plays a critical role in controlling the activity of the endogenous E2F-4 complexes.

Our previous studies showed that E2F-4 accounts for a large proportion of the endogenous E2F complexes, including the majority of pRB-, p107- or p130-associated E2F activity (57). This result raised the possibility that a significant proportion of these complexes might localize to the cytoplasm at one or more stages of the cell cycle. We addressed this issue by examining the localization of the individual E2F complexes in actively dividing HL60 cells (Fig. 9). As predicted by our overexpression experiments, the endogenous free E2F-4 complexes were found to be predominantly cytoplasmic. Surprisingly, we did not detect any obvious redistribution of free E2F-4 from the cytoplasm to the nucleus in any of the cell cycle fractions. This observation suggests that we can rule out a model in which the activation of E2F-responsive genes is triggered by the wholesale nuclear import of free E2F-4.

The p107-E2F and p130-E2F complexes, which consist primarily of E2F-4, are also predominantly cytoplasmic in actively dividing cells. As with free E2F-4, we did not detect any obvious change in the ratio of nuclear to cytoplasmic forms of these species at any particular stage of the cell cycle. This finding seems at odds with the recent report that coexpression with p107 or p130 can increase the nuclear uptake of E2F-4 in transient transfection assays (51). We have conducted similar experiments and, under these overexpression conditions, have also found that p107 and p130 can trigger the nuclear localization of E2F-4 (unpublished data). Given the difference between transient transfection and in vivo assays, we assume that the overexpression of these proteins must somehow perturb the mechanism(s) that establishes their subcellular localization. The analysis of this difference may provide the key to understanding how the localization of the endogenous E2F complexes is regulated. As with the E2F proteins, these observations suggest that we need to be cautious in interpreting the results of experiments performed with overexpressed p107 or p130 protein.

Consistent with the role of E2F as a cellular transcription factor, some of the endogenous E2F complexes were detected in the nucleus. However, supershift experiments indicated that most of the nuclear E2F activity was generated by a single E2F species, the pRB-E2F complex. In contrast to the other E2F complexes, pRB-E2F was found to be exclusively nuclear despite the fact that E2F-4 was its major component. The nuclear pRB-E2F complex was present at high levels during G_1 but not in S-phase cells. The disappearance of this species correlates closely with the known timing of phosphorylation of pRB and is sufficient to account for the reduction in nuclear E2F-4 protein levels at later stages of the cell cycle.

Our finding that the transcriptional potential of a given E2F complex is dependent upon its nuclear localization strongly suggests that the endogenous E2F-responsive genes are regulated by the subset of E2F species that can localize to the nucleus. We therefore conclude that the transcriptional regulation of E2F-responsive genes in actively dividing cells is largely dependent upon the properties of the pRB-E2F complex. Overexpression studies have indicated that the pRB-E2F complex mediates the basal repression of E2F-responsive genes through sequestration of other transcription factors that are bound at the promoter (2, 10, 62, 65, 69, 70). Therefore, the current model of E2F action suggests that dissociation of the pRB-E2F complex leads to the induction of target genes by both relieving basal repression and releasing free, transcriptionally active E2F. Our observation that the pRB-E2F complex was present in the nucleus at high levels during G_1 strongly supports the notion that the dissociation of the pRB-E2F complex contributes to the induction of E2F-responsive genes by relieving their repression. However, although we did detect low levels of free E2F activity in the nuclei of S-phase cells, the dissociation of the pRB-E2F complex did not yield a commensurate increase in the levels of nuclear free E2F activity. Moreover, supershift experiments argued that the free E2F-4 complexes were selectively lost from the nuclear compartment after release from pRB. It is unclear whether this free E2F-4 is translocated to the cytoplasm or is subject to ubiquitin-mediated proteolysis (30, 37). Whatever the mechanism, dissociation of the high levels of the nuclear pRB-E2F complex resulted in surprisingly little nuclear free E2F activity. The reduction in the levels of nuclear E2F activity was consistent with in vivo footprinting studies that demonstrated that the E2F-responsive elements of known target genes are only occupied during G_0-G_1 (75). We assume that the low levels of nuclear free E2F species must contribute to the activation of E2F-responsive genes. However, our data suggest that we need to reconsider the relative contributions that pRB-E2F repression and free E2F activation make to the transcriptional regulation of E2F-responsive genes.

The pRB-E2F, p107-E2F, and p130-E2F complexes are preferentially sequestered in different subcellular compartments. Analysis of both human tumors and mutant mouse strains suggests that pRB plays a critical role in the regulation of cellular proliferation that cannot be fulfilled by either p107 or p130 or both. Our investigation of endogenous E2F activity has

led to the unexpected finding that the p107-E2F and p130-E2F species are preferentially localized in a different subcellular compartment than the pRB-E2F complex in actively dividing cells. As discussed above, the nuclear localization of the pRB-E2F complex suggests that this complex plays a major role in repressing the transcription of E2F-responsive genes prior to the G_1-S transition. In contrast, it seems likely that the cytoplasmic retention of the p130-E2F and p107-E2F species significantly reduces the ability of these complexes to repress transcription. These data raise the possibility that the different localization properties of these species contribute to the distinct biological consequences of pRB, p107, and p130 action.

At this point, we have restricted our analysis of E2F complexes to actively dividing cells. The resultant findings raise questions about the role of E2F in quiescent cells. It has been shown that p130-E2F is the sole E2F species in G_0 cells, and it was therefore assumed that this complex would mediate the repression of E2F-responsive genes in this setting (13, 57, 64). Given the findings of this study, it will be important to determine the localization of p130-E2F in quiescent cells. Similarly, when these cells are stimulated to reenter the cell cycle, the pRB-E2F complex accumulates at high levels during S, G_2, and M (57, 64). If nuclear, this complex may contribute to the down-regulation of E2F-responsive genes after the G_1-S transition. Alternatively, it is possible that localization is regulated by cell cycle staging rather than being an intrinsic property of the individual E2F complexes and that the pRB-E2F complex will be found to be cytoplasmic when cells reenter the cell cycle from G_0. Further analysis of quiescent cells should lead to a better understanding of both the mechanism and biological consequences of the subcellular localization of the individual E2F complexes.

In addition to E2F, pRB, p107, and p130 have been reported to regulate many other transcription factors (reviewed in reference 8). Clearly, our data only address the localization of the pRB-E2F, p107-E2F, and p130-E2F species. Consistent with our findings, immunofluorescence studies have confirmed that the endogenous pRB protein localizes to the nucleus (56). In contrast, we are unaware of any analysis of the localization of the endogenous p107 and p130 proteins. It is therefore unclear whether significant proportions of these proteins are localized in the cytoplasm in vivo or whether this property is specific to the pool of p107 and p130 that is bound to E2F. A comparison of the relative levels and localization of E2F-associated versus total p107 and p130 proteins may provide critical information about the relative importance of E2F in either p107 or p130 function.

Potential mechanisms of E2F-4 localization. There are two mechanisms that could account for the cytoplasmic localization of the E2F-4 protein, the lack of an NLS or the presence of an NES. Although we cannot rule out the possibility that E2F-4 contains a weak NES, our analysis of both E2F-4 deletion mutant and chimeric proteins suggests that the predominant cytoplasmic localization of this protein results from its lack of an NLS. In contrast, this deletion strategy identified a short motif, P(A/V)KR(K/R)L(D/E)L(D/E), that is both necessary and sufficient to mediate the nuclear localization of E2F-1, -2, and -3. The presence or absence of these motifs explains the localization properties of the monomeric E2F proteins. However, E2F-4 complexes are able to enter the nucleus in some situations, most noticeably when bound to pRB. How this occurs is unclear, but associated proteins almost certainly play a role in this process. Our preliminary studies suggest that coexpression of pRB is unable to induce the nuclear uptake of E2F-4. Instead, our experiments implicate DP proteins in the control of E2F-4 localization. While interaction with DP-1 does not significantly alter the localization of E2F-4, DP-2 binding is sufficient to drive E2F-4 into the nucleus. The monomeric DP proteins also localize to different compartments of the cell, and this localization correlates with the presence of a stretch of basic residues within DP-2 that is absent in DP-1. These observations suggest that the DP proteins act to establish the localization of the associated E2F-4 complex. Future experiments will focus on comparing the DP components of the nuclear and cytoplasmic E2F-4 complexes.

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