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E2F-3 accumulation is regulated by polypeptide stability

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E2F is a complex family of transcription factors which appears to regulate the transcription of genes required for the S phase of the mammalian cell cycle. In the present work, we have examined the mechanisms regulating E2F-3 accumulation in mouse fibroblasts. We have determined that E2F-3 DNA binding activity is restricted to the G_1/S transition and S phase in both normal BALB/c-3T3 fibroblasts and in an SV40 virus-transformed BALB/c-3T3 derivative. Immunoblot analysis indicates that G_0 and G_1 cells have little or no E2F-3 polypeptide and that the increase in the DNA binding activity of E2F-3 at the G_1/S boundary is reflected by an increase in total E2F-3 protein. In contrast to the E2F-3 polypeptide, RNAse protection assays demonstrate that the E2F-3 mRNA is clearly present in G_0 and G_1 cells. Finally, pulse/chase experiments indicate that the half-life of E2F-3 is approximately 40-fold greater in cells blocked in S phase relative to asynchronously growing cells. Together, these results indicate that the accumulation E2F-3 at S phase may be regulated, at least in part, at the level of protein stability.

Keywords: cell cycle; E2F; protein stability; Rb

Introduction

The E2F family of transcription factors appears to regulate gene expression during the mammalian cell cycle through interactions with members of the Rb family. The E2F/Rb families regulate promoters which are transcriptionally activated at the G_1/S boundary and include a number of cell cycle regulatory proteins, as well as gene products which are required for DNA synthesis (reviewed in reference Slansky and Farnham, 1996). E2F sites within E2F-regulated promoters may contribute regulation either by cell cycle-specific repression or by cell cycle-specific activation (or both) depending on the promoter context. For example, an E2F binding site in the *B-myb* promoter acts as a transcriptional repressor element during G_0 and early G_1 (Lam *et al*., 1995; Lam and Watson, 1993), whereas an E2F binding site in the *dihydrofolate reductase* promoter functions as a positive element during late G_1 and S phase (Blake and Azizkhan, 1989; Fry *et al*., 1997). Members of the Rb family have been shown to possess a strong transcriptional repression activity when tethered to a promoter (Adnane *et al*., 1995; Sellers *et al*., 1995; Weintraub *et al*., 1992, 1995), and thus, cell cycle-specific repression conferred by an E2F site is likely mediated by trimeric E2F complexes which contain one member of the Rb family. Cell cycle-specific activation by E2F is likely mediated by dimeric E2F which possesses a potent transcriptional activation domain (Helin *et al*., 1992; Kaelin *et al*., 1992; Shan *et al*., 1992), as well as the ability to bend DNA (Cress and Nevins, 1996), both of which may contribute to promoter activation.

Cellular E2F activity is formed by heterodimers of one member of the E2F family and one member of the DP family or heterotrimers which also include one member of the Rb family (for a review see reference Slansky and Farnham, 1996). At present the E2F family is known to contain at least five members, the DP family contains two members, and the Rb family contains three members. Heterodimers containing E2F-4 and E2F-5 appear to form trimeric complexes with all three members of the Rb family, whereas E2F-1, -2 and -3 are more selective and interact with pRb alone (Beijersbergen *et al*., 1994; Dyson *et al*., 1993; Ginsberg *et al*., 1994; Hijmans *et al*., 1995; Ikeda *et al*., 1996; Lees *et al*., 1993; Moberg *et al*., 1996). Recent evidence suggests that it is likely that different members of the E2F family may perform distinct functions in an organism. First, mice nullizygous for the E2F-1 gene display a strong predisposition to hyperplastic growth and tumor formation (Field *et al*., 1996; Yamasaki *et al*., 1996). These results suggest that other members of the E2F family cannot compensate for the functional loss of E2F-1. Second, it has been demonstrated that the overexpression of specific E2F family members leads to the activation of subsets of E2F-regulated promoters. For instance, expression of E2F-1 with a recombinant adenovirus stimulates the transcription of E2F-regulated genes such as *thymidylate synthase* and *ribonucleotide reductase*, while other well characterized E2F-regulated genes such as *DHFR* and *thymidine kinase*, were not affected (DeGregory *et al*., 1995). Finally, our recent work has demonstrated that different E2F family members have different DNA binding specificities and that E2F family member-specific DNA sequences placed within the context of a *B-myb* promoter appear to have different *cis*-regulatory activities (Tao *et al*., 1997).

Previous work has demonstrated various modes of regulation for the members of the E2F family during the course of a mammalian cell cycle. Specifically, when serum-starved rodent fibroblasts are stimulated to enter DNA synthesis it is observed that the mRNAs for both E2F-1 and E2F-2 are sharply upregulated at the G_1/S boundary, whereas the mRNAs for both E2F-3 and DP-1 are clearly present in the quiescent cells

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and show only a gradual and modest (4–5-fold) increase following serum treatment (Slansky and Farnham, 1996). Likewise, when resting human lymphocytes are stimulated to proliferate there is a dramatic upregulation of the E2F-1 and E2F-2 mRNAs, however, the mRNAs for E2F-3 and E2F-4 are already abundant in the unstimulated cells and show only a modest increase following PHA treatment (Moberg *et al*., 1996). Finally, we have examined the regulation of the E2F family expression in differentiating HL60 cells (Kassatly and Cress, manuscript submitted). We find that both E2F-1 and E2F-2 are downregulated at the transcriptional level in the differentiating HL60 cells. In contrast, the messages for E2F-3 and E2F-4 are abundant in differentiated HL60 cells and yet the E2F-3 protein is dramatically downregulated. Together these results suggest that E2F-3 accumulation may be primarily regulated post-transcriptionally.

In the current work we have utilized BALB/c-3T3 fibroblasts (BALBs) and an SV40 virus-transformed version of these cells (SV40s) as a system to examine the post-transcriptional regulation of E2F-3 activity. We have determined that E2F-3 DNA binding activity is restricted to the G_1/S transition and S phase in both normal and transformed cells. Immunoblot analysis with E2F-3 specific antibodies indicate that the increase in the DNA binding activity of E2F-3 at the G_1/S boundary is reflected by an increase in total E2F-3 protein. Finally, pulse-chase experiments and immunoprecipitations indicate that the half-life of E2F-3 increases 40-fold during S phase relative to its half-life in asynchronously growing cells. Together these results indicate that the accumulation of the E2F-3 polypeptide is regulated, at least in part, at the level of protein stability.

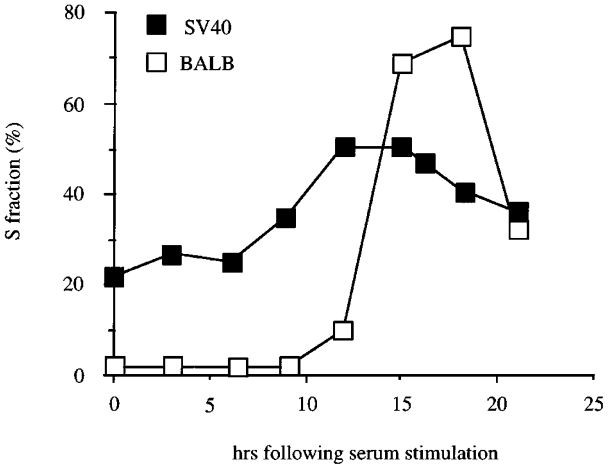


Figure 1 SV40 virus-transformed BALB/c-3T3 fibroblasts rapidly enter DNA synthesis following growth stimulation. BALB/c-3T3 fibroblasts (BALBs) and an SV40 virus-transformed derivative (SV40s) were grown and synchronized as described in Materials and methods. The fraction of total cells in S phase was estimated from DNA content and is plotted for each cell line as a function of time following serum addition

Results

E2F-3 accumulation peaks during DNA synthesis in both normal and transformed fibroblasts

In order to examine the potential mechanism(s) regulating E2F-3 accumulation we have examined the expression of E2F-3 in BALB/c-3T3 cells and an SV40 virus-transformed BALB/c-3T3 derivative. Figure 1 demonstrates that both of these cell lines can be growth arrested in G_0/G_1 and induced to synchronously enter DNA synthesis following the addition of fresh media containing 20% FCS (fetal calf serum), as was previously shown (Yu *et al*., 1993). The normal BALB/c-3T3 cells reached peak DNA synthesis approximately 15–18 h following serum stimulation, whereas the SV40 virus-transformed cells reached peak DNA synthesis more rapidly at 12–15 h. We then examined the activities of the E2F family in these cells using a high resolution E2F-specific EMSA (electrophoretic mobility shift assay) which resolved several individual E2F/DP family heterodimers, as well as trimeric E2F complexes containing members of the Rb family (Ikeda *et al*., 1996).

Our greatest interest in this experiment was a relatively minor form of E2F in the normal BALBs which did not become apparent until 12–15 h following treatment with serum. Two observations support the conclusion that this band corresponds to a complex containing E2F-3. First, this S phase-specific band comigrates with authentic E2F-3 transiently overexpressed as a heterodimer with DP-1. Second, antibody addition (Figure 2b and c) revealed that this band was abolished by an E2F-3 antibody and not by antibodies against E2F-1, E2F-2 or E2F-4. The SV40 virus-transformed cells also displayed a temporal pattern of E2F-3 activation following serum stimulation. E2F-3 activity was present at very low levels in the starved cells, appeared at 6 h following serum stimulation, peaked during DNA synthesis and then decreased again as cells reached G_2/M. However, it is clear that the transformed cells accumulated much higher levels of total E2F-3 than their untransformed counterparts during the S phase. To be certain that these commercially-obtained antibodies were in fact functional and selective, they were tested against authentic E2F-1, E2F-2, E2F-3 and E2F-4 transiently overexpressed in mammalian cells as heterodimers with DP-1 as previously described (Cress and Nevins, 1994). Figure 2d reveals that each antibody selectively abolished (and in some cases 'supershifted') the appropriate complex, but did not cross react with other family members.

A second form of E2F was present at all times and in both cell lines. This band corresponded to a dimeric complex containing E2F-4 according to its relative mobility and antibody addition experiment (Figure 2b and c). Unlike E2F-3, E2F-4 did not appear to be dramatically upregulated in the transformed cells relative to the normal cells. The normal BALBs also contained additional slower migrating E2F complexes which corresponded to trimeric complexes containing members of the Rb family. The major species of E2F present in the quiescent BALB/c-3T3 cells consisted of a trimeric complex containing p130 (as determined by antibody addition experiments [data not shown] and reference Bennet *et al*., 1996). At 9 h following serum

addition the p130 complex was greatly diminished and by 12 h the G_0 p130 complex was replaced by a new complex with a slower mobility. Based upon antibody addition experiments (data not shown and Bennet *et al*., 1996), this 12 h complex contains the p107 protein. As expected E2F trimeric complexes were not present in the transformed cells due to the activity of the T antigen which is known to disrupt E2F/Rb family complexes (Chellappan *et al*., 1992). We were not able to identify several other E2F-site-specific binding activities (indicated as ? in the Figures). These activities may correspond to additional E2F family members, such as E2F-5, or other proteins with E2F-like DNA-binding specificity.

The activation of E2F-3 DNA binding activity correlates with an accumulation of the polypeptide, but not the mRNA

To determine if the absence of E2F-3 DNA binding activity in serum-starved cells could be accounted for by an absence of the E2F-3 protein we performed immunoblot analysis. Figure 3a demonstrates that the E2F-3 polypeptide was not observed in starved cells, peaked during DNA synthesis and then fell again following DNA synthesis. In contrast the level of E2F-4 polypeptide was relatively constant throughout the cell cycle (Figure 3b).

To determine if the absence of the E2F-3 polypeptide in serum-starved cells corresponded to an absence of the E2F-3 mRNA we used an RNAse protection assay to measure the total levels of E2F-3 mRNA in starved cells and following serum stimulation (Figure 4a). As a control we used the GAPDH gene (Figure 4b) which displays only modest cell cycle variations in rodent fibroblast (Johnson *et al*., 1994). As anticipated from previous results with other mouse fibroblasts (Slansky and Farnham, 1996), there was significant E2F-3 mRNA present in the quiescent cells. In addition there was a 4–5-fold increase in the total E2F-3 mRNA following serum stimulation. Thus an increase in the total E2F-3 mRNA may contribute to E2F-3 polypeptide accumulation following serum stimulation, but mRNA levels cannot account for the complete absence of E2F-3 in quiescent cells.

To determine if activation of E2F-3 DNA binding activity following serum stimulation could occur even in the absence of the accumulation of new E2F-3 mRNA following serum stimulation, we serum stimulated starved SV40 virus-transformed cells in the presence and absence of DRB (5,6-dichlorobenzimidazole riboside), an RNA polymerase II inhibitor. Figure 4b reveals that in untreated cells E2F-3 was not activated at 1 or 2 h following serum addition, but was then activated by 3 h. In contrast, in DRB-treated cells E2F-3 activation was not evident until 6 h, indicating a delay of up to 3 h. Thus DRB addition delayed, but did not block, the activation of E2F-3 following serum stimulation. As a control in these experiments we also included cells treated with the protein synthesis inhibitor cycloheximide. Figure 4b reveals that activation of E2F-3 did require new protein synthesis. Together these results support the conclusion that E2F-3 activation is, in part, independent of new E2F-3 mRNA accumulation, but is dependent upon new protein synthesis.

The half-life of E2F-3 increases during S phase

Our results suggest that at least part of the observed regulation of E2F-3 accumulation was at the post-transcriptional level. This accumulation could be regulated by an increased translational rate at the G_1/S border and during S. Alternatively, E2F-3 accumulation could be regulated at the level of protein turnover. To further examine the mechanism(s) responsible for the accumulation of E2F-3 we performed metabolic labeling experiments coupled with immunoprecipitations to determine the turnover rate of E2F-3. Our first step was to examine the potential of our E2F-3 antibody to selectively immunoprecipitate E2F-3 from extracts of asynchronous cells cultured overnight in media containing ^{35}S-methionine/cysteine. Figure 5a demonstrates that the rabbit polyclonal antibody sc-878X (Santa Cruz Biotechnology) selectively immunoprecipitated a 60 kDa polypeptide which comigrates with authentic hE2F-3 transcribed *in vitro*. We next compared the amount of ^{35}S-label incorporated into E2F-3 overnight to a 3 h pulse in asynchronously growing cells. Figure 5b reveals that a significant amount of E2F-3 was synthesized in a 3 h pulse in asynchronous cells, but that little E2F-3 accumulated in the serum-starved cells, as expected. Having determined that a short 3 h labeling period was appropriate for growing cells we then synchronized cells in S phase by the use of a thymidine block. The thymidine block was very successful in that 80% of the cells were in S phase as estimated by DNA content. Synchronized cells and asynchronous cells were labeled by a 3 h pulse in the presence of ^{35}S-methionine and then chased with fresh complete media for 1, 2 and 4 h. Figure 5c shows the results of immunoprecipitation of E2F-3 from the two different cell populations. Figure 5d shows the decay curves for E2F-3 as measured using a PhosphorImager and ImageQuant Software. The slope of the decay curve indicates that in asynchronous cells the half-life of E2F-3 was just under 1 h. However, for the cells synchronized in S phase the half-life of E2F-3 was estimated to be 40 h, since there was no diminution in the E2F-3 signal over the 4 h period of time. Similar stabilization of the E2F-3 protein in S phase was observed in repetitions of this experiment. These results clearly indicate that E2F-3 was greatly stabilized during S phase relative to other states of the cell cycle.

Discussion

E2F-3 (along with E2Fs -1 and -2) is one of the three Rb-specific E2Fs. The Rb-specific E2Fs may be particularly important in development and in tumor suppression since within the Rb family only pRb appears to have an irreplaceable role in development and tumor suppression. pRb is the only Rb family member commonly found to be mutated in human cancers. In fact, the inheritance of a single dysfunctional Rb allele strongly predisposes children to the loss of the second functional Rb allele and subsequently to retinoblastoma (Knudson, 1993; Weinberg, 1995). Likewise, $Rb^{+/-}$ mice have a strong predisposition to tumor formation (Jacks *et al*., 1992; Lee *et al*., 1992). pRb is also essential for normal development

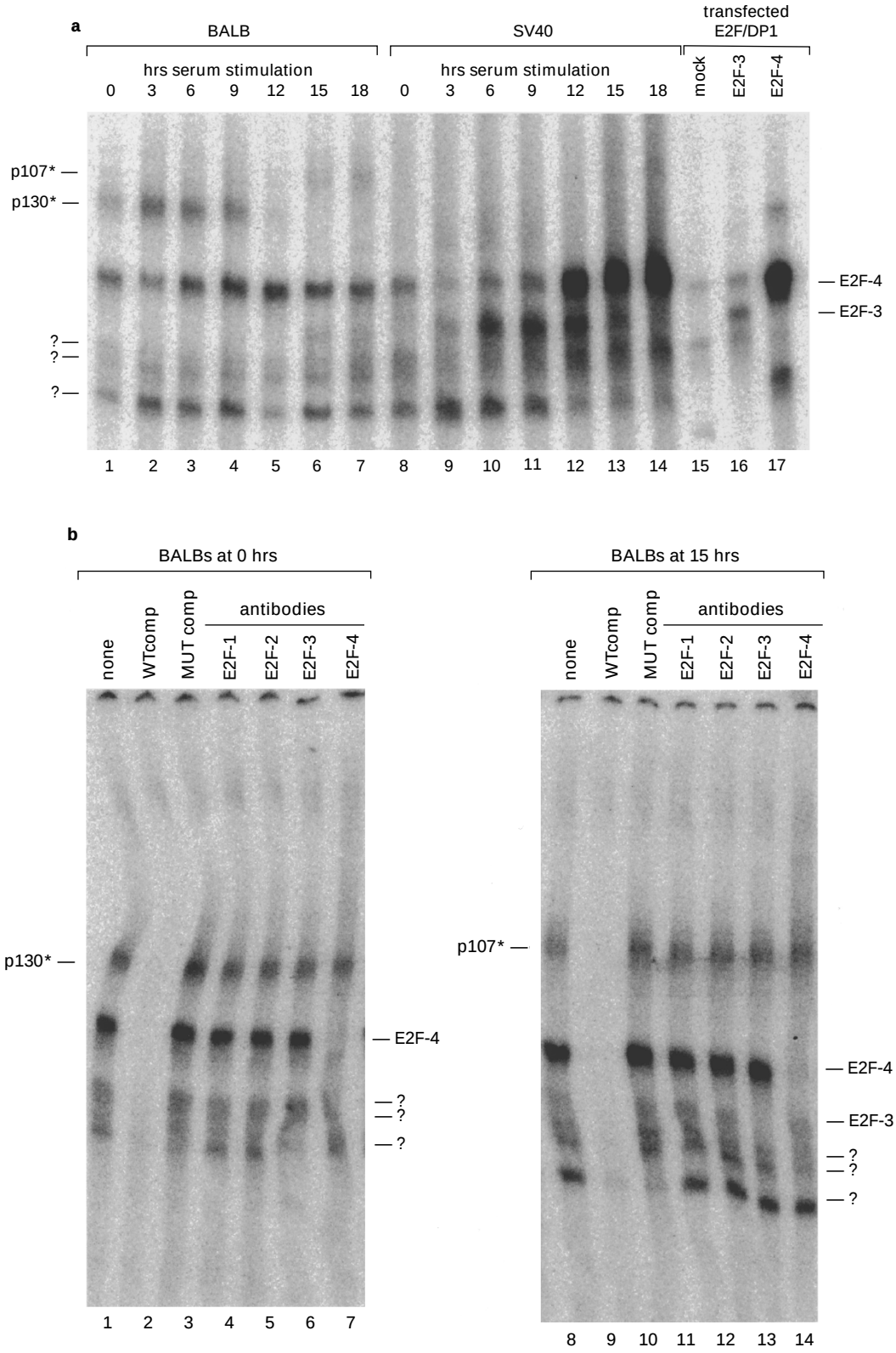
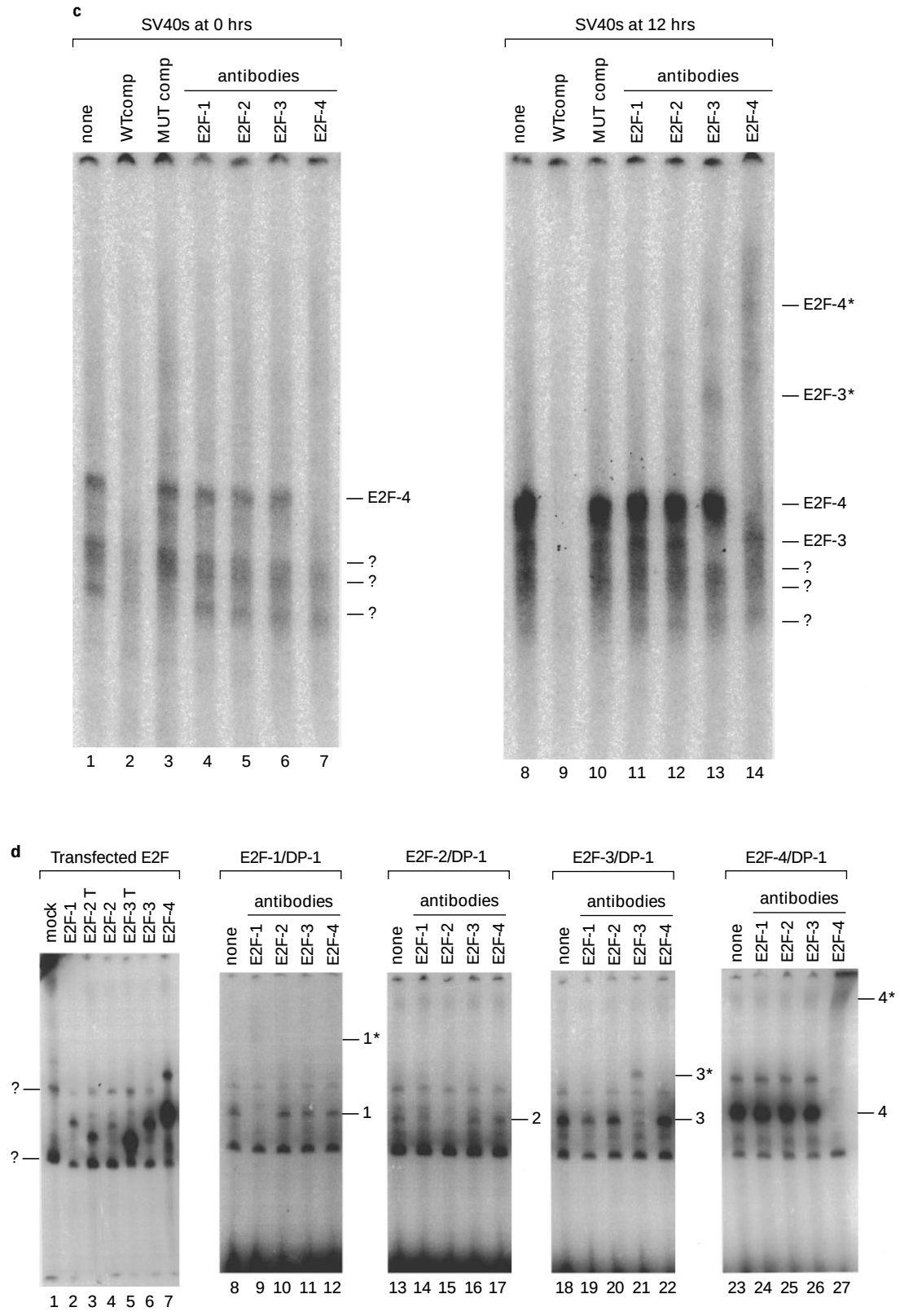


Figure 2 E2F-3 DNA binding activity is coincident with the G_1/S boundary in both normal and transformed BALB/c-3T3 fibroblasts. (**a**) Normal lanes (1–7) and SV40 virus-transformed (lanes 8–14) BALB/c-3T3 fibroblasts were growth arrested as previously described (Yu *et al*., 1993) and stimulated to enter DNA synthesis as described in Figure 1. Whole cell extracts were prepared at the indicated times following treatment with 20% FCS. An EMSA reveals E2F-specific DNA binding activity. The putative identities of complexes are indicated. Lanes 15–17 represent C-33A cells either mock transfected (lane 15) or transfected with 10 μg each of plasmids expressing E2F-3 (lane 16) or E2F-4 (lane 17) together with DP-1 which serve as mobility reference as previously described (Cress and Nevins, 1994). An * indicates that this complex was defined in other work (Bennet *et al*., 1996; Dong *et al*., manuscript submitted). The symbol '?' indicates that the complex could not be identified. (**b**) E2F-specific oligonucleotides and polypeptide-specific antibodies were added to the indicated EMSA reactions in order to reveal the identities of specific bands. Lanes 1–7 correspond to growth-arrested BALB cells. Lanes 8–14 correspond to an extract from BALB cells stimulated for 15 h with 20% FCS which are in peak DNA synthesis. WT comp (lanes 2 and 9) corresponds to the addition of 20 ng of an unlabeled oligonucleotide containing a E2F binding sequence. MUT comp (lanes 3 and 10) corresponds to the addition



of 20 ng of an unlabeled oligonucleotide containing an inactivating dinucleotide substitution in the E2F binding sequence. One μg of the indicated antibodies were included in the binding reactions. The antibodies used were sc-193X, sc-633X, sc-878X, and sc-886X for E2F-1 through -4, respectively and all were from Santa Cruz Biotechnology. (**c**) Lanes 1–7 correspond to growth-arrested SV40 cells. Lanes 8–14 correspond to an extract from SV40 cells stimulated for 12 h with 20% FCS which are in peak DNA synthesis. Putative supershifted complexes are indicated by E2F*. Otherwise (**c**) is identical to (**b**). (**d**) E2F complexes of defined polypeptide composition were overexpressed by transfecting C-33A cells with plasmids expressing DP-1 together with the indicated E2F partner as we have previously described (Cress and Nevins, 1994). Lane 1 corresponds to 2 μg of extract from C-33A cells which were mock transfected and indicates endogenous levels of E2F activity in those cells. Lanes 2, 4, 6 and 7 correspond to the transfection of wildtype E2F-1, E2F-2, E2F-3 and E2F-4, respectively. Lanes 3 and 5 correspond to transfection of N-terminal deletion mutants of E2F-2 and E2F-3, respectively (Lees *et al*., 1993). The mobility difference observed between the deletion mutants and full-length proteins demonstrate that the indicated EMSA band corresponds to the exogenous gene and not to the activation of an endogenous E2F by the exogenous E2F. Note that all of the exogenous E2Fs may activate endogenous E2F-4 to some extent

since pRb$^{-/-}$ mice die *in utero* (Jacks *et al*., 1992; Lee *et al*., 1992). In contrast, p107$^{-/-}$ or p130$^{-/-}$ mice develop normally and have no detectable increase in tumor formation (Cobrinik *et al*., 1996; Lee *et al*., 1996).

The E2F-3 protein has several unique characteristics among the three Rb-specific E2F family members. First, we have discovered that the E2F-3 polypeptide is prominent in HL60 cells undergoing apoptosis suggesting that a failure to down-regulate E2F-3

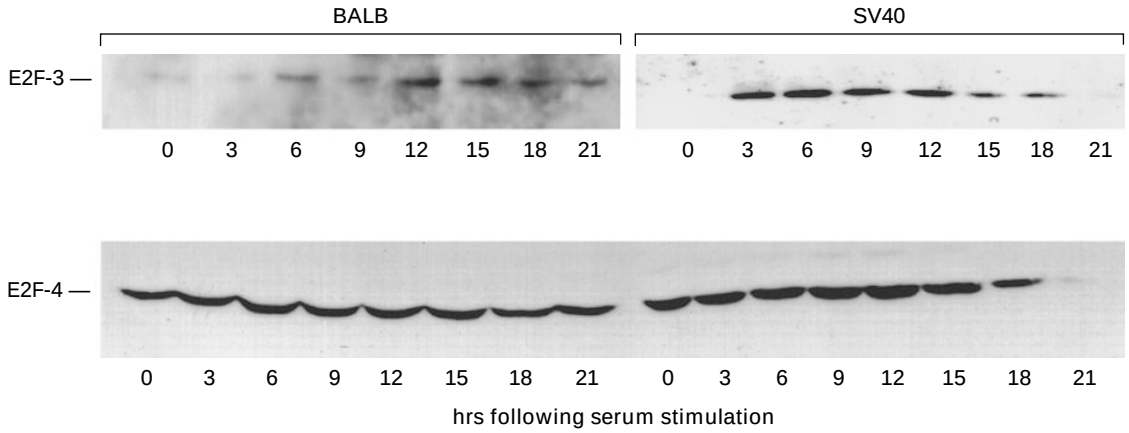


Figure 3 The increase in E2F-3 DNA binding at the G_1/S boundary activity is accompanied by an increase in total E2F-3 polypeptide. Whole cell extracts were prepared and subjected to immunoblot analysis. For the BALB cells 200 μg of protein was loaded per lane for the immunoblot. For the SV40 virus-transformed cells 50 μg of protein was loaded

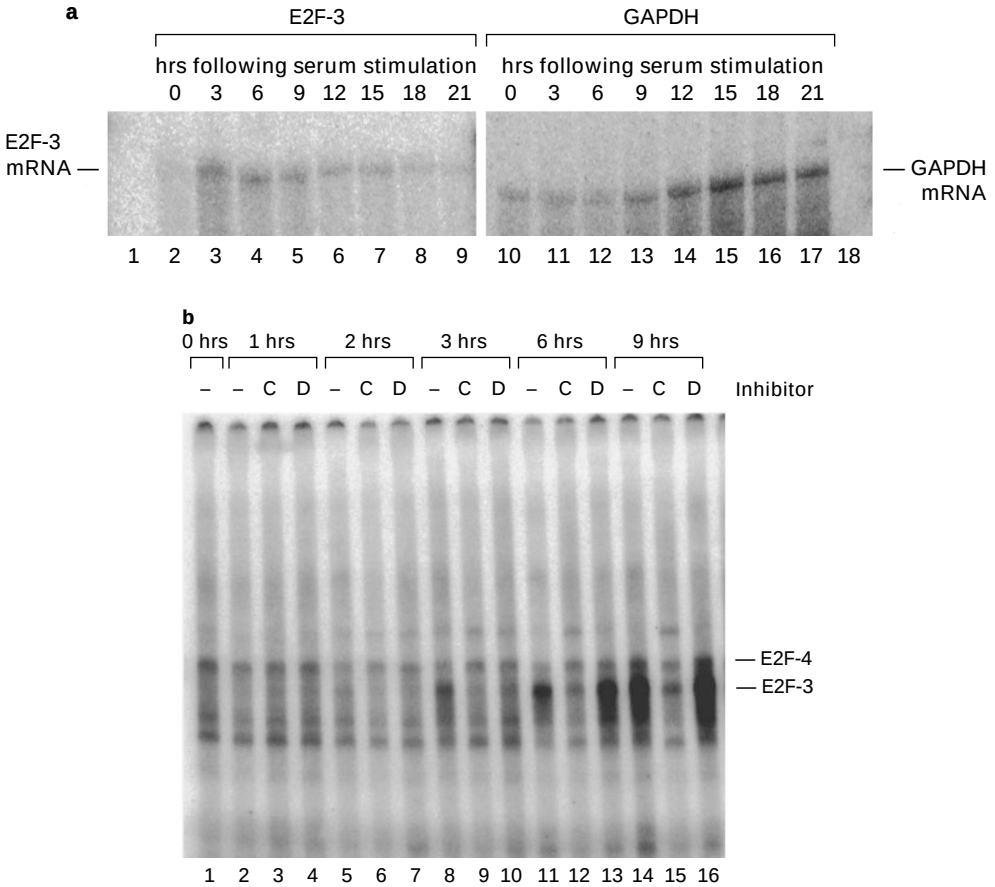


Figure 4 The increase in the E2F-3 polypeptide is independent of new RNA synthesis. (**a**) SV40 virus-transformed cells were growth arrested and serum-stimulated as in Figures 1 and 2 and total RNA was harvested at the indicated times following serum stimulation. An RNAse protection assay reveals the levels of the E2F-3 mRNA (lanes 2–9) and the GAPDH mRNA (lanes 10–17) as indicated. Lanes 1 and 18 represent no added RNA. (**b**) SV40 virus-transformed cells were growth arrested and serum-stimulated as in Figures 1 and 2. At 1 h following addition of 20% FCS, 100 μM DRB (**d**), or 10 μg/ml cyclohexamide (**c**) were added to the indicated cells. Whole cell extracts were prepared at the indicated times and an EMSA was performed. The E2F-3 and E2F-4 specific complexes are indicated. Lane 1 represents growth arrested cells. Lanes 2, 3 and 4 represent cells treated or not and immediately harvested. Lanes 5–7, 8–10, 11–13 and 14–16 represent cells harvested at 2, 3 6 and 9 h following serum stimulation, respectively

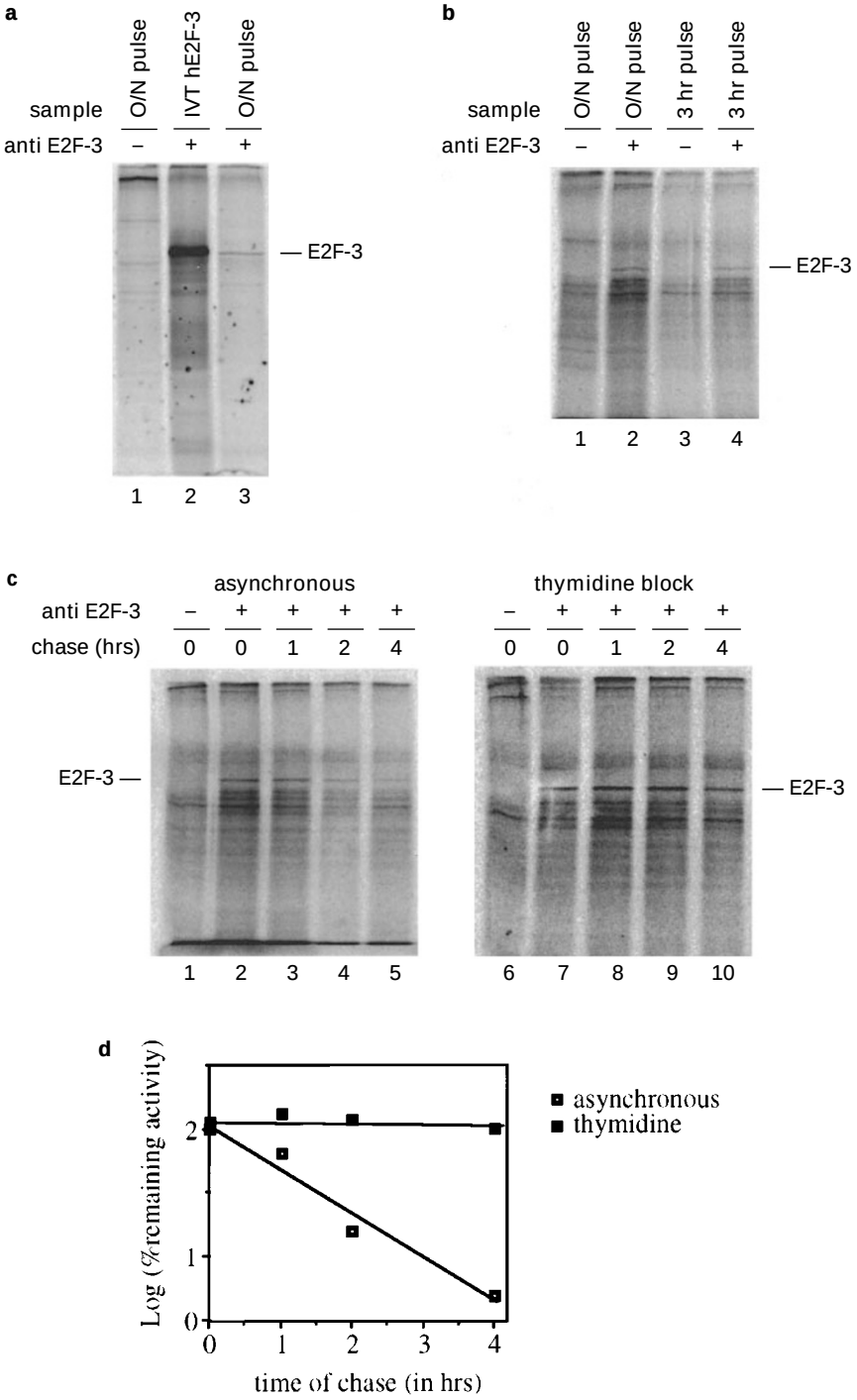


Figure 5 The increase in the E2F-3 polypeptide at the G1/S boundary is likely due to an increase in the half-life of the polypeptide during S phase. (**a**) E2F-3 antibody sc-878X is functional and specific in immunoprecipitations. Lane 1, 50 μg of extract from cells labeled overnight with ^{35}S-methionine/cysteine was incubated with anti-rabbit IgG beads conjugated magnetic beads without E2F-3 antibody to indicate background binding: lane 2, hE2F-3 translated *in vitro* in the presence of ^{35}S-methionine/cysteine was immunoprecipitated quantitatively with E2F-3 antibody sc-878X and indicates expected mobility of mouse E2F-3. Lane 3, E2F-3 antibody sc-878X selectively immunoprecipitates a polypeptide which comigrates with authentic human E2F-3 translated *in vitro*. (**b**) A 3 h pulse is sufficient to label E2F-3. Lane 1, immunoprecipitation using extract from overnight labeled SV40 virus-transformed cells without E2F-3 antibody. Lane 2, immunoprecipitation using extract from overnight labeled cells with E2F-3 antibody included. Lane 3, immunoprecipitation using extract from cells labeled with a 3 h pulse without E2F-3 antibody. Lane 4, immunoprecipitation using extract from cells labeled with a 3 h pulse with E2F-3 antibody included. (**c**) The E2F-3 half-life is 40-fold greater in S phase cells relative to asynchronous cells. Lanes 1–5, asynchronously growing SV40 virus-transformed cells were labeled in a 3 h pulse. The labeled cells were then incubated in fresh media for chase periods of 1, 2 and 4 h as indicated. Lane 1, 3 h pulse with no antibody. Lane 2, 3 h pulse with antibody included. Lanes 3, 4 and 5 correspond to 1, 2 and 4 h chase periods, respectively, including E2F-3 antibody. Lanes 6–10, correspond to cells blocked in S phase by thymidine. Otherwise, lanes 6–10 are parallel to lanes 1–5. (**d**) Quantitation of immunoprecipitation results. The amount of ^{35}S remaining in the E2F-3 immunoprecipitated band at 1, 2 and 4 h following a chase with cold methionine was quantified using a PhosphorImager and ImageQuant Software and plotted as a log of the percentage of the total labeled E2F-3 following a 3 h pulse. The slopes of these plots indicate an E2F-3 half-life of 0.9 h for asynchronous cells and 40 h for S phase cells

may have a contributory role in the process of cell death (Kassatly and Cress, manuscript submitted). Second, E2F-3 is the only Rb-specific E2F that accumulates to an EMSA-measurable level in fibroblasts suggesting that E2F-3 may have a dominant role in some cells. Third, E2F-3 is the only Rb-specific E2F which is not transcriptionally silent in quiescent or terminally differentiated cells; both E2F-1 and E2F-2 are transcriptionally silent in such cells (Moberg *et al*., 1996); (Kassatly and Cress, manuscript submitted). Finally, while the abundance of other E2F family members remains much the same, E2F-3 activity is much more abundant in transformed fibroblasts than in their normal counterparts. This observation suggests that E2F-3 activation may correlate with transformed growth characteristics.

In other work, we discovered that differentiated HL60 cells possess no E2F-3 polypeptide, but did have E2F-3 message suggesting that E2F-3 has the potential to be downregulated post-transcriptionally. Unfortunately, for mechanistic studies, the HL60 cell line expressed relatively low levels of the E2F-3 polypeptide and DNA binding activity making it difficult to measure the synthesis or degradation of E2F-3 using pulse/chase experiments. In the present work, we have examined the mechanism responsible for the regulation of E2F-3 accumulation in normal BALB/c-3T3 fibroblasts and in an SV40 virus-transformed derivative. The SV40 virus-transformed cells were ideal for these mechanistic experiments since E2F-3 is very abundant in extracts of these cells.

We have determined that E2F-3 and E2F-4 represent the predominant forms of free E2F in BALB/c-3T3 fibroblasts as well as in the SV40 virus-transformed BALB/c-3T3 cells. As has been observed in other experiments with different cell lines, the E2F-4 polypeptide and DNA binding activity are apparent at all stages of the cell cycle. On the other hand, E2F-3 DNA binding activity is restricted to the G_1/S transition and S phase in both cell lines. As anticipated from previous results (Bennet *et al*., 1996), E2F-4 was observed to be in interactions with members of the Rb family in the normal BALBs, but no trimeric complexes were observed in the SV40 virus-transformed cells. It is clear, however, that high levels of E2F-3 expression are not essential for DNA synthesis in the SV40 virus-transformed cells. Even when serum-starved for several days, 20% of these SV40 virus-transformed cells continue to enter DNA synthesis (Figure 1) even though they demonstrate undetectable levels of E2F-3 (Figures 2 and 3).

RNAse protection demonstrates upregulation of the E2F-3 mRNA following serum stimulation. Thus, mRNA accumulation likely contributes to some extent to the accumulation of the E2F-3 polypeptide. However, it is clear that the E2F-3 mRNA is preexisting in the serum-starved cells, yet they have no or very little protein. To formally demonstrate that the activation of E2F-3 following serum stimulation could be achieved in the absence of new RNA synthesis we performed a serum stimulation experiment in which we added an RNA PolII inhibitor, DRB, 1 h following serum stimulation. DRB was chosen as an inhibitor, since extensive work has demonstrated 100 μM DRB to be highly effective in these cells (Olson *et al*., 1993). The 1 h delay was included to allow the expression of necessary immediate early genes. This 1 h of RNA synthesis should allow insufficient time for any significant accumulation of E2F-3 message, since the activation of E2F-3 DNA binding activity did not occur until 3 h following serum stimulation. It is clear from Figure 3 that DRB did not block E2F-3 activation, although it does cause a delay of up to 3 h. This is likely due to an attenuation of RNA synthesis-dependent signals upstream of E2F-3 activation.

Immunoblot analysis with E2F-3 specific antibodies indicates that the increase in the DNA binding activity of E2F-3 at the G_1/S boundary is reflected by an increase in total E2F-3 protein. This result was identical to our findings in synchronized HL60 cells in which we observed the E2F-3 polypeptide only during peak DNA synthesis and together these results suggest that the accumulation of the E2F-3 polypeptide during S phase is common to different cell types. Close inspection of Figures 2 and 3, however, reveals that the temporal presence of the E2F-3 polypeptide may be broader than E2F-3's DNA-binding activity. This observation would suggest an additional level of regulation, in addition to polypeptide accumulation. One possibility is that the newly accumulated E2F-3 takes some time to associate with a DNA binding partner. A second possibility is that E2F-3 (or its DP partner) must be modified in order to bind DNA. The most likely modification would be phosphorylation/dephosphorylation. It has been shown that each of the Rb-specific E2Fs possess a binding motif for cyclin A (Lees *et al*., 1993). This cyclin A-binding motif allows cyclin A to associate with E2F-1, -2 or -3 and phosphorylate the DP partner of the heterodimer in late S phase (Krek *et al*., 1994; Xu *et al*., 1994). This phosphorylation event leads to diminished DNA binding as the cells exit S phase.

Finally, pulse/chase/immunoprecipitation experiments indicate that the half-life of E2F-3 changes in different cell cycle stages. In addition to E2F-3, several additional proteins appear to coimmunoprecipitate with E2F-3. Some of these bands are clearly binding nonspecifically to the magnetic beads, since they appear in IPs including no antibody (for example lanes 1 and 6 of Figure 5c). However, at least two of the major bands are likely authentic E2F-3-associated proteins for two reasons. First these bands do not quantitatively appear in immunoprecipitations, for example compare Figure 5 (a) with (b) indicating that they are not proteins directly recognized by the antibody. Second, when E2F-3 is stabilized in S phase (thymidine-blocked cells) its associations with these other labeled proteins appears to be increased (Figure 5c), i.e. their apparent half-lives increase as the half-life of E2F-3 increases. We predict that these E2F-3 associated proteins will correspond to known E2F-3 partners such as DP-1 and DP-2 which have the appropriate molecular weights.

The observation that the half-life of E2F-3 changes in different cell cycle stages supports the hypothesis that E2F-3 polypeptide accumulation is regulated, at least in part, at the level of polypeptide stability. The lack of E2F-3 accumulation in growth arrested cells could be entirely due to rapid degradation, however, it is likely that other mechanisms may also contribute.

There is an obvious increase in the E2F-3 mRNA following serum stimulation which may contribute to increases accumulation of the polypeptide. Because the half-life of E2F-3 is changing during the course of a cell cycle it is difficult to address the possibility that the translational efficiency of the E2F-3 mRNA may also contribute to the accumulation of E2F-3 at the G_1/S boundary.

Previous work in which E2F proteins were highly overexpressed by transient transfection have demonstrated that E2F-1 and E2F-4 have the potential to be degraded by the ubiquitin–proteosome pathway (Hateboer *et al*., 1996; Hofmann *et al*., 1996). It is quite possible that the rapid degradation of E2F-3 in cell cycle stages other than S phase is mediated by the ubiquitin–proteosome pathway. It will be of great interest in the future to examine the biochemical mechanisms regulating the half-life of E2F-3.

Materials and methods

Cells, tissue culture and flow cytometry

BALB/c-3T3 cells (BALBs) and the SV40 virus-transformed derivative cell line (SV40s) were a kind gift from Richard Jove (Moffitt Cancer Center, Tampa, FL) and are described in reference (Yu *et al*., 1993). Cells were cultured and growth arrested as previously described (Yu *et al*., 1993). Briefly, BALB/c-3T3 cells were grown to confluence in Dulbecco's Modified Eagle's Media containing 5% calf serum. The media was then removed and the cells were cultured in media containing 0.5% calf serum for 48 h. The cells were then stimulated to enter DNA synthesis by the addition of fresh media containing 20% FCS. The SV40 virus-transformed BALBs were arrested by splitting confluent plates 1/10 in media containing 5% calf serum and allowing the cells to grow to confluence and simultaneously expend the available growth factors over the course of 4–5 days. The cells were then stimulated to enter DNA synthesis by replacing spent media with fresh media containing 20% FCS. For flow cytometry 10^6 trypsinized cells were washed once with PBS (phosphate-buffered saline), resuspended in 100 μl PBS, fixed with 1 ml cold ethanol, washed again with 1 ml PBS, treated with 10 μg/ml RNAse A in 200 μl PBS, and stained with propidium iodide. Flow data was acquired on a Becton Dickinson FACScan and analysed using CELLFIT 2.01.2.

Electrophoretic mobility shift assays

Whole cell protein extracts were prepared as previously described (Ikeda *et al*., 1996). Adherent cells were washed with PBS and then scraped off plates into PBS prior to collection by centrifugation. Protein concentrations were determined using a Bradford protein assay (Biorad, Hercules, CA). High resolution electrophoretic mobility shift assays were performed using a *dihydrofolate reductase* promoter fragment as probe as previously described (Ikeda *et al*., 1996) with the exception that Nonidet P-40 and Mg (Huber *et al*., 1994) were omitted from binding reactions to improve binding affinities.

Immunoblots

Immunoblot analysis was performed using standard protocols in which cell extracts were subjected to SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred membranes and the membranes immunoblotted with the specific polyclonal antibodies which were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. An ECL Detection System and Rainbow Molecular weight markers (Amersham, Arlington Heights, IL) were used for detection and as molecular weight standards, respectively, according to the manufacturer's protocols.

RNAse protection assays

Total RNA was harvested from 100 mM dishes using Tri-Reagent (MRC, Inc., Cincinnati, OH). Radiolabeled RNA probes were prepared by *in vitro* transcription in the presence of α^{32}P-UTP using T7 RNA polymerase and linearized templates. For mouse E2F3, a cRNA probe was prepared from plasmid, mE2F3B1 (Dagnino *et al*., 1997), linearized with *Hind*III. For GAPDH, a cRNA probe was prepared from plasmid pBlu-GAPDH (Tso *et al*., 1985), linearized with Bbs I (Bpu1A). Plasmids mE2F3B1 and pBlu-GAPDH were kind gifts from Christopher Fry and Peggy Farnham, U Wisconsin, Madison, WI and Tere Muñoz-Antonia, U South Florida, Tampa, FL, respectively. One hundred μg of total RNA were used in each assay.

Pulse/chase and immunoprecipitations

For metabolic labeling experiments cells were cultured in media supplemented with 20% FCS in six well plates. Cells were synchronized in S phase by addition of 2 mM thymidine for 24 h. During the labeling period, cells were incubated in methionine free media (Life Technologies, Gaithersburg, MD) supplemented with 100 μCi/ml ^{35}S-methionine (Dupont NEN, Boston, MA). During the chase period, the labeling media was removed and replaced with fresh complete media containing 20% FCS and thymidine as appropriate. Following pulse/chase, cell monolayers were washed once with PBS and lysed in 250 μl RIPA buffer (containing 150 mM NaCl, 1.0% Nonidet-P40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate and 50 mM Tris, at pH 8.0). Extracts were cleared by centrifugation and stored at -80°C. Immunoprecipitations were performed using 50 μg total cell extract, 1 μg of an E2F-3 rabbit polyclonal antibody sc-878X (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Rabbit IgG conjugated to magnetic beads (Dynal, Oslo, Norway).

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