The EMBO Journal vol.13 no.14 pp.3329 – 3338, 1994

Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation

Paramjeet Singh, Siew Heng Wong and Wanjin Hong[1]

Membrane Biology Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511, Singapore
[1]Corresponding author

Communicated by P.Chambon

The transcription factor E2F has been implicated in controlling the activation of multiple genes associated with cell proliferation. E2F-1, which is a component of E2F, can promote oncogenesis when transfected into REF cells. The transformation caused by E2F-1 correlates with constitutive overexpression of the transgene, increased transcription of E2F-dependent genes and the enhancement of two E2F DNA binding complexes containing the retinoblastoma susceptibility gene product (Rb) and E2F-1. The oncogenic potential of E2F-1 is dependent on functional DNA binding and transactivation domains but does not require the ability to interact directly with Rb. These findings provide the first direct evidence that sustained unregulated expression of E2F-1 can lead to the loss of cell proliferation control and that E2F-1 is a key component in cell cycle control.

Key words: E2F-1/neoplastic transformation/retinoblastoma protein/

Introduction

The transcription factor E2F, also called DRTF1 (differentiation-regulated transcription factor 1), was initially identified as a cellular factor participating in the activation of the adenovirus E2 promoter through interactions with two identical elements within the promoter (Nevins, 1986; Kovesdi *et al.*, 1986, 1987; Yee *et al.*, 1987; Reichel *et al.*, 1989; Marton *et al.*, 1990). Its normal cellular function is likely to be the regulation of the cell cycle, since it is implicated in the transcriptional regulation of several growth-responsive genes that participate in DNA synthesis. The E2F binding sequence has been identified in the promoters of c-*myc*, c-*myb*, N-*myc*, dihydrofolate reductase (DHFR), thymidine kinase, DNA polymerase α and *cdc2* genes (Blake and Azizkhan, 1989; Thalmeier *et al.*, 1989; Hiebert *et al.*, 1989, 1991; Mudryj *et al.*, 1990; Pearson *et al.*, 1991; Dalton, 1992; Nevins, 1992); the products of which are associated with entry into S phase and DNA synthesis. Significantly, the E2F binding sites have proved necessary for the activation of c-*myc* transcription in response to serum stimulation (Mudryj *et al.*, 1990) and for the increased expression of the DHFR gene at the G_1/S boundary (Means *et al.*, 1992). This variation of E2F site-containing gene expression with the cell cycle would require a corresponding regulation of E2F activity. This regulation is presumably achieved through the interaction of E2F with multiple cellular proteins (Nevins, 1992). E2F has been shown to form specific multiprotein complexes with the retinoblastoma susceptibility protein (Rb), p107 (a homologue of Rb), cyclins and cdk2; the formation and dissociation of these complexes correlate with progression through the cell cycle (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Pagano *et al.*, 1992; Shirodkar *et al.*, 1992). Rb and p107, both of which exhibit anti-proliferative activity, have been shown to inhibit the transactivation activity of E2F (Hiebert *et al.*, 1992; Schwarz *et al.*, 1993; Zhu *et al.*, 1993). Growth inhibition by Rb also appears to be mediated, at least in part, through interactions with E2F (Qin *et al.*, 1992). Furthermore, conditions that prevent Rb from interacting with E2F, e.g. mutations in the E2F binding 'pocket' of Rb and the adenovirus E1A (or other viral oncoproteins)-mediated dissociation of the Rb–E2F complex (Bandara and La Thangue, 1991), are associated with human tumours (Marshall, 1991) and cell transformation (Raychaudhuri *et al.*, 1991). Together, these observations suggest a growth promoting role for E2F which is negatively regulated by Rb and p107 through the suppression of its transactivation activity.

E2F itself appears to be a multicomponent transcription factor (Shivji and La Thangue, 1991), requiring a heterodimeric complex for efficient binding of DNA in a sequence-specific manner (Huber *et al.*, 1993). Recently, two distinct polypeptides of comparable size and structural organization, E2F-1 and DP-1, were each shown to bind E2F sites on DNA (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992; Girling *et al.*, 1993) and together form at least part of the transcriptionally active E2F complex (Helin *et al.*, 1993b; La Thangue and Johnston, 1993). Like the E2F complex, transactivation mediated by E2F-1 is also inhibited by direct association with Rb (Flemington *et al.*, 1993). However, E2F-1 appears to be significantly less abundant than DP-1 and exists in only a subset of the E2F complexes (La Thangue and Johnston, 1993). The expression of E2F-1 is also linked to the cell cycle, occurring predominantly at the G_1/S boundary and in the S phase (Shan *et al.*, 1992). All these observations raise the possibility that, besides the interactions of pre-existing complexes with cellular proteins like Rb and p107, *de novo* synthesis of E2F-1 might contribute to the overall regulation of E2F activity during the cell cycle. We investigated whether the deregulated overexpression of E2F-1 could by itself lead to neoplastic transformation. Our results provide a direct demonstration that sustained overexpression of E2F-1 results in loss of cell proliferation control and cell transformation. Furthermore, our biochem-

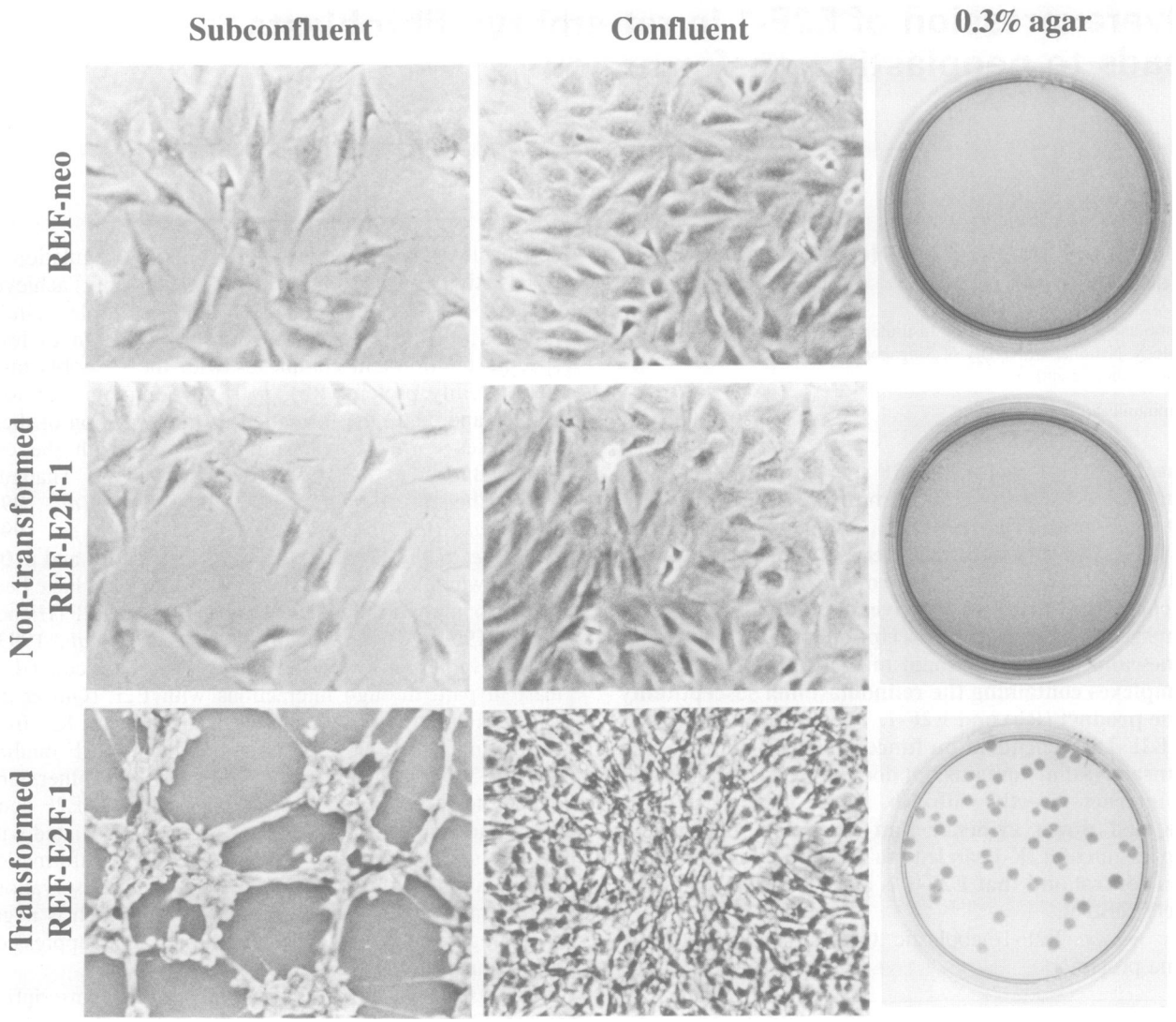

Fig. 1. Morphology and colony-forming ability of transfected rat embryo fibroblasts. Cultures growing in DME with 10% fetal calf serum were photographed at subconfluent and confluent stages. Colony formation in 60 mm diameter dishes shown here is 21 days after 100 cells were seeded in 0.3% agarose in DME with 10% fetal calf serum.

ical characterization of the transformed cells as well as mutagenesis of E2F-1 provide significant insight into the mechanistic process underlying the E2F-1-mediated cell transformation.

Results

Transformation of REF by E2F-1

Rat embryo fibroblast (REF) cells were chosen for this study because they display the characteristics of normal fibroblastic cells, namely a flat morphology and anchorage-dependent growth which is inhibited in a density-dependent manner. The REF cells also take up DNA fairly well and display a low background transformation rate during cloning and drug selection for markers on the introduced extraneous DNA. The E2F-1 cDNA was placed under the control of the CMV promoter by introducing it as an *Eco*RI–*Sac*I fragment into the G418-selectable mammalian expression vector $pXJ41_{neo}$ (Zheng *et al.*, 1992). This plasmid was transfected into REF cells which were replated at one tenth density after 2 days and selected for DNA uptake by including G418 in the growth medium. Individual colonies were isolated and replated at cloning densities to obtain secondary clones which were expanded into cell lines. In all cases, the initial and secondary clones exhibited identical morphology and growth characteristics.

In four independent transfections, the CMV vector and CMV–E2F-1 construct yielded approximately equal numbers of stable transfectants, a total of 61 REF-neo and 72 REF-E2F-1 respectively. Of these, only the 14 REF-neo and 16 REF-E2F-1 obtained from the first transfection were investigated further. All 14 REF-neo clones displayed the adherent flat cell morphology of the parental cell line and remained as tight monolayers when the cultures were confluent (Figure 1). On the other hand, only seven of the 16 REF-E2F-1 clones displayed the normal morphology of REF. The others exhibited a smaller, spindle-shaped morphology with many rounded and refractile cells and a tendency to form foci at subconfluent densities (Figure 1). These clones adhered less strongly to the culture vessel and also tended to form short strings

Table I. Phenotype of transfected rat embryo fibroblasts

	Neomycin resistant colonies	Contact inhibition	Growth in soft agarose	Growth in 0.1% serum	Doubling time in 1% serum (h)	Tumorigenicity in nude mice
REF-neo Untransformed	14	+	–	–	120	0/5
REF-E2F-1 Transformed	9	+	–	–	120	0/5
REF-E2F-1	7	–	+	+	48	5/5

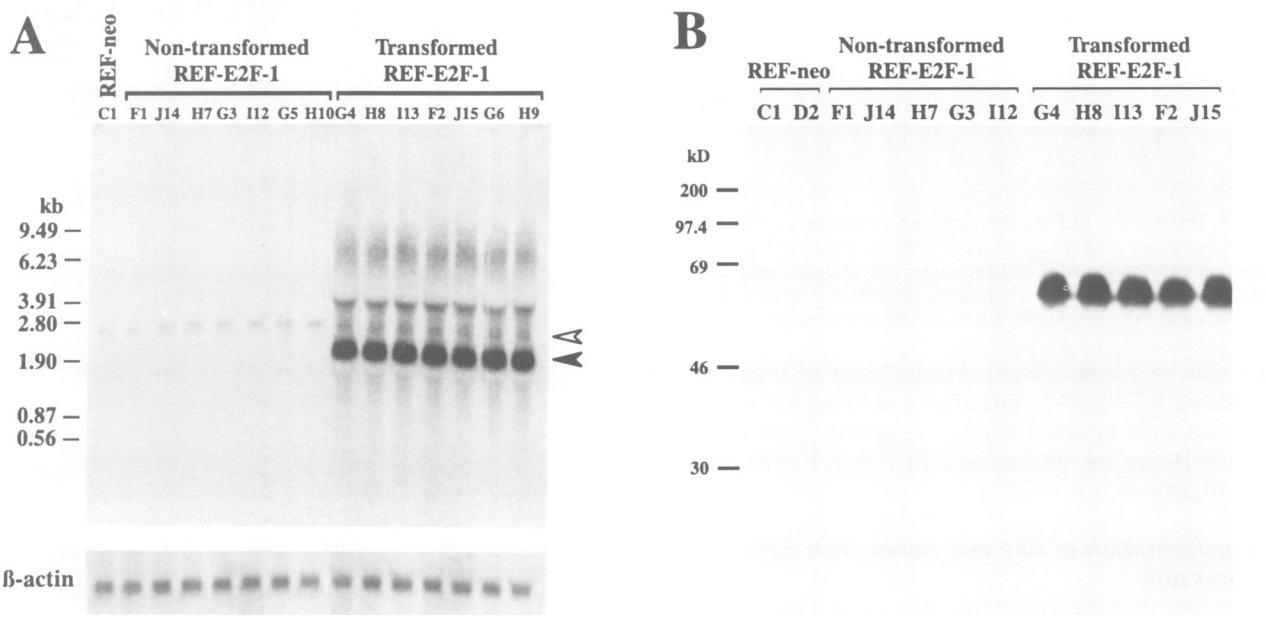

Fig. 2. Transformation of REF correlates with E2F-1 expression. (**A**) Twenty micrograms of total RNA from each transfected REF cell line were fractionated by agarose gel electrophoresis, blotted onto a nylon membrane and hybridized with ^{32}P-labelled E2F-1 cDNA. The hollow arrow indicates the endogenous 2.6 kb E2F-1 message and the filled arrow indicates the transcript from the transfected E2F-1 cDNA. The blot was stripped of bound E2F-1 probe and rehybridized with labelled actin cDNA to normalize for loading inequalities across the tracks. (**B**) Ninety micrograms of protein in total cell extracts from each transfected REF cell line were resolved by SDS–PAGE, transferred to nitrocellulose and probed with monoclonal antibody against E2F-1. The membrane was developed with ^{125}I-labelled protein A and visualized through autoradiography at –70°C. E2F-1 appears as a broad band of ~60 kDa size.

and bunches of cells, the latter often growing as free-floating masses. Confluent cultures rapidly progressed to disorganized multilayers resulting from continued growth of the cells.

The morphologically transformed REF-E2F-1 clones also displayed anchorage-independent growth as assessed by growth in soft agar. These clones were able to form colonies in agarose (Figure 1) ranging in concentration from 0.3% to 1.2% with an apparent cloning efficiency of ~50%. Neither the REF-neo clones nor the morphologically non-transformed REF-E2F-1 transfectants formed detectable colonies in the soft agar assay. From these results, we arrived at a preliminary conclusion that a fraction of the REF-E2F-1 transfectants exhibited the characteristics of uncontrolled growth and transformation.

E2F-1 transformed cells are tumorigenic

The transformation of the CMV-E2F-1 transfectants was further supported by their growth characteristics. The morphologically transformed REF-E2F-1 cells displayed a reduced requirement for serum growth factors evidenced by their continued proliferation in media with 0.1% fetal calf serum, whereas the unaltered REF-E2F-1 and REF-neo cell lines became quiescent (Table I). Under reduced serum conditions (1%), the altered REF-E2F-1 cells also displayed an accelerated rate of proliferation with a doubling time of only 48 h compared with 120 h for the unaltered REF cell lines, although this difference was less marked in 10% serum. Unlike the REF-neo and unaltered REF-E2F-1 cells, no apparent saturation density could be recorded for the morphologically transformed REF-E2F-1 lines as proliferation in culture continued beyond confluence and the cells eventually lifted off the dishes in sheets. This indicated that these cells were not inhibited by contact with neighbouring cells and grew in a density-independent manner. Taken together, the anchorage-independent growth in low serum and their lack of contact inhibition qualify the morphologically altered REF-E2F-1 cells as fully transformed.

To determine if the transformed REF-E2F-1 cells were also tumorigenic, 5×10^5 cells from each clone were introduced subcutaneously into 4-week-old female athymic mice. After a latency of 2 weeks, tumours appeared at the site of injection of the transformed REF-E2F-1 cells,

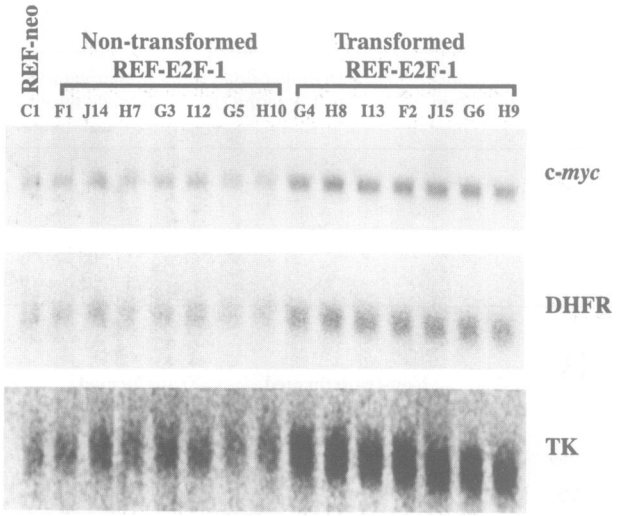

Fig. 3. Transformed REF exhibit increased expression of E2F-dependent genes. The nitrocellulose membrane shown in Figure 2 was reprobed sequentially with labelled c-*myc*, dihydrofolate reductase and thymidine kinase cDNAs after stripping the previous probe. The hybridized probe was quantified with the PhosphorImager.

whereas no tumours were detected with REF-neo or the unaltered REF-E2F-1 cells after 2 months. No secondary tumours were detected during the period of these experiments. Hence the transformed REF-E2F-1 cells are also tumorigenic.

Transformation of REF correlates with E2F-1 expression

To resolve the observation that only slightly more than half of the E2F-1 transfected clones displayed a transformed phenotype, the expression of the transgene in each clone was analysed. Analysis of total RNA with the E2F-1 cDNA as a hybridization probe revealed that although all clones exhibited low levels of the 2.6 kb endogenous E2F-1 mRNA transcript, only the transformed REF-E2F-1 lines showed expression of the transgene (Figure 2A). This was seen as an intense band of ~1.9 kb and appeared to be expressed at similar levels in all the transformed REF-E2F-1 clones. This E2F-1 transgene expression, which was quantified to be >10 times in excess of the endogenous gene, did not affect the expression of the endogenous E2F-1 gene in the transformed cells compared with the REF-neo or unaltered REF-E2F-1 transfectants. Thus the endogenous E2F-1 gene expression is not subject to autoregulation.

Immunoblot analysis for the presence of the E2F-1 polypeptide yielded results consistent with the RNA analysis. Specific monoclonal antibodies detected large amounts of E2F-1 in total extracts of transformed REF-E2F-1 cells, whereas the same protein was barely detectable in identical extracts of REF-neo and unaltered REF-E2F-1 (Figure 2B). The overexpressed E2F-1, estimated by densitometric scanning to be ~20 times in excess of the endogenous levels, migrated in SDS–PAGE with an apparent molecular weight of ~60 kDa and exhibited some trailing. In some experiments, especially when significantly less protein was loaded, E2F-1 was detected as a doublet with another band of ~57 kDa (Figure 7B, lane 10) consistent with earlier observations (Helin *et al.*,

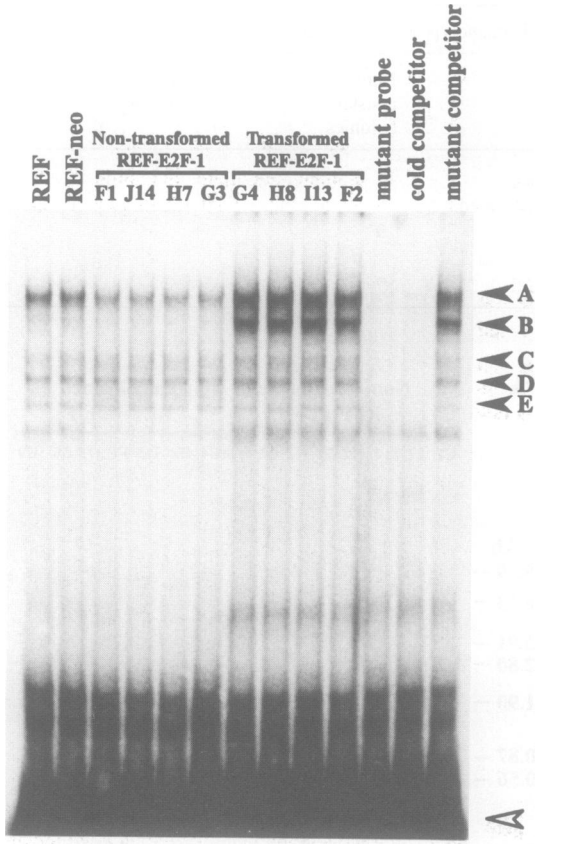

Fig. 4. Transformed REF exhibit enhancement of selected E2F DNA binding complexes. Twenty micrograms of total cell protein were incubated with a ^{32}P-labelled synthetic E2 promoter fragment, resolved by polyacrylamide electrophoresis and the DNA binding complexes detected and quantified with the PhosphorImager. Specificity of the complexes was determined with labelled mutant E2 promoter DNA (lane 11) and competition with excess unlabelled E2 promoter and mutant DNA fragments (lanes 12 and 13). The filled arrows indicate the specific E2F DNA binding complexes and the hollow arrow shows the excess unbound probe.

1992; Kaelin *et al.*, 1992). Thus the overexpressed E2F-1 protein in the transformed REF-E2F-1 cells exhibits physical properties similar to the endogenous protein. Furthermore, the transformation of REF cells clearly correlates with the overexpression of E2F-1.

Overexpression of E2F-1 results in increased expression of E2F-dependent genes

The increased levels of E2F-1 in the transformed cells might be expected to affect the overall E2F transactivation activity in these cells. We assessed this by analysing the expression of selected E2F-dependent genes. Total RNA from the various transfectants was hybridized sequentially with cDNA probes for c-*myc*, thymidine kinase and DHFR. All three genes examined were expressed at increased levels in the transformed REF-E2F-1 cells compared with REF-neo and the unaltered REF-E2F-1 (Figure 3). The enhanced expression in the transformed cells was most notable with thymidine kinase where a 6-fold increase in its message was detected, whereas the expression of c-*myc* and DHFR genes was increased ~2-fold. Thus the presence of high levels of E2F-1 in the transformed REF cells apparently results in the increased expression of the E2F-dependent genes, although the nominal changes

observed may very well reflect the increased growth rates of the transformed cells.

Enhancement of two E2F DNA binding complexes in transformed cells

We analysed the E2F DNA binding activity in the various REF clones to determine if increased levels of the transcription factor were present to account for higher expression of the target genes. Total cell extracts were mixed with a ^{32}P-labelled double-stranded oligonucleotide probe representing the E2F binding site within the adenovirus E2 promoter (Shirodkar *et al.*, 1992) and the protein–DNA complexes were resolved by non-denaturing acrylamide gel electrophoresis. In all the REF-neo and REF-E2F-1 cell lines, the same five distinct E2F complexes (A, B, C, D and E) were detected and these were specific for the E2F probe, as determined through the use of a labelled non binding mutant probe and competition with unlabelled homologous probe (Figure 4). However, the levels of the slower migrating complexes A and B were significantly increased in the transformed REF-E2F-1 as compared with the non-transformed lines (Figure 4, compare lanes 1–6 with lanes 7–10). This increase in the DNA binding capacity was estimated by densitometric scanning to be five times for complex A and 15 times for complex B, which in some experiments appeared as a doublet (data not shown). Both complexes A and B were converted to C, D and E in the presence of 0.6% DOC, suggesting that the latter were components of A and B (Figure 5, lanes 2 and 7). Consistent with earlier reports (Kaelin *et al.*, 1992; Shan *et al.*, 1992), antibodies specific for E2F-1 had little effect on the complexes in the non-transformed cells, suggesting that little, if any, E2F-1 was present in these complexes. However, in the transformed REF-E2F-1, the complexes A and B were both essentially abolished (Figure 5, lanes 3–4 and 8–9), indicating that E2F-1 is a major component of these complexes. These complexes, which are present in the non-transformed cells at significantly lower amounts, appear to have incorporated the overexpressed E2F-1 in the transformed cells. Thus the other components of these complexes are not limiting and are available for complexing with the overexpressed E2F-1. Antibodies specific for Rb abolished complex B and reduced complex A, with the concomitant appearance of a complex migrating more slowly than complex A (Figure 5, lanes 5 and 10). Thus both complexes A and B also contain Rb in addition to E2F-1. Treatment with antisera specific for other components known to exist in E2F complexes did not yield any conclusive results, but their presence in complexes A and B cannot be dismissed, since faint, severely retarded complexes were detected with antibodies specific for cyclin E and cdc2 but without abolishing or reducing any of the complexes (data not shown).

Transforming activity of E2F-1 is independent of its Rb binding domain but requires the DNA binding and transactivation domains

The enhancement of the Rb-containing complexes A and B in the transformed cells raised the possibility that high levels of E2F-1 could compete effectively for Rb by binding directly to it (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). This might eliminate free Rb or

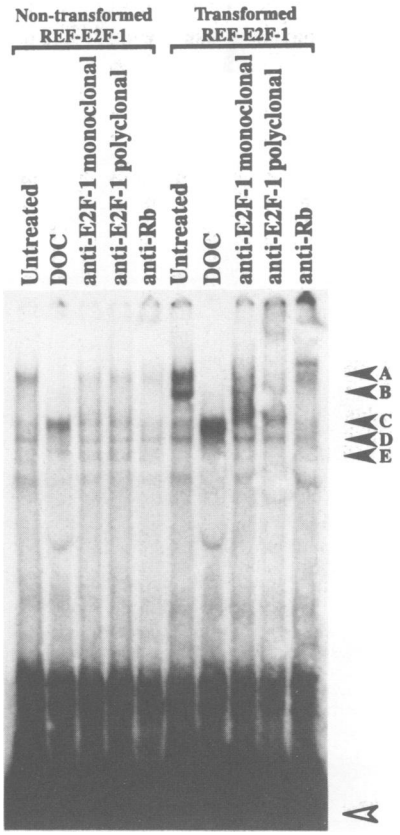

Fig. 5. The enhanced E2F DNA binding complexes A and B in transformed REF contain E2F-1 and Rb. Total cell extracts were treated for 30 min with 0.6% DOC or antibodies against E2F-1 or Rb before analysis for E2F DNA binding activities. The filled arrows indicate the specific E2F DNA binding complexes and the hollow arrow shows the excess unbound probe.

even extract it from a complex or complexes where it is required for regulation, with the overall effect of lifting the Rb suppression on E2F activity and causing uncontrolled cell growth. We tested this possibility by transfecting REF cells with cDNA coding for an E2F-1 mutant, E2F-1(1–416) which lacks the C-terminal 21 amino acids. This mutant has been documented as being incapable of binding Rb by itself but retains considerable transactivation activity (Shan *et al.*, 1992; Helin *et al.*, 1993a). Twelve independent REF clones with stable integration of E2F-1(1–416) were isolated and analysed for characteristics of transformation. Of these, five were unaltered in morphology and growth characteristics, whereas the remaining seven displayed a morphology indistinguishable from the transformed REF-E2F-1 (Figure 6A). These morphologically altered cells were confirmed to be indeed transformed by virtue of their growth in low serum and soft agarose and tumorigenicity in nude mice (data not shown). Interestingly, when the REF-E2F-1(1–416) clones were examined for their E2F DNA binding activities, the pattern obtained was similar to that seen with REF-E2F-1 (Figure 6B). The non-transformed REF-E2F-1(1–416) displayed the five complexes with relative levels similar to unaltered REF, whereas the transformed clones exhibited increased amounts of complexes A and B. Both complexes A and B contained E2F-1(1–416), since both polyclonal and monoclonal antisera to E2F-1 were able to disrupt these

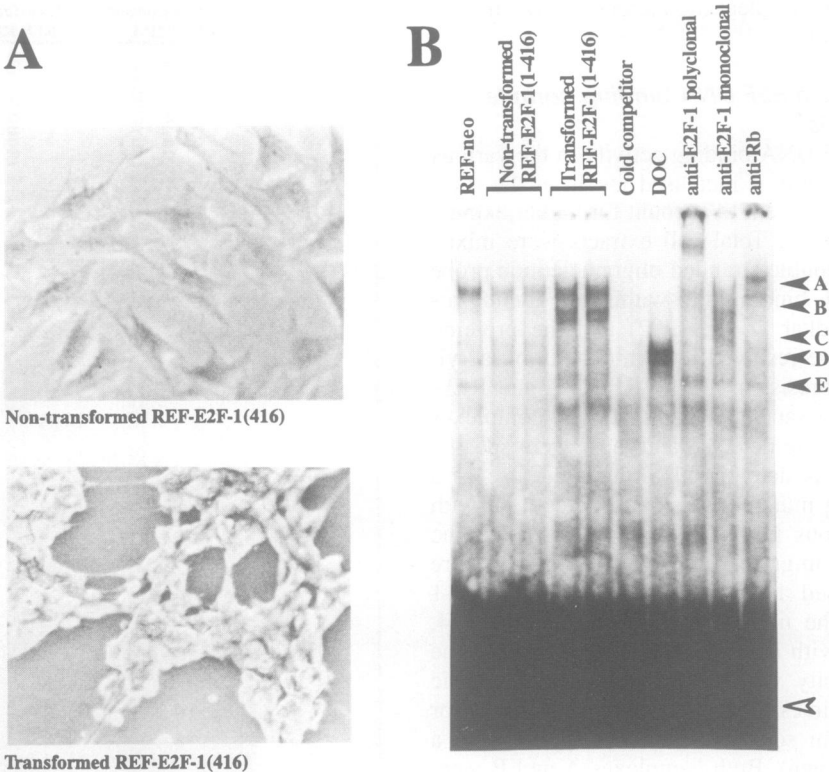

Fig. 6. REF expressing E2F-1(1–416) are transformed and exhibit enhanced E2F DNA binding complexes A and B. (**A**) Morphology of transformed and non-transformed REF-E2F-1(1–416) at subconfluent density. Northern analysis indicated that the transformed REF-E2F-1(1–416) expressed the transfected E2F-1(1–416) cDNA but the non-transformed REF-E2F-1(1–416) did not (data not shown). (**B**) Total cell extracts from two clones each of transformed and non-transformed REF-E2F-1 were analysed for E2F DNA binding activities. Specificity of the DNA binding complexes was determined with excess unlabelled E2 promoter DNA. The presence of E2F-1(1–416) and Rb was assessed by pretreating the cell extracts with specific antibodies. The filled arrows indicate the specific E2F DNA binding complexes and the hollow arrow shows the excess unbound probe.

complexes. Surprisingly, both complexes A and B were abolished by antibodies to Rb, indicating that Rb was again present in these complexes. Since E2F-1(1–416) does not bind directly to Rb (Shan *et al.*, 1992), its association with Rb could be mediated by other components in the complex. Thus the transforming potential of E2F-1 does not require its direct binding to Rb.

We further transfected REF cells with cDNA for another mutant of E2F-1, E2F-1(DBM), which is altered from amino acids 124 to 134 within the DNA binding domain and was designed to be deficient in DNA binding. This was confirmed earlier by mixing the *in vitro* translated E2F-1(DBM) with the ^{32}P-labelled E2 promoter fragment. No DNA binding was detected (Figure 7A) although the wild type E2F-1 clearly bound DNA under the same conditions. When nine stable clones of REF-E2F-1(DBM) were examined, none displayed an altered morphology, ability to grow in soft agarose or low serum, foci formation or tumorigenicity in nude mice (data not shown). Immunoblot analysis revealed that most clones were expressing E2F-1(DBM), with at least four exhibiting levels equal to or higher than E2F-1 in the transformed REF-E2F-1 (Figure 7B). In addition to the absence of transformation, the DNA binding pattern was identical to that of untransformed REF and enhancement of complexes A and B was not detected (Figure 7C). Thus, although E2F-1(DBM) is overexpressed, its inability to bind DNA renders it unable to transform REF cells, even with a functional Rb binding domain. Therefore, the depletion of Rb and the consequent derepression of endogenous E2F activity cannot be the mechanism for transformation of REF cells by deregulated overexpression of E2F-1. Hence the transforming potential of E2F-1 is associated with its DNA binding activity but not a functional Rb binding domain.

Since E2F-1 possesses a functional transactivation domain within residues 368–437 (Kaelin *et al.*, 1992; Shan *et al.*, 1992), we investigated its involvement in the transformation of REF caused by overexpression of E2F-1. REF cells were transfected with a truncated cDNA of E2F-1, E2F-1(1–284), which codes for the first 284 amino acids of E2F-1. This completely removes the transactivation domain (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992) but the resulting polypeptide is still capable of binding DNA (data not shown). Of the total of 18 stable transfectants isolated and analysed, none exhibited any characteristics of cell transformation. Using a subset of nine randomly selected REF-E2F-1(1–284) clones, it was obvious that some of these expressed the E2F-1(1–284) transcript at levels equal to or higher than the E2F-1 message in the transformed REF-E2F-1 (data not shown). Furthermore, the E2F DNA binding activity profile of five out of these nine clones was indistinguishable from that of the transformed REF-E2F-1, complete with increased levels of complexes A and B (Figure 8A). These complexes clearly contained E2F-1(1–284), since antisera directed against amino acids 369–437 at the C-terminus of E2F-1 (Kaelin *et al.*, 1992) was unable to disrupt them (Figure 8B). In the event, polyclonal antibodies against

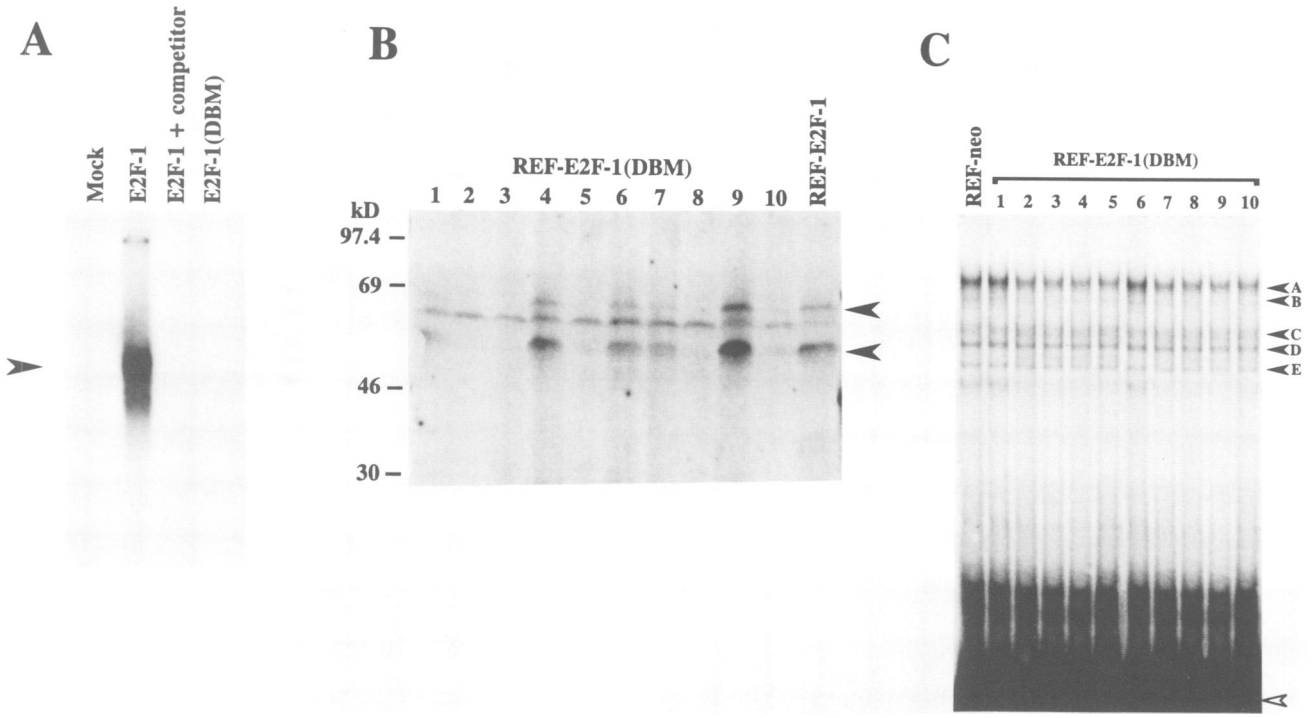

Fig. 7. Expression of the non-transforming, DNA binding deficient E2F-1(DBM) does not lead to enhancement of E2F complexes A and B. (**A**) Ten microliters of *in vitro* translated E2F-1 and E2F-1(DBM), altered across residues 124–150, were tested for E2F DNA binding activity against mock translated extract. Specificity of the DNA binding complex was determined with excess unlabelled E2 promoter DNA. The arrow indicates the specific E2F-1/DNA complex. (**B**) Twenty micrograms of total protein from 10 REF-E2F-1(DBM) clones and one transformed REF-E2F-1 were resolved by SDS–polyacrylamide electrophoresis, blotted onto nitrocellulose, probed with monoclonal antibody against E2F-1 and developed with ^{125}I-labelled protein A. The blot was visualized and quantified with the PhosphorImager. The arrows indicate the 60 kDa and 57 kDa doublet resulting from expression of E2F-1 and E2F-1(DBM). The band across all tracks between the 60 and 57 kDa proteins was observed in some experiments. (**C**) Total cell extracts of REF-E2F-1(DBM), containing 20 μg protein, were tested for E2F DNA binding activity. The filled arrows indicate the specific E2F DNA binding complexes and the hollow arrow shows the excess unbound probe.

the full length E2F-1 also did not affect the complexes (Figure 8), although they reacted with E2F-1(1–284) in immunoblot analysis of the *in vivo* and *in vitro* translated protein. The residual portion of the truncated E2F-1(1–284) in complexes A and B could possibly be inaccessible to antibodies, probably because the remaining domains are those involved in dimerization and DNA binding. However these complexes were still sensitive to 0.6% DOC and contained Rb since they were disrupted by antibodies against Rb. Thus the incorporation of the transactivation deficient E2F-1(1–284) into complexes A and B enables DNA binding functionality but does not lead to transformation of the host cells, suggesting that cell transformation by sustained levels of E2F-1 is associated with its direct transactivation activity.

Discussion

The data presented in this report relate to whether the deregulated overexpression of E2F-1 in primary cells could cause cell transformation and its underlying mechanism. We chose rat embryo fibroblasts for our studies because these cells exhibit the growth characteristics of primary fibroblasts. In addition, REF cells have a low background transformation rate and are refractory to transformation by most single oncogenes (Weinberg, 1989). This property is particularly attractive, since transformation by a single factor must then result from the perturbation of a process fundamental to the maintenance of the normal cellular state, rather than the additive or synergistic effects of a subpotent stimulus and a pre-existing latent defect. Although our study is based on overexpression, this does not limit our interpretations and conclusions, because only the wild-type E2F-1 and E2F-1(1–416), but not E2F-1(DBM) or E2F-1(1–284) expressed at similar levels, could lead to cell transformation. Our preliminary studies also suggest that a comparable expression of DP-1, another component of E2F, has no effect on the growth characteristics (data not shown).

The E2F-1 gene was placed under the control of the strong constitutive promoter of CMV and introduced into REF cells in a neomycin-selectable vector. The stable transfectants were analysed for cell transformation based on changes in morphology, altered growth characteristics and tumorigenicity in nude mice. In the event, about half of the transfectants were confirmed to be transformed with an altered morphology. The transformation of REF-E2F-1 cells was clearly associated with overexpression of the transfected E2F-1 cDNA, the expression of which was similar in all the transformed clones. Since only E2F-1 was transfected and overexpressed, it follows then that this is sufficient to cause uncontrolled growth and transformation of REF cells. The overexpression of E2F-1, in all probability, resulted in increased E2F activity, detected as enhanced expression of the E2F-dependent genes associated with entry into and progression through the S phase. This increased expression of the E2F-dependent genes suggests that the provision of E2F-1 alone is sufficient to

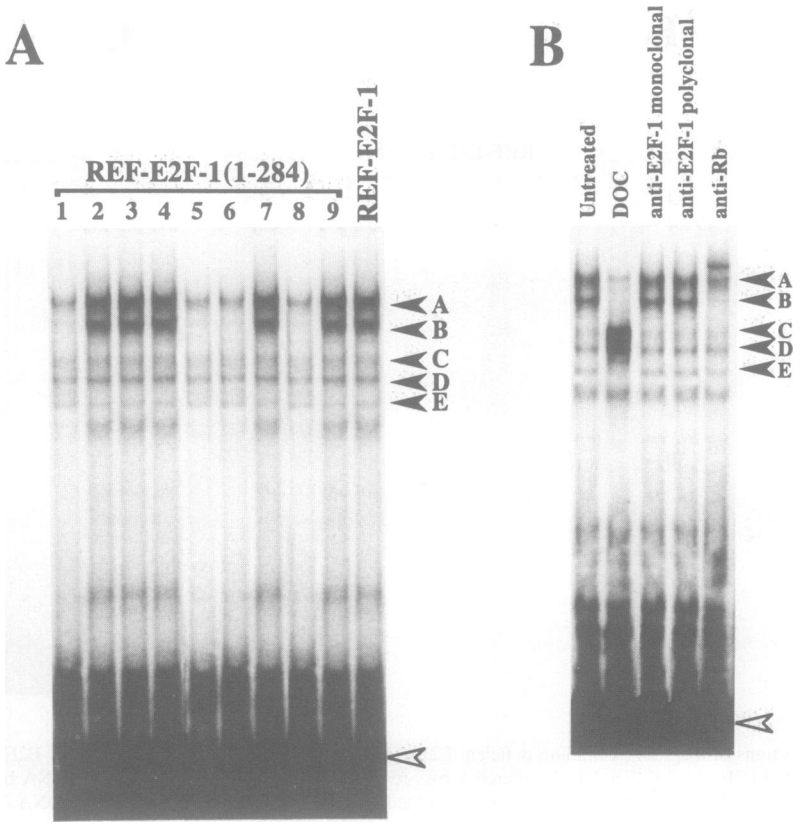

Fig. 8. The non-transforming E2F-1(1–284) lacking the Rb binding and transactivation domains incorporates into and enhances the E2F complexes A and B. (**A**) Total cell extracts of REF-E2F-1(1–284), containing 20 μg protein, were tested for E2F DNA binding activity. The filled arrows indicate the specific E2F DNA binding complexes and the hollow arrow shows the excess unbound probe. (**B**) The cell extract from clone 7 of REF-E2F-1(1–284) was pretreated with DOC or antibodies against E2F-1 or Rb. The arrows indicate the specific E2F DNA binding complexes.

elicit E2F activity. It is unclear whether the increased expression of the E2F-dependent genes we detect is a cause or consequence of the entry into S phase, but it appears that the constitutive expression of E2F-1 drives REF cells that would otherwise have remained quiescent to enter S phase independently of mitogenic signals and would be sufficient for the observed uncontrolled growth of these cells. It is tempting to extrapolate from this that one of the normal physiological functions of E2F-1 may be to trigger the entry of cells into the S phase in response to appropriate growth signals. This is consistent with a report that appeared while this manuscript was in preparation, which showed that transient overexpression of E2F-1 alone is sufficient to cause quiescent cells to enter S phase (Johnson *et al.*, 1993), although this study did not evaluate the effect of E2F-1 on endogenous gene expression, E2F DNA binding complexes or cell growth. Our work extends this by showing unequivocally that sustained levels of E2F-1 do not merely induce cells to enter S phase, but are also capable of driving the cell division cycle through all stages.

Two potential explanations for the E2F-1-mediated cell transformation are the direct activation of E2F-responsive genes by overexpressed E2F-1 or the sequestration of Rb which results in the release of other Rb-bound factors. Several lines of evidence suggest that the latter is unlikely. First, E2F-1(1–416), in which the Rb binding domain was deleted, is still transforming. Secondly, mutation of the DNA binding region of E2F-1 resulted in a mutant which contains the Rb binding domain but is no longer transforming. Thus the transformation activity of E2F-1 is closely associated with the DNA binding but not the Rb binding activity of E2F-1, supporting the first hypothesis. Furthermore, the transactivation domain of E2F-1 is essential for this transforming activity, since the transactivation domain deletion mutant E2F-1(1–284) could be incorporated into complexes A and B but was non-transforming. It thus emerges that direct binding of E2F-1 to the target genes and the direct interaction of its transactivation domain with the cellular transcriptional machinery are the major events leading to the transformation of the transfected cells. However, this does not eliminate the possibility that Rb could also be a target of the transforming event.

Although five distinct E2F DNA binding complexes are detected in the transformed cells, only complexes A and, especially, B contain E2F-1 as their principal component, since they could be selectively abolished by antibodies against E2F-1. The correlation between E2F-1-mediated transformation and the marked enhancement of complexes A and B indicates that these complexes may be directly involved in the enhanced E2F-responsive gene expression and cell transformation. The presence of Rb, a reported suppressor of E2F-1-mediated transactivation (Flemington *et al.*, 1993), raises the query as to how these complexes could participate in such activities. It seems that incorporation of Rb into complexes A and B does not involve its direct interaction with E2F-1, because deletion of the Rb

binding domain of E2F-1 in both E2F-1(1–416) and E2F-1(1–284) has no apparent effect on the incorporation of Rb into complexes A and B. It is thus possible that other endogenous proteins associated with complexes A and B play a dominant role in recruiting Rb into these complexes, leaving the DNA binding and transactivation domains of E2F-1 accessible for extraneous interactions. The resulting complexes could bind effectively to E2F sites on target genes and *trans*-activate their expression. This is possible because additional cellular Rb binding proteins, including other components of E2F complexes, have been identified (DeCaprio *et al.*, 1988; Dyson *et al.*, 1989, 1990; Münger *et al.*, 1989; Defeo-Jones *et al.*, 1991; Lees *et al.*, 1993; Ivey-Hoyle *et al.*, 1993) and more are expected to be found. Our observations raise the possibility that the Rb-containing complexes A and B may be transcriptionally active and responsible for the transformed state of REF cells overexpressing E2F-1, especially since Rb has been observed to exert a positive effect on transcription (Kim *et al.*, 1992a,b; Bocco *et al.*, 1993; Udvadia *et al.*, 1993). This explanation is not consistent with the current view that 'free' E2F is the major driving complex for E2F-responsive gene expression (Moran, 1991; Mudryj *et al.*, 1991; Nevins, 1992; Schwarz *et al.*, 1993). This 'free E2F' concept is derived mainly from correlation of 'free' E2F with transcriptional activation of a limited and very well defined E2F-responsive reporter construct in transient transfection assays with Rb and p107 (Dalton, 1992; Hiebert *et al.*, 1992; Weintraub *et al.*, 1992) or following dissociation of E2F complexes with viral oncoproteins (Mudryj *et al.*, 1991). The response of such a transiently transfected reporter system could be compromised by its presence in excessive copies, absence of a proper chromosomal environment and the lack of flanking sequences, and thus not represent the complicated cellular process of E2F-responsive gene expression. This is especially so since E2F-responsive genes exhibit differential transcriptional activation with respect to the cell cycle (Helin and Harlow, 1993). Further investigations of the regulation of endogenous E2F-dependent genes with regard to E2F DNA binding complexes should provide a clearer insight into the important but complicated cell cycle regulatory events.

Materials and methods

Cell culture and transfection

REF cells (a gift from C.J.Pallen's laboratory, IMCB) were maintained in Dulbeco's modified Eagle's minimal essential medium (DME) supplemented with 10% fetal bovine serum. DOTAP (*N*-[1-(2,3-dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; Boehringer Mannheim) was used according to the manufacturer's instructions for transfection of cells. Forty-eight hours after transfection, the cells were replated at one tenth density in media with 400 μg/ml G418 sulfate (Life Technologies). Well-isolated colonies were selectively removed with cloning cylinders and replated at low densities to obtain secondary colonies which were then expanded into cell lines.

Growth in soft agar

One thousand or one hundred cells were plated on separate 60 mm diameter dishes in 0.3–1.2% agar in complete medium on a bed of 1.5% agar in complete medium. After 21 days, the colonies were scored and photographed.

Saturation density, serum requirement and growth in low serum

Approximately 5×10^4 cells were plated on 60 mm diameter dishes and incubated overnight. The old medium was then removed and the cells were washed twice with serum-free medium and fed with DME containing fetal calf serum at 0.1% (serum requirement studies), 1% (low serum growth studies) or 10% (saturation density studies). Over the next 7 days, triplicate dishes were scored daily by dispersing the cells with trypsin, staining with trypan blue and counting with a haemocytometer. Doubling times were determined by analysis of semilogarithmic plots, using data from the exponential phase of growth. Saturation density was estimated from dishes that exhibited no increase in cell numbers over 3 consecutive days.

Tumorigenicity in nude mice

Cells were dispersed with trypsin, washed three times with phosphate-buffered saline (PBS) to remove serum and resuspended at 1×10^6 cells/ml in PBS. About 1×10^5 cells were then injected subcutaneously into 3-week-old athymic mice. Tumour occurrence and size was monitored weekly for up to 3 months.

Construction of E2F-1 plasmids

The mammalian expression vector used was $pXJ41_{neo}$ (Zheng *et al.*, 1992) which carries the strong constitutive CMV promoter for high level expression of transgenes and the neomycin gene to confer resistance to G418 sulfate for selection of stable transfectants. The cDNA for E2F-1 has been described previously (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). The E2F-1 cDNA was placed under the control of the CMV promoter by ligating the *Eco*RI–*Sac*I(blunt-ended) cDNA fragment with the *Eco*RI–*Xho*I(blunt-ended) $pXJ41_{neo}$ to form pXJ41-E2F-1. pXJ41-E2F-1(1–416), which expresses a truncated E2F-1, amino acids 1–416, was created by replacing the *Xho*I–*Xho*I fragment of pXJ41-E2F-1 with a *Xho*I linker carrying translation termination codons in all three frames. pXJ41-E2F-1(1–284), which expresses another truncated form of E2F-1 consisting of the N-terminal 284 amino acids, was constructed by cloning the *Eco*RI–*Bgl*II fragment of the cDNA into the *Eco*RI–*Xho*HI treated $pXJ41_{neo}$ via a *Bgl*II–*Xho*I adapter with translation termination codons. The DNA binding-deficient mutant E2F-1(DBM), with a frameshift mutation (codons 124–150) in the putative DNA binding domain, was created by replacing the *Sma*I–*Sal*I fragment of the E2F-1 cDNA with a homologous synthetic DNA. This fragment was designed with an additional guanine nucleotide after codon 123 but lacked the cytosine nucleotide of codon 150 and the two termination codons (TGA) that would have appeared in the frame if the E2F-1 cDNA were changed to tryptophan codons (TGG).

Analysis of gene expression

Messenger RNA levels were assessed by Northern blot analysis of total RNA (Ausubel *et al.*, 1993) with ^{32}P-labelled probes generated from appropriate cDNA fragments. For determination of protein levels of E2F-1, cells were lysed in 10 mM Tris, pH 8.0, 0.5% SDS and 5 mM EDTA and the protein content determined with the BCA protein assay reagent (Pierce). Equal amounts of protein were fractionated in each track of denaturing polyacrylamide gels, electroblotted onto nitrocellulose membranes, probed with monoclonal and polyclonal E2F-1 antibodies (Upstate Biotechnology), which have been described before (Kaelin *et al.*, 1992), and developed with ^{125}I-labelled protein A (Ausubel *et al.*, 1993). Radioactive membranes were quantified with storage phosphor screens (PhosphorImager, Molecular Dynamics) and the electronic images output directly to a film recorder (Lasergraphics Personal LFR).

In vitro translation

About 5 μg plasmid DNA that had been linearized with an appropriate restriction enzyme was transcribed in a 50 μl reaction with the Promega kit using T7 RNA polymerase. The RNA transcripts from each reaction were purified and translated in rabbit reticulocyte lysate. Both procedures were according to protocols provided by Promega.

E2F DNA binding assays

E2F electrophoretic mobility shift assays were performed with total cell extracts (Shirodkar *et al.*, 1992) and a ^{32}P-labelled synthetic probe corresponding to the adenovirus promoter (–70 to –32) or a non binding mutant as previously described (Qin *et al.*, 1992; Shirodkar *et al.*, 1992). Where used, 0.6% DOC, followed 20 min later by 1.2% NP-40, was added to the total cell extracts prior to complex formation with the labelled probe. Similarly, the cell extracts were reacted with the E2F-

1 and Rb antibodies (Rb-Ab2, Oncogene Science) 30 min before probe addition.

Acknowledgements

We thank Kristian Helin, Bei Shan and Wen-Hwa Lee for essential reagents used in this study, Zheng Xinmin and Bor Luen Tang for helpful discussions and Sree Devi Menon for considerable assistance with various experiments and critical reading of the manuscript. This work is supported by a research fund (to WH) from IMCB, NUS.

References

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1993) *Current Protocols in Molecular Biology.* Greene Publishing and Wiley Interscience, New York.

Bagchi,S., Weinmann,R. and Raychaudhuri,P. (1991) *Cell*, **65**, 1063–1072.

Bandara,L.R. and La Thangue,N.B. (1991) *Nature*, **351**, 494–497.

Blake,M.C. and Azizkhan,J.C. (1989) *Mol. Cell. Biol.*, **9**, 4994–5002.

Bocco,J.L., Reimund,B., Chatton,B. and Kedinger,C. (1993) *Oncogene*, **8**, 2977–2986.

Cao,L., Faha,B., Dembski,M., Tsai,L.H., Harlow,E. and Dyson,N. (1992) *Nature*, **355**, 176–179.

Chellappan,S.P., Hiebert,S., Mudryj,M., Horowitz,J.M. and Nevins,J.R. (1991) *Cell*, **65**, 1053–1061.

Chittenden,T., Livingston,D.M. and Kaelin,W.G.,Jr (1991) *Cell*, **65**, 1073–1082.

Dalton,S. (1992) *EMBO J.*, **11**, 1797–1804.

DeCaprio,J.A., Ludlow,J.W., Figge,J., Shew,J.-Y., Huang,C.-M., Lee,W.-H., Marsilio,E., Paucha,E. and Livingston,D.M. (1988) *Cell*, **54**, 275–283.

Defeo-Jones,D., Huang,P.S., Jones,R.E., Haskell,K.M., Vuocolo,G.A., Hanobik,M.G., Huber,H.E. and Oliff,A. (1991) *Nature*, **352**, 251–254.

Devoto,S.H., Mudryj,M., Pines,J., Hunter,T. and Nevins,J.R. (1992) *Cell*, **68**, 167–176.

Dyson,N., Howley,P.M., Münger,K. and Harlow,E. (1989) *Science*, **243**, 934–937.

Dyson,N., Bernards,R., Friend,S.H., Gooding,L.R., Hassell,J.A., Major,E.O., Pipas,J.M., Vandyke,T. and Harlow,E. (1990) *J. Virol.*, **64**, 1353–1356.

Flemington,E.K., Speck,S.H. and Kaelin,W.G.,Jr (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6914–6918.

Girling,R., Partridge,J.F., Bandara,L.R., Burden,N., Totty,N.F., Hsuan,J.J. and La Thangue,N.B. (1993) *Nature*, **362**, 83–87.

Helin,K. and Harlow,E. (1993) *Trends Cell Biol.*, **3**, 43–46.

Helin,K., Lees,J.A., Vidal,M., Dyson,N., Harlow,E. and Fattaey,A. (1992) *Cell*, **70**, 337–350.

Helin,K., Harlow,E. and Fattaey,A. (1993a) *Mol., Cell Biol.*, **13**, 6501–6508.

Helin,K., Wu,C.-L., Fattaey,A.R., Lees,J.A., Dynlacht,B.D., Ngwu,C. and Harlow,E. (1993b) *Genes Dev.*, **7**, 1850–1861.

Hiebert,S.W., Lipp,M. and Nevins,J.R. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 3594–3598.

Hiebert,S.W., Blake,M., Azizkhan,J. and Nevins,J.R. (1991) *J. Virol.*, **65**, 3547–3552.

Hiebert,S.W., Chellappan,S.P., Horowitz,J.M. and Nevins,J.R. (1992) *Genes Dev.*, **6**, 177–185.

Huber,H.E., Edwards,G., Goodhart,P.J., Patrick,D.R., Huang,P.S., Ivey-Hoyle,M., Barnett,S.F., Oliff,A. and Heimbrook,D.C. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3525–3529.

Ivey-Hoyle,M., Conroy,R., Huber,H.E., Goodhart,P.J., Oliff,A. and Heimbrook,D.C. (1993) *Mol. Cell. Biol.*, **13**, 7802–7812.

Johnson,D.G., Schwarz,J.K., Cress,W.D. and Nevins,J.R. (1993) *Nature*, **365**, 349–352.

Kaelin,W.G.,Jr, Krek,W., Sellers,W.R., DeCaprio,J.A., Ajchenbaum,F., Fuchs,C.S., Chittenden,T., Li,Y., Farnham,P.J., Blanar,M.A., Livingston,D.M. and Flemington,E.K. (1992) *Cell*, **70**, 351–364.

Kim,S.J., Onwuta,U.S., Lee,Y.I., Botchan,M.R. and Robbins,P.D. (1992a) *Mol. Cell. Biol.*, **12**, 2455–2463.

Kim,S.J., Wagner,S., Liu,F., O'Reilly,M.A., Robbins,M.A. and Green,M.R. (1992b) *Nature*, **358**, 331–334.

Kovesdi,I., Reichel,R. and Nevins,J.R. (1986) *Cell*, **45**, 219–228.

Kovesdi,I., Reichel,R. and Nevins,J.R. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 2180–2184.

La Thangue,N.B. and Johnston,L.H. (1993) *Curr. Biol.*, **3**, 554–557.

Lees,E., Faha,B., Dulic,V., Reed,S.I. and Harlow,E. (1992) *Genes Dev.*, **6**, 1874–1885.

Lees,J.A., Saito,M., Vidal,M., Valentine,M., Look,T., Harlow,E., Dyson,N. and Helin,K. (1993) *Mol. Cell. Biol.*, **13**, 7813–7825.

Marshall,C.J. (1991) *Cell*, **64**, 313–326.

Marton,M.J., Baim,S.B., Ornelles,D.A. and Shenk,T. (1990) *J. Virol.*, **64**, 2345–2359.

Means,A.L., Slansky,J.E., McMahon,S.L., Knuth,M.W. and Farnham,P.J. (1992) *Mol. Cell. Biol.*, **12**, 1054–1063.

Moran,E. (1991) *Curr. Biol.*, **1**, 281–283.

Mudryj,M., Hiebert,S.W. and Nevins,J.R. (1990) *EMBO J.*, **9**, 2179–2184.

Mudryj,M., Devoto,S.H., Hiebert,S.W., Hunter,T., Pines,J. and Nevins,J.R. (1991) *Cell*, **65**, 1243–1253.

Münger,K., Werness,B.A., Dyson,N., Phelps,W.C., Harlow,E. and Howley,P.M. (1989) *EMBO J.*, **8**, 4099–4105.

Nevins,J.R. (1986) *Crit. Rev. Biochem.*, **19**, 307–322.

Nevins,J.R. (1992) *Science*, **258**, 424–429.

Pagano,M., Draetta,G. and Jansen Durr,P. (1992) *Science*, **255**, 1144–1147.

Pearson,B.E., Nasheuer,H.P. and Wang,T.S. (1991) *Mol. Cell. Biol.*, **11**, 2081–2095.

Qin,X.-Q., Chittenden,T., Livingston,D.M. and Kaelin,W.G.,Jr (1992) *Genes Dev.*, **6**, 953–964.

Raychaudhuri,P., Bagchi,S., Devoto,S.H., Kraus,V.B., Moran,E. and Nevins,J.R. (1991) *Genes Dev.*, **5**, 1200–1211.

Reichel,R., Neill,S.D., Kovesdi,I., Simon,M.C., Raychaudhuri,P. and Nevins,J.R. (1989) *J. Virol.*, **63**, 3643–3650.

Schwarz,J.K., Devoto,S.H., Smith,E.J., Chellapan,S.P., Jakoi,L. and Nevins,J.R. (1993) *EMBO J.*, **12**, 1013–1020.

Shan,B., Zhu,X., Chen,P.L., Durfee,T., Yang,Y., Sharp,D. and Lee,W.H. (1992) *Mol. Cell. Biol.*, **12**, 5620–5631.

Shirodkar,S., Ewen,M., DeCaprio,J.A., Morgan,J., Livingston,D.M. and Chittenden,T. (1992) *Cell*, **68**, 157–166.

Shivji,M.J. and La Thangue,N.B. (1991) *Mol. Cell. Biol.*, **11**, 1686–1695.

Thalmeier,K., Synovzik,H., Mertz,R., Winnacker,E.L. and Lipp,M. (1989) *Genes Dev.*, **3**, 527–536.

Udvadia,A.J., Rogers,K.T., Higgins,P.D.R., Murata,Y., Martin,K.H., Humphrey,P.A. and Horowitz,J.M. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3265–3269.

Weinberg,R.A. (1989) *Cancer Res.*, **49**, 3713–3721.

Weintraub,S.J., Prater,C.A. and Dean,D.C. (1992) *Nature*, **358**, 259–261.

Yee,A.S., Reichel,R., Kovesdi,I. and Nevins,J.R. (1987) *EMBO J.*, **6**, 2061–2068.

Zheng,X.M., Wang,Y. and Pallen,C.J. (1992) *Nature*, **359**, 336–339.

Zhu,L., van den Heuvel,S., Helin,K., Fattaey,A., Ewen,M., Livingston,D., Dyson,N. and Harlow,E. (1993) *Genes Dev.*, **7**, 1111–1125.

Received on March 18, 1994; revised on April 22, 1994