Interactions of the p107 and Rb proteins with E2F during the cell proliferation response

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The E2F transcription factor is found in complexes with
a variety of cellular proteins including the retinoblas-
toma tumor suppressor protein. Various assays have
demonstrated a tight correlation between the functional
capacity of Rb as a growth suppressor and its ability to
bind to E2F. Moreover, only the underphosphorylated
form of Rb, which appears to be the active species,
interacts with E2F. Despite the fact that the majority of
Rb becomes hyperphosphorylated at the end of G_1, we
now show that the E2F–Rb interaction persists through
the G_1/S transition and into S phase. A distinct E2F
complex does appear to be regulated in relation to the
transition from G_1 to S phase. We now demonstrate that
this complex contains the Rb-related p107 protein.
Moreover, like the Rb protein, p107 inhibits E2F-
dependent transcription in a co-transfection assay. This
result, together with the observation that free,
uncomplexed E2F accumulates as cells leave G_1 and
enter S phase, suggests that the p107 protein may regulate
E2F-dependent transcription during G_1. In contrast,
although Rb does regulate the transcriptional activity of
E2F, this association does not coincide with the G_1 to S
phase transition.
Key words: E2F/p107/proliferation/Rb protein/transcription

Introduction

The E2F transcription factor was originally identified as a
cellular DNA binding protein that recognized two identical
elements within the adenovirus E2 promoter (Kovesdi et al.,
1986; Yee et al., 1987). Subsequent experiments demon-
strated the importance of the E2F sites for E1A-induced E2
transcription (Loeken and Brady, 1989) and the ability of
E2F to stimulate transcription dependent on these sites (Yee
et al., 1989). During an adenovirus infection, E2F is found
in association with a 19 kDa product of the early viral E4
gene (Hardy et al., 1989; Hardy and Shenk, 1989; Huang
and Hearing, 1989; Reichel et al., 1989). The DNA binding
properties of this E2F–E4 heteromeric complex are quite
different from that of the uncomplexed E2F molecule. E2F
binding to the E2 promoter results in the formation of an
unstable DNA–protein complex, whereas the E2F–E4

interaction is cooperative and generates a very stable
DNA–protein complex (Hardy et al., 1989; Hardy and
Shenk, 1989; Huang and Hearing, 1989; Neill et al., 1990;
Raychaudhuri et al., 1991). Although the majority of E2F
in a HeLa cell is in a form that allows interaction with the
E4 protein, analysis of E2F in extracts of a variety of other
cell types revealed the existence of E2F-containing
complexes that prevented the interaction of E4 with E2F
(Bagchi et al., 1990). The adenovirus E1A protein could
dissociate these complexes, releasing a free E2F that could
then interact with E4 (Bagchi et al., 1990).

Recent experiments have identified the components of
these E2F complexes as important cellular regulatory
proteins. These include the retinoblastoma gene product
(Whyte et al., 1988), the Rb-related p107 protein (Ewen
et al., 1991), the cyclin A protein (Pines and Hunter, 1990)
and the p33[cdk2] protein kinase (Tsai et al., 1991). These are
also proteins found in association with the adenovirus E1A
protein and it would appear that the E1A-mediated
dissociation of E2F complexes containing these proteins
results in the transfer of these proteins to E1A (for review,
see Nevins, 1992). Moreover, SV40 T antigen and the
human papillomavirus E7 protein share the ability to disrupt
the E2F interactions (Chellappan et al., 1992). These results
thus implicate E2F as an important target for the action of
tumor suppressor proteins, cell cycle regulatory proteins and
viral oncoproteins.

Transfection assays have shown that the free E2F molecule
is able to stimulate transcription of the E2 promoter, whereas
the association of E2F with Rb impairs the activation
potential (Dalton, 1992; Hamel et al., 1992; Hiebert et al.,
1992; Zamanian and La Thangue, 1992). Moreover, E2F
appears to be a critical component of the activation of
transcription of the cellular dihydrofolate reductase (DHFR)
gene (Blake and Azizkhan, 1989) as cells progress from G_1
into S phase (Means et al., 1992). Although previous
experiments demonstrated the cell cycle regulation of
complex formation involving E2F with cyclin A specifically
during S-phase, several questions remained unanswered. In
particular, the identity of G_1-specific E2F interactions that
may control E2F activity and the role of the Rb protein in
such control have not been addressed. We have now
extended these initial studies to answer these questions.

Results

Previous experiments have documented the existence of
multiple E2F-containing protein complexes in cell extracts
(Chellappan et al., 1991; Bagchi et al., 1991; Bandara and
La Thangue, 1991; Bandara et al., 1991; Mudryj et al.,
1991; Cao et al., 1992; Devoto et al., 1992; Shirodkar
et al., 1992). Two of these complexes are regulated as a
function of the cell cycle (Mudryj et al., 1991; Shirodkar
et al., 1992). Analysis of E2F in extracts of synchronized

NIH 3T3 cells revealed a G_1-specific E2F complex. As cells progressed towards S phase, the G_1 complex was lost, leaving only free E2F in the extract. Then, as cells entered S phase, a second E2F complex was formed; this complex accumulated during S phase and then was lost at the end of S phase or in G_2. A variety of experiments have now demonstrated that the S phase complex contains the Rb-related p107 protein, the cyclin A polypeptide and the cyclin-dependent cdk2 kinase in addition to E2F (Cao et al., 1992; Devoto et al., 1992; Shirodkar et al., 1992). In contrast, the components of the G_1-specific complex have not been identified.

Additional experiments have detected a third distinct E2F complex that contains the retinoblastoma gene product (Rb). This E2F−Rb complex was, however, not detected in the NIH 3T3 extracts, but rather in a variety of human cell extracts (Bandara and La Thangue, 1991; Chellappan et al., 1991). Analysis of the Rb protein that co-purified with E2F revealed that only the fastest migrating species of Rb was in a complex with E2F (Chellappan et al., 1991). This form of Rb, which has been demonstrated to be the underphosphorylated form, has been proposed to be the active growth-repressing Rb species (DeCaprio et al., 1989). Since the Rb protein becomes hyperphosphorylated as cells traverse G_1/S (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989), it seemed possible that the E2F−Rb interaction is restricted to G_1, with the phosphorylation of Rb possibly being responsible for the release of E2F.

Proliferation-dependent changes in E2F interactions

In order to define the cellular control of each of the E2F interactions including the E2F−Rb complex, we have analyzed extracts of human cells in which the E2F−Rb complex can be detected. For these assays, we have employed human peripheral lymphocytes that have been stimulated in culture with PHA or we have used human diploid fibroblast cultures that were brought to quiescence by starvation and then stimulated by serum addition. As depicted in Figure 1, the analysis of extracts from these cultures revealed similarities to the previous analyses of NIH 3T3 cells. Clearly evident is a G_1-specific E2F complex in both the T cells (Figure 1A) as well as the fibroblasts (Figure 1B). That this complex is likely the same as that previously detected in the NIH 3T3 cells is based on the equivalent gel mobility as well as the G_1-specific appearance. Also evident in this experiment is the previously defined S phase-specific E2F complex that contains p107, cyclin A and the cdk2 kinase. As seen previously, this complex begins to accumulate as cells enter S phase (defined by the kinetics of thymidine incorporation) (Figure 1B). In addition to these two specific E2F complexes, we also detect an accumulation of free, uncomplexed E2F near the G_1/S transition. Finally, the nature of the slower migrating complex that also has G_1 kinetics is at present unclear.

Somewhat surprising were the kinetics of appearance and accumulation of the E2F−Rb complex. As described above, we anticipated that the E2F−Rb complex might be regulated at the G_1/S transition, based on the fact that only the underphosphorylated Rb protein was found in the E2F complex (Chellappan et al., 1991) and much of the Rb in the cell is phosphorylated at the end of G_1 (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). However, as shown in Figure 1, the E2F−Rb interaction (defined by Rb antibody addition, data not shown) forms during G_1 and then persists into S phase. Moreover, the persistence of the E2F−Rb complex at a time when cells appear to be entering S phase does not appear
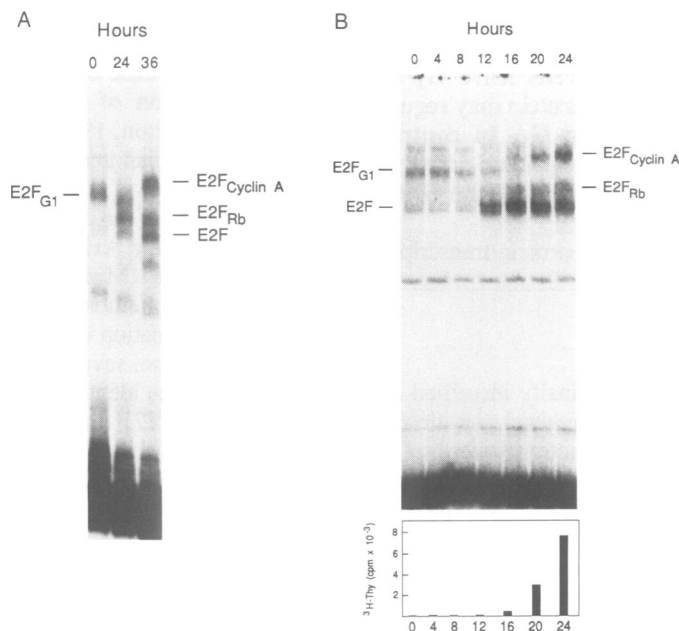
Fig. 1. Identification of E2F interactions following stimulation of quiescent human cells. A. Human peripheral lymphocytes were isolated and cultured with the addition of PHA to stimulate proliferation. Extracts were prepared at the indicated times following PHA addition and assayed for E2F binding activity, as described in Materials and methods. The various E2F−DNA complexes are indicated. The identity of the E2F−cyclin A complex and the E2F−Rb complex were confirmed by the addition of antibodies specific to either cyclin A or Rb. B. Cultures of the human foreskin fibroblast cell line Hs68 were starved into quiescence by serum deprivation. Proliferation was then induced by the addition of fresh media containing 10% fetal calf serum. Samples were taken at the indicated times, extracts prepared and assays for E2F binding activity were performed. Shown at the bottom are assays for [^{3}H]thymidine incorporation. Plates of cells were labeled for 1 h beginning at the indicated time. Thymidine incorporation was measured as described in Materials and methods.

to be due to the presence of a significant fraction of cells that failed to enter S phase based on the fact that by 24 h, 85% of the cells are replicating DNA as indicated by BrdU incorporation (Table I). The presence of the E2F−Rb complex in S phase extracts is also consistent with a recent analysis of E2F complexes in elutriated cell fractions (Shirodkar *et al.*, 1992).
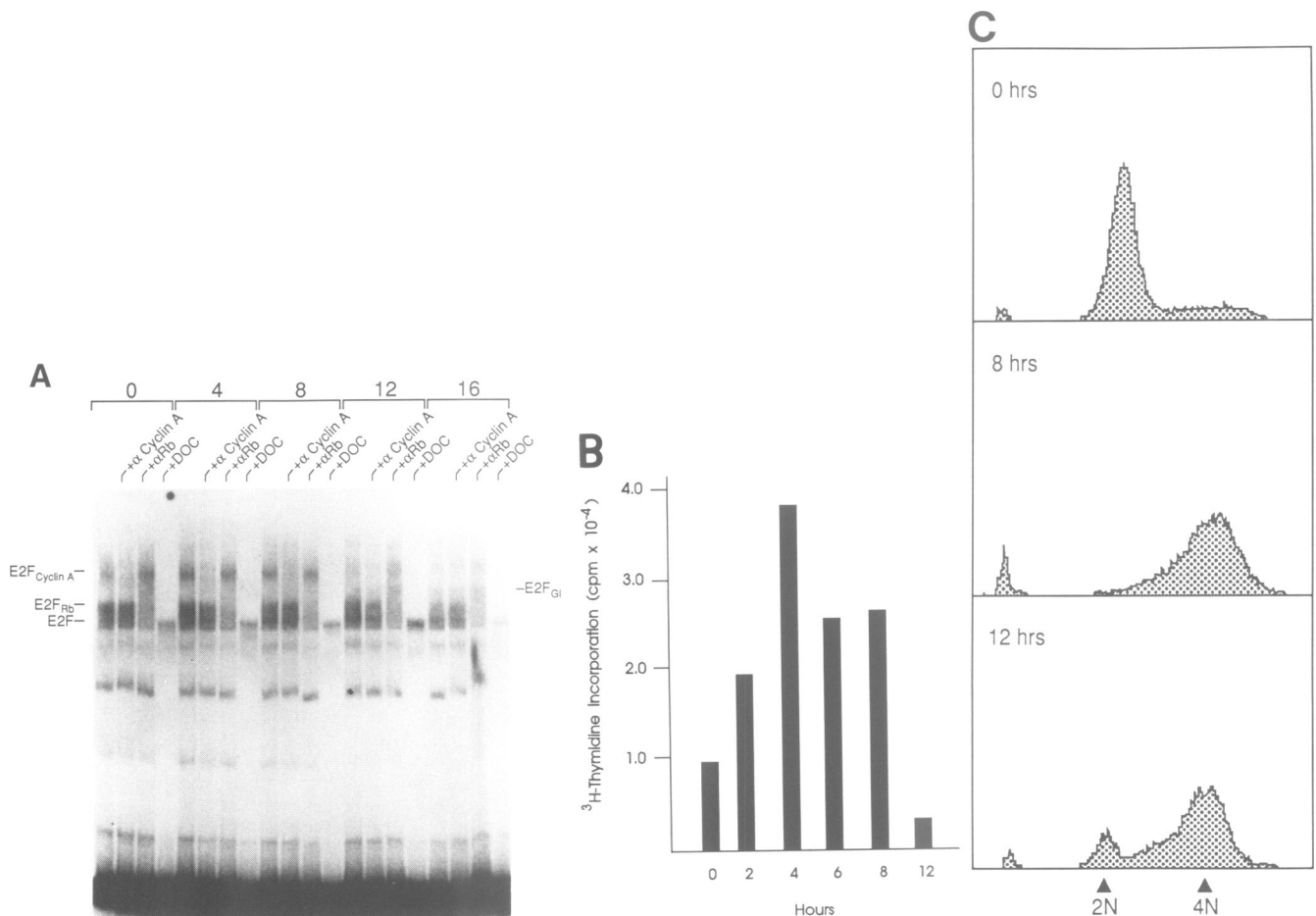
An additional analysis of the cell cycle control of the E2F−Rb interaction was provided by an assay of E2F in

Table I. BrdU incorporation in synchronized human fibroblasts

Labeling time (hours after serum addition)	BrdU positive (%)
0−12	4
12−18	27
18−24	69
0−24	85
0−24 (no serum addition)	<1

Cultures of the human diploid fibroblast cell line Hs68 were serum-starved to quiescence and then stimulated by the addition of fresh media and serum. BrdU was added to the media at the indicated time. Incorporation of BrdU was measured as described in Materials and methods.

extracts of cells that were synchronized by a double hydroxyurea block. Since we were unable to obtain highly synchronized cultures of the human fibroblasts at G_1/S, we have utilized CV1 cells as an alternative. The efficiency of synchronization was indicated by an analysis of [^{3}H]thymidine incorporation (Figure 2B), FACS analysis of the cell population (Figure 2C) and BrdU incorporation. At 6 h after the release from the hydroxyurea block, 96% of the cells were BrdU positive (data not shown). This degree of synchrony is also reflected in the FACS analysis at the 8 h time point in which it is evident that the vast majority of cells are replicating DNA. By 12 h following release from the block, DNA synthesis is complete and a fraction of the cells can be seen to re-enter G_1. As seen in Figure 2A, the E2F−Rb complex was clearly detected in the S phase cells, consistent with the assays of growth stimulated cell extracts. Moreover, the E2F−Rb complex persisted throughout S phase and remained beyond the time that the E2F−cyclin A complex was lost. There was a decline in the final sample, suggesting that the level of the E2F−Rb complex may fluctuate as a function of the cell cycle, again consistent with analyses of elutriated cell fractions (Shirodkar *et al.*, 1992). It is clear, however, that the interaction of E2F with Rb does not change as cells pass from G_1 into S phase. Thus, at a


Fig. 2. The E2F−Rb complex persists through S phase. **A.** CV1 cell cultures were arrested at the beginning of S phase by a double hyroxyurea block as described in Materials and methods. Cells were then released from the block and samples taken at the indicated times. Extracts were prepared and assayed for E2F binding activity. Each sample was assayed alone or in the presence of a cyclin A antisera, an Rb antisera or following incubation with deoxycholate (DOC). **B.** Thymidine incorporation. Cultures were labeled with [^{3}H]thymidine for 1 h beginning at the indicated time following release from the HU block. Incorporation was measured as described in methods. **C.** FACS analysis. Aliquots of cells taken at the indicated times following release from the HU block were stained with propridium iodide and then analyzed by FACS as described in Materials and methods.
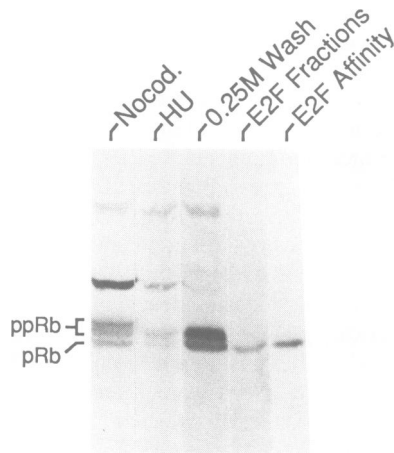
Fig. 3. Only the underphosphorylated Rb protein is found in the S phase E2F−Rb complex. An extract was prepared from CV1 cells synchronized by hydroxyurea as detailed in Materials and Methods and then applied to a heparin agarose column. The column was washed and then eluted with 0.25 M KCl. Following the 0.25 M wash, the column was eluted with a KCl gradient ranging from 0.25−0.5 M. All of the E2F activity eluted with the gradient at ~0.4 M KCl. The E2F-containing fractions were then applied to an E2F DNA affinity column. The column was washed and then eluted as described in Materials and methods. Aliquots of each fraction were concentrated by TCA precipitation and applied to a 10% acrylamide SDS gel. An extract of CV1 cells blocked with nocodazole, which yields a more highly phosphorylated population of Rb, was analyzed in parallel. A blot of the gel was then probed with an RB-specific antibody. pRB, underphosphorylated Rb polypeptide; ppRb, phosphorylated Rb polypeptide.

time when Rb is becoming hyperphosphorylated (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989), the E2F−Rb interaction does not appear to be affected.

One interpretation of the experiments shown in Figures 1 and 2 is that the majority of Rb is not in association with E2F; rather, only the small percentage of Rb that remains underphosphorylated through S phase would be bound to E2F. To address this specifically, we have prepared an extract of CV1 cells that were blocked in S phase with hydroxyurea, fractionated the extract on a heparin−agarose column and then assayed for the Rb protein as well as E2F activity. As shown in Figure 3, analysis of the Rb protein by a Western blot assay revealed the presence of multiple Rb species that previous experiments have shown to reflect the phosphorylation state of Rb. The extract was applied to a heparin−agarose column, which was then washed with 0.25 M KCl and eluted with a KCl gradient. All of the E2F was eluted between 0.4 and 0.5 M KCl. Analysis of the Rb protein in the 0.25 M wash (no E2F activity) or in the fractions containing E2F, revealed that the bulk of the Rb protein eluted in the 0.25 M wash. Moreover, the Rb protein that co-eluted with E2F in the high salt fractions was only the rapidly migrating, presumably underphosphorylated species. That this underphosphorylated Rb was indeed in a complex with E2F was demonstrated by further purifying the E2F on a DNA affinity column. As seen in Figure 3, the underphosphorylated Rb co-purified with E2F. Although we cannot eliminate the possibility that the Rb in association
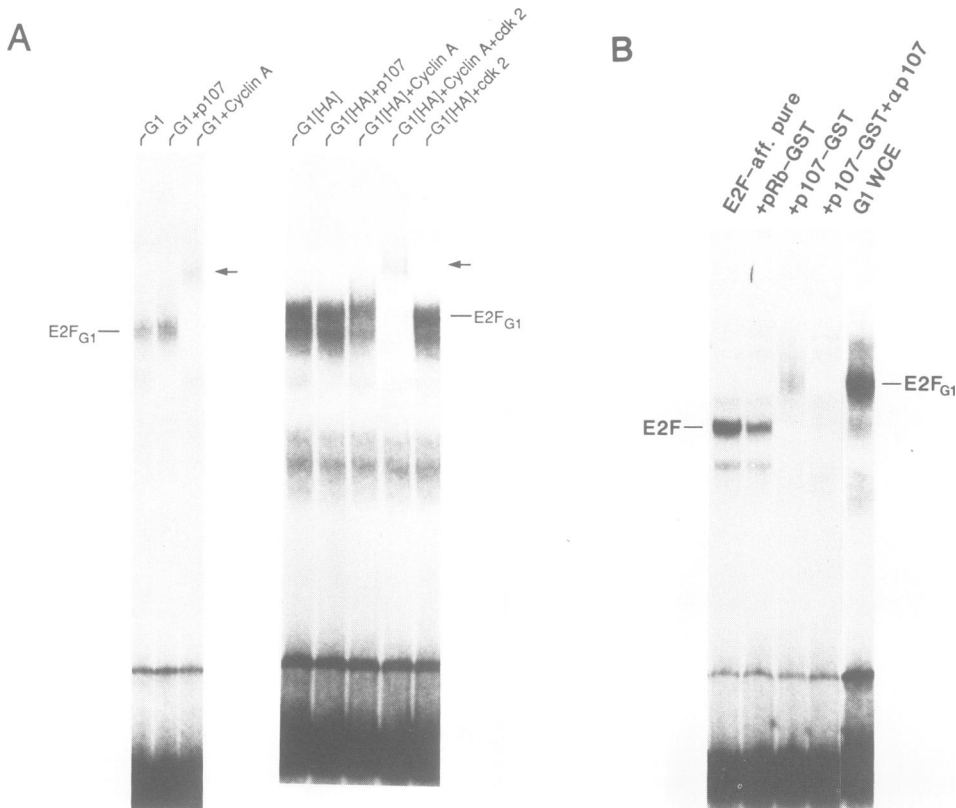
Fig. 4. Role of the p107 protein in formation of E2F complexes. **A.** Reconstitution of an E2F complex with a G₁ extract. Left: an extract prepared from NIH 3T3 cells in G₁ was assayed for E2F DNA binding activity alone or after incubation with a GST−p107 protein or a GST−cyclin A protein. Right: the NIH 3T3 G₁ extract was fractionated on a heparin agarose column and the E2F-containing fractions were isolated. This material [G₁(HA)] was then assayed for E2F DNA binding activity alone, in the presence of GST−p107, GST−cyclin A, GST−cyclin A and GST p33cdk2 or GST−p33cdk2. **B.** Reconstitution of an E2F complex with purified E2F and p107. Affinity-purified E2F was assayed by gel retardation either alone or in the presence of GST−Rb, GST−p107 or GST−p107 plus and αGST−p107 antibody. A sample of a G₁ NIH 3T3 extract was assayed separately to provide a marker for the G₁-specific E2F complex.
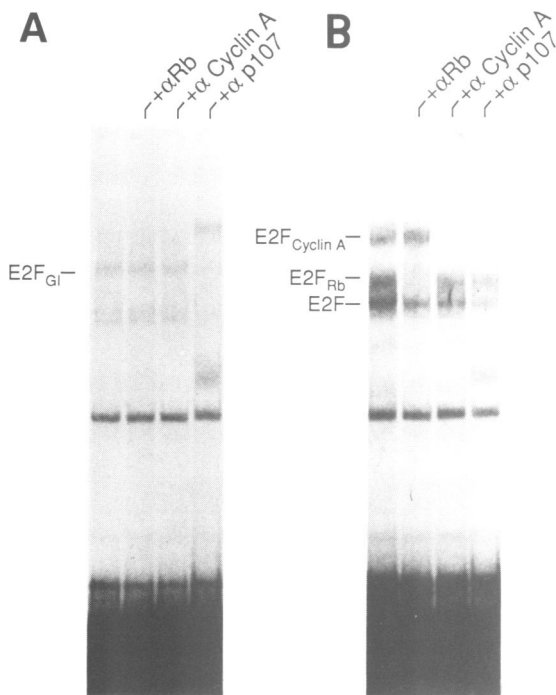
Fig. 5. The E2F$-$G$_1$ complex contains the p107 protein. **Left:** an extract of human Hs68 cells in G$_1$ (8 h following serum stimulation) was assayed for E2F DNA binding activity either alone or in the presence of an Rb antibody, a cyclin A antibody or a p107 antibody. **Right:** An S phase extract of human Hs68 cells (24 h following serum stimulation) was assayed for E2F as in the left panel.

with E2F does become partially phosphorylated in late G$_1$ and S phase, we can say that the phosphorylation events that cause the decrease in electrophoretic mobility do not occur on E2F-bound Rb. We conclude that the Rb protein in association with E2F, even during the S phase of the cell cycle, is the underphosphorylated form of Rb and at that time, represents a small fraction of the Rb protein in the cell, most of which is hyperphosphorylated.

The E2F $-$ G$_1$ complex contains the Rb-related p107 protein

Several observations suggested that the Rb-related p107 protein might be a component of the G$_1$-specific E2F complex. First, addition of a purified GST$-$cyclin A protein to a G$_1$ extract of NIH 3T3 cells resulted in a conversion of the E2F$-$G$_1$ complex to a complex with mobility equivalent to the S phase E2F complex that is known to contain p107 (Figure 4A). In contrast, the addition of a GST$-$p107 protein to the extract had no effect. If the E2F G$_1$ complex was first partially purified by heparin$-$agarose chromatography and then used for the assay, the addition of the cyclin A protein was without an effect. However, the addition of a GST$-$cdk2 protein along with cyclin A again resulted in the formation of the complex. Since the only component of the previously characterized S phase complex that was not required for formation of this complex was the p107 protein, this result strongly implies that the G$_1$ complex contains the p107 protein.

A direct assay for the interaction of p107 with E2F is shown in Figure 4B. The addition of a purified GST$-$p107
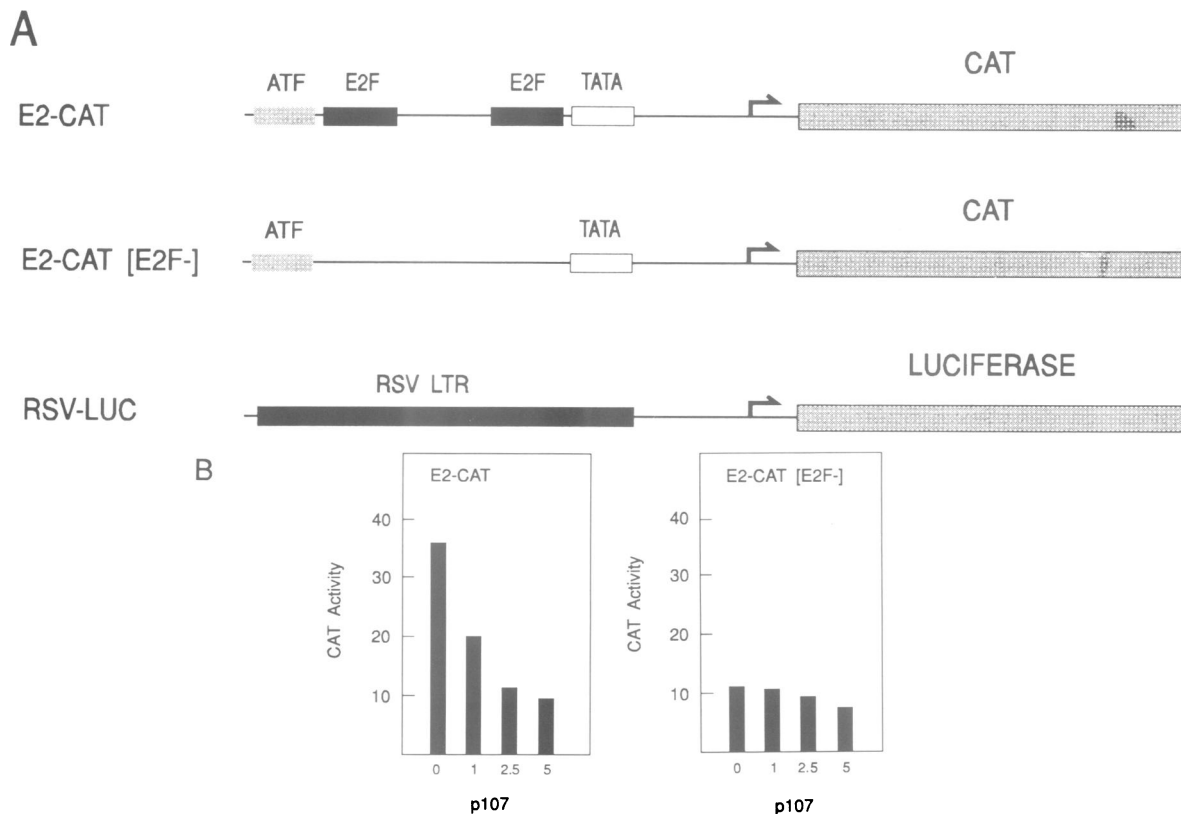
Fig. 6. The p107 protein inhibits E2F-dependent transcription activation. **A.** Plasmids. The E2$-$CAT and E2$-$CAT (E2F$-$) plasmids are depicted and have been described previously by Hiebert *et al.* (1992). The RSV$-$luc plasmid contains the luciferase gene under the control of the RSV LTR. **B.** CAT assays. The human cervical carcinoma C33A cell line was transfected with E2$-$CAT or E2$-$CAT (E2F$-$) (9.7 μg) together with RSV$-$luc (4 μg) and increasing amounts as indicated (in μg) of the p107-expressing plasmid. Extracts were prepared 48 h after transfection and assayed for CAT activity as well as luciferase activity. CAT activity is expressed as a percentage conversion, normalized to the luciferase activity in the same sample.

protein to a preparation of affinity-purified E2F from HeLa cells generated a complex with mobility identical to that of the E2F$-$G$_1$ complex. A caveat in this experiment is the use of a GST$-$p107, rather than native p107. Nevertheless, the apparent molecular weight of the p107 fusion protein is very close to the same as that of the native cellular p107. The observation that affinity-purified free E2F and p107$-$GST form a complex of identical mobility to the native E2F$-$G$_1$ complex suggests that E2F and p107 may be the only components of the G$_1$ complex. The observation that the purified G$_1$ complex only binds cyclin A in the presence of cdk2 is consistent with this interpretation. Moreover, the ability of the GST$-$p107 protein to form a complex with affinity-purified E2F is in contrast to the result with the Rb protein. Although Rb can form the E2F$-$Rb complex with a partially purified E2F preparation, it is unable to do so with affinity-purified E2F (Hiebert *et al.*, 1992). This result suggests the need for an additional component to allow the formation of the E2F$-$Rb complex. In contrast, p107 and E2F alone appear to be sufficient for complex formation.

To provide direct evidence for the presence of p107 in the G$_1$ complex, we have assayed an extract that contains the G$_1$ complex in the presence of antiserum raised against the p107 protein. As shown in Figure 5, the addition of a control antiserum had no effect on the E2F$-$G$_1$ complex. However, addition of the p107-specific antiserum reduced the intensity of the complex and resulted in the appearance of a slower migrating complex. This effect was eliminated by incubating the antiserum with the GST$-$p107 protein, but not with the GST$-$Rb protein (data not shown). We therefore conclude that the E2F complex found in quiescent cells and cells that progress during G$_1$ contains the Rb-related p107 protein.

The p107 protein inhibits E2F-dependent transcription

Previous experiments have shown that the interaction of the Rb protein with E2F coincides with an inhibition of E2F-dependent transcription in a co-transfection assay (Dalton, 1992; Hamel *et al.*, 1992; Hiebert *et al.*, 1992; Weintraub *et al.*, 1992). We have employed a similar assay to determine whether the expression of the p107 protein also affects E2F-dependent transcription. To assay for E2F$-$dependent transcription, we used a CAT gene that is under the control of the adenovirus E2 promoter and compared the activity from this construct with one in which the two E2F sites are mutated (Figure 6A). A human cervical carcinoma cell line (C33A) that lacks a functional Rb gene and that contains a significant amount of free, uncomplexed E2F (Chellappan *et al.*, 1992) was transfected with the E2$-$CAT or E2$-$CAT(E2F$-$) plasmid together with increasing amounts of a p107 expression vector. As a control for transfection efficiency and non-specific effects of p107, an internal control was included in each transfection consisting of a luciferase gene under the control of the RSV LTR. As shown in Figure 6B, co-transfection of the p107 plasmid resulted in an inhibition of expression of the E2$-$CAT gene. In contrast, there was little effect on expression of the E2$-$CAT gene lacking E2F sites. We therefore conclude that p107 inhibits E2F-dependent transcription, at least when assayed on the adenovirus E2 promoter.

Discussion

The experiments we report here describe a complex series of changes in the interactions involving the E2F transcription factor as a function of the cell proliferative response. These data, together with previous results, now define three distinct E2F$-$containing complexes: one complex contains the p107 protein, another contains the Rb protein and the third contains p107 together with cyclin A and cdk2. Moreover, these experiments demonstrate that the p107 protein regulates the transcriptional activity of E2F.

Control of E2F During G$_1$

Our previous experiments identified an E2F complex that accumulated during the G$_1$ phase of the cell cycle and then disappeared in late G$_1$ (Mudryj *et al.*, 1991). The experiments reported here demonstrate that the Rb-related p107 protein is a component of this G$_1$ complex and may in fact be the only component along with E2F. The fact that the two other E2F-containing complexes contain either the p107 protein or the related Rb protein, together with the observation that purified E2F and purified p107 can interact to form a complex, suggests that it is the p107 protein or the Rb protein that make direct contact with E2F. In this context, p107 may then serve the function of a scaffold during S phase by bringing the cyclin A$-$cdk2 complex to E2F. Indeed, other experiments demonstrate a direct interaction between p107 and cyclin A that is dependent on sequences that are unique to p107 and that are not involved in the binding to viral oncoproteins (Ewen *et al.*, 1992). Moreover, recent experiments have also demonstrated an E2F complex, restricted to the G$_1$ phase of the cell cycle, which contains p107, cyclin E and the cdk2 kinase (Lees *et al.*, 1992). This finding thus raises the possibility the one role of p107 is to bring distinct cyclin-dependent kinase complexes in association with E2F at specific points in the cell cycle. Nevertheless, our experiments demonstrate that this must not be the only function for the p107 protein in that the G$_1$ complex we detect appears to contain only p107 in association with E2F.

Previous experiments have shown that the release of E2F from an interaction with p107$-$cyclin A$-$cdk2 stimulated the capacity of E2F to activate transcription (Mudryj *et al.*, 1991). The experiments we report here now directly demonstrate that the p107 protein inhibits E2F-dependent transcription. Based on these results, we speculate that the function of the G$_1$-specific interaction of p107 with E2F may be to control the capacity of E2F to function as a transcription factor.

Given the fact that the G$_1$ complex involves the p107 protein and the S phase cyclin A$-$cdk2 complex also involves the p107 protein, one might imagine that upon the synthesis of cyclin A at the beginning of S phase, the G$_1$ complex would simply convert into the S phase complex. Although this simple mechanism remains a possibility, we have also observed instances in which the G$_1$ complex is lost and the S phase complex has yet to be formed (Mudryj *et al.*, 1991). This result might suggest a more complicated mechanism requiring the disassembly of the G$_1$ complex and then the re-assembly of the S phase complex. Possibly there is an activity that functions at the end of G$_1$ in a manner similar to the E1A protein, targeting the G$_1$

complex and causing its dissociation. Of course, such an activity would have to possess specificity for the E2F−p107 complex and not dissociate the E2F−Rb complex.

The E2F−Rb interaction

A series of recent studies have demonstrated an interaction between the E2F transcription factor and the Rb protein. Other reports have identified interactions between Rb and other proteins, such as c-myc (Rustgi *et al.*, 1991), but the only evidence for an *in vivo* Rb target, as defined by the existence of a complex in a cell extract, is with E2F. Moreover, a variety of experiments have shown a close correlation between Rb function and its capacity to interact with E2F, implicating E2F as an important functional target for Rb (Qin *et al.*, 1992). Initial speculation centered on a role of Rb in controlling the availability of active E2F at the G_1/S transition. Specifically, since only the underphosphorylated Rb is found in the E2F complex and given the change in phosphorylation of the Rb protein at the G_1/S transition (Buchkovich *et al.*, 1989; Chen et al., 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989), it appeared that E2F might be released from the interaction with Rb as a result of this phosphorylation. However, as we now show here, E2F is not released from the interaction with Rb at the end of G_1, but rather persists through S phase. It is true that uncomplexed E2F accumulates at the end of G_1, but this cannot be due to the release of E2F from the Rb interaction. A more likely source for the generation of the free E2F at late G_1 is the release from the p107-containing G_1-specific complex. Alternatively, new synthesis of E2F at this time could contribute as well.

As already discussed, the phosphorylation of Rb is unlikely to be a mechanism for releasing E2F from an inactive complex, since at the time that the majority of the Rb protein in the cell is undergoing phosphorylation, the E2F−Rb complex persists. It would appear that the Rb protein in association with E2F is not a substrate for the kinase that phosphorylates Rb resulting in the observed electrophoretic mobility shift. Although this phosphorylation of Rb may not disrupt the E2F−Rb complex, it is likely that the hyperphosphorylation of Rb would prevent the interaction with E2F, a conclusion consistent with the recent analyses of the interaction of the product of a presumptive E2F clone with Rb (Helin *et al.*, 1992). A further indication of the preferential interaction of underphosphorylated Rb with E2F comes from an analysis of an Rb mutant that cannot be phosphorylated. This mutant was found to be significantly more efficient in repressing E2F-dependent transcription than the wild type Rb protein (Hamel *et al.*, 1992). We note that there is a substantial accumulation of uncomplexed E2F at the time when the bulk of the Rb protein becomes phosphorylated. Clearly, the results presented here lead to the conclusion that the Rb−E2F complex is not the source of this E2F. The G_1-specific p107−E2F complex is certainly a potential source, although it is also possible that under some circumstances new synthesis of E2F contributes to the accumulation, since there is an accumulation of the RNA products of the E2F clone following stimulation of cell proliferation (Kaelin *et al.*, 1992). Regardless, if this uncomplexed E2F is indeed functionally significant for cell cycle transcription, a purpose of the phosphorylation of Rb

at this time may be to prevent further interaction with E2F and thus preserve the pool of active E2F.

Finally, what might be the functional significance of the interaction of Rb with E2F? Previous experiments have shown that the interaction of Rb with E2F in transfection assays correlates with the inhibition of the transcriptional activity of E2F (Hiebert *et al.*, 1992). Moreover, recent experiments have demonstrated that Rb can repress transcription of the c-myc promoter (Hamel *et al.*, 1992) as well as the cdc2 promoter (Dalton, 1992) and that in each case this repression is dependent on the E2F site found in the respective promoters. Interestingly, the kinetics of accumulation of the E2F−Rb complex during the proliferative response are roughly coincident with the kinetics of decline in transcription of the c-myc gene following activation after serum addition. Thus, one potential role for the interaction of Rb with E2F may be as a negative regulator of transcription of cellular genes such as c-myc. In this view, the E2F−Rb complex is not inert, but rather plays an active role in transcription control, a suggestion consistent with recent experiments, which have shown that the E2F site can act as a negative *trans* element, dependent on Rb function (Weintraub *et al.*, 1992). It is therefore possible that the role of the E2F−Rb interaction is to regulate the level of expression of genes such as c-myc that are important for cellular proliferation.

Materials and methods

Cells

Extracts

E2F assays

Affinity column isolation of E2F

GST fusion proteins

Plasmids encoding the p107–GST (aa 252–936), cyclin A–GST and cdk2–GST fusion proteins were kindly provided by M.Ewen, E.Lees and L.Tsai, respectively. Bacteria were grown at 23°C to an A_{600} of 0.4–0.6 and expression-induced with 0.1 mM IPTG for 90 min. Bacteria were lysed with lysozyme, DNase I, Tween and Triton, and bacterial debris was removed by centrifugation. To isolate fusion proteins, the lysate was applied to glutathione (GST)–Sepharose (Pharmacia), the column washed in 10 mM DTT, 1% Tween, 250 μM PMSF, PBS and the proteins eluted with 10 mM GST in 50 mM Tris, pH 9.6. The proteins were then extensively dialyzed into 0.5 M NaCl, 5 mM DTT and 0.2 M Tris, pH 8.2 and stored as frozen aliquots.

In vitro reconstitution of E2F complexes

Both whole cell extracts and biochemically fractionated extracts were used in reconstitutions. The purified GST fusion proteins (10–100 ng) were added to the standard E2F binding assay reaction mixture. The conditions for assaying E2F binding were as described previously by Chellappan et al. (1991).

Antibodies

Polyclonal antiserum were raised in rabbits using GST–cyclin A and GST–p107 fusion proteins. Antisera to GST–p107 was partially purified using protein A–Sepharose chromatography. Monoclonal antibody to Rb was purchased from Oncogene Science (Rb AB-1).

Transfection assays

The human cervical carcinoma cell line C33A, which lacks functional Rb, was employed for calcium phosphate transfection assays, using the E2–CAT and E2 (E2F–) CAT plasmids as described previously by Hiebert et al. (1992). The amounts indicated in Figure 6 were brought to a total DNA concentration of 24 μg with the addition of pGEM4 DNA. CAT activity was measured as described previously by Gorman et al. (1982). An RSV–luciferase plasmid was included in each transfection as an internal control. Luciferase activity was measured as described previously by deWet et al. (1987) with the exception that cells were lysed in 0.25 M Tris to which was added a similar volume of 200 mM potassium phosphate buffer, pH 7.8 at the time of the assay. The luciferase activity of the E2–CAT sample was used to normalize the remaining samples. The p107 expression vector (pCMV4/p107) was constructed as follows. The p107 insert (aa 122 to the C-terminus) was obtained via PCR of a GST–p107 expression plasmid kindly provided by Dr M.Ewen. The 5' PCR primer (5'-GCGCGCGCG-CGCGTCGACAGATCTACCATGGAACTGCATGATGGACTTCTCG-TAGAA-3') contained SalI and BglII restriction sites as well as a start codon and a Kozak consensus sequence for translation initiation. The 3' PCR primer (5'-GCGCGCGCGCGCGCAAGCTTTTAATGATTTGCTCTTTCACT-3') contained a Hind III restriction site. The BglII and HindIII sites were used to clone the insert into the pCMV4 expression vector (Andersson et al., 1989).

Acknowledgements

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