The EMBO Journal vol.12 no.11 pp.4317–4324, 1993

Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F

Lasantha R.Bandara[1], Victoria M.Buck[1,2], Maryam Zamanian[1], Leland H.Johnston[2] and Nicholas B.La Thangue[1,3]

Laboratories of [1]Eukaryotic Molecular Genetics and [2]Yeast Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[3]Corresponding author

Communicated by P.W.J.Rigby

It is widely believed that the cellular transcription factor DRTF1/E2F integrates cell cycle events with the transcription apparatus because during cell cycle progression in mammalian cells it interacts with molecules that are important regulators of cellular proliferation, such as the retinoblastoma tumour suppressor gene product (pRb), p107, cyclins and cyclin-dependent kinases. Thus, pRb, which negatively regulates early cell cycle progression and is frequently mutated in tumour cells, and the Rb-related protein p107, bind to and repress the transcriptional activity of DRTF1/E2F. Viral oncoproteins, such as adenovirus E1a and SV40 large T antigen, overcome such repression by sequestering pRb and p107 and in so doing are likely to activate genes regulated by DRTF1/E2F, such as *cdc2*, c-*myc* and *DHFR*. Two sequence-specific DNA binding proteins, E2F-1 and DP-1, which bind to the E2F site, contain a small region of similarity. The functional relationship between them has, however, been unclear. We report here that DP-1 and E2F-1 exist in a DNA binding complex *in vivo* and that they bind efficiently and preferentially as a heterodimer to the E2F site. Moreover, studies in yeast and *Drosophila* cells indicate that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation.

Key words: cell cycle/DNA binding proteins/transcription factors

Introduction

Several lines of evidence suggest that the cellular transcription factor DRTF1/E2F plays an important role in regulating the cell cycle of mammalian cells. For example, DRTF1/E2F DNA binding activity is periodically induced during cell cycle progression, peaking during S phase (Mudryj *et al.*, 1991; Shirodkar *et al.*, 1992), and negatively regulated during cellular differentiation (La Thangue and Rigby, 1987). This binding activity correlates with the transcriptional activity of certain genes that are necessary for cellular proliferation, such as DHFR, DNA polymerase α and p34^{cdc2}, which contain DRTF1/E2F binding sites in their promoters (Blake and Azizkhan, 1989; Dalton, 1992; Means *et al.*, 1992). Furthermore, the retinoblastoma tumour suppressor gene product (pRb), which negatively regulates cell cycle progression from G_1 into S phase and is frequently mutated in tumour cells, binds to DRTF1/E2F (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991). The functional consequence of this interaction is that pRb prevents DRTF1/E2F from activating transcription (Zamanian and La Thangue, 1992). Several other molecules that are implicated in cell cycle control, such as Rb-related p107, cyclins A and E, and p33^{cdk2} also associate with DRTF1/E2F during cell cycle progression (Bandara *et al.*, 1991, 1992; Mudryj *et al.*, 1991; Devoto *et al.*, 1992; Lees *et al.*, 1992). Taken together, these observations suggest that DRTF1/E2F integrates cell cycle events with the transcription apparatus, ensuring that the cell makes the appropriate changes in gene expression at the correct time during cell cycle progression.

Further evidence for the importance of DRTF1/E2F has come from studies on the mechanism of action of viral oncoproteins. Thus, certain oncoproteins, such as adenovirus E1a, SV40 large T antigen and human papilloma virus E7 regulate the activity of DRTF1/E2F by sequestering pRb and the other associated proteins, converting it from a transcriptionally inactive to an active form (Hiebert *et al.*, 1992; Zamanian and La Thangue, 1992, 1993). Because this effect requires regions in these viral oncoproteins previously shown to be necessary for cellular immortalization and transformation (Bandara and La Thangue, 1991; Zamanian and La Thangue, 1992), it is likely that DRTF1/E2F plays an important role in these processes.

Although progress has been made in identifying the cellular proteins that interact with DRTF1/E2F, until recently, relatively little was known about its molecular details. Two distinct polypeptides which are both DNA binding components of DRTF1/E2F have now been molecularly characterized. The first, referred to as E2F-1, was isolated through its ability to bind directly to pRb, which it does through a C-terminal region (Helin *et al.*, 1992; Kaelin *et al.*, 1992). In contrast, DP-1 was defined as a component of DRTF1/E2F DNA binding activity after biochemically purifying DRTF1 from F9 embryonal carcinoma (EC) stem cells, a cell system in which DRTF1/E2F is down-regulated during the differentiation process (La Thangue and Rigby, 1987; La Thangue *et al.*, 1990). cDNAs that encode DP-1 were isolated after obtaining amino acid sequence from affinity purified DP-1 (Girling *et al.*, 1993).

Both E2F-1 and DP-1 contain a region that allows each polypeptide to bind in a sequence-specific fashion as a homodimer to the E2F motif (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Girling *et al.*, 1993). Although the DNA binding domains are not closely related to any previously defined DNA binding structure they are, nevertheless, distantly related to the DNA binding domains in some yeast cell cycle-regulating transcription factors (La Thangue and Taylor, 1993). The functional relationship between DP-1 and E2F-1 has, however, remained unclear. In this study, we show that DP-1 and E2F-1 exist as a complex *in vivo* which

© Oxford University Press

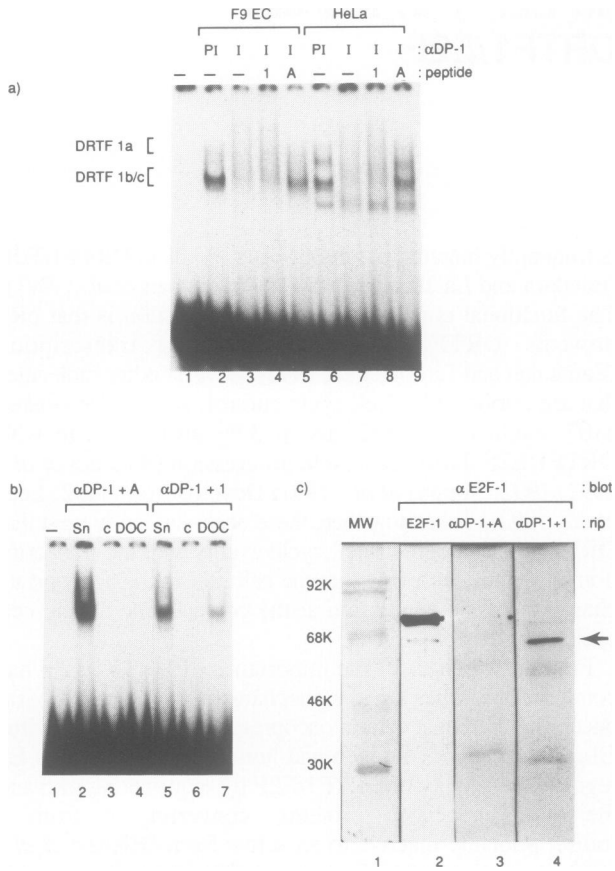

Fig. 1. DP-1 and E2F-1 exist in the same protein complex *in vivo*. (**a**) DP-1 is in DRTF1/E2F DNA binding complexes formed in HeLa cell extracts: gel retardation was performed using F9 EC and HeLa whole cell extracts (in which DRTF1 resolves as three distinct complexes, a, b and c; indicated in figure) with the E2F binding site taken from the adenovirus E2A promoter (nucleotides −71 to −50) in the presence of either preimmune (PI; tracks 2 and 6) or immune (I; tracks 3−5 and 7−9) anti-DP-1 (peptide A) antiserum with the addition of either unrelated peptide 1 (tracks 4 and 8) or peptide A (tracks 5 and 9). In both F9 EC and HeLa cell extracts, all the DRTF1/E2F DNA binding complexes were affected by the anti-DP-1 antibody. (**b**) Anti-DP-1 immunoprecipitates DRTF1/E2F DNA binding activity: immunoprecipitation was performed from HeLa cell extracts with anti-DP-1 in the presence of either homologous peptide A (tracks 2−4) or unrelated peptide 1 (tracks 5−7). The immunoprecipitates were treated with 1% deoxycholate (DOC) and 1.5% NP40, and the detergent-released material assayed for DRTF1/E2F DNA binding activity; the depleted HeLa cell extract is also indicated (Sn; tracks 2 and 5). No DNA binding activity was released in the absence of detergent (indicated by c; tracks 3 and 6). (**c**) Immunoblotting DP-1 immunoprecipitates with anti-E2F-1: anti-DP-1 immunoprecipitates performed in the presence of either peptide A (track 3) or peptide 1 (track 4) were immunoblotted with the anti-E2F-1 monoclonal antibody SQ41; the E2F-1 polypeptide, present in track 4, is indicated by the arrow. As a positive control, ~100 ng of the E2F-1 fusion protein, GST−E2F-1^{89-437}, was immunoblotted in track 2. Track 1 shows standard molecular weights.

recognizes the E2F binding site. Moreover, *in vitro* assays demonstrate that DP-1 and E2F-1 bind efficiently and preferentially as a complex to the E2F site, an interaction which requires the region of similarity between the two proteins. Furthermore, reconstructing DRTF1/E2F in *Drosophila* and yeast cells suggests that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation. These data indicate that DP-1 and E2F-1 can functionally interact and that such an interaction is likely to be physiologically relevant in mammalian cells.

Results

DP-1 and E2F-1 exist as a complex in HeLa cells

DP-1 is a component of DRTF1/E2F which is present in murine developmentally regulated and cell cycle regulated DRTF1/E2F complexes (L.R.Bandara, T.S.Sørensen, M.Zamanian and N.B.La Thangue, in preparation) and thus is likely to be a general component of DRTF1/E2F DNA binding activities. Furthermore, DP-1 is the product of a conserved gene since a closely related protein is expressed in amphibians and *Drosophila* (R.Girling and N.B.La Thangue, in preparation; F.-H.Xu and N.B.La Thangue, in preparation). DP-1 thus appears to be a frequent and evolutionarily conserved DNA binding component of DRTF1/E2F. E2F-1, which was isolated through its ability to bind directly to pRb, also interacts in a sequence-specific fashion with the E2F site (Helin *et al.*, 1992; Kaelin *et al.*, 1992). Both proteins contain a small region of similarity that overlaps domains previously shown to be necessary for sequence-specific DNA binding activity (Girling *et al.*, 1993).

We assessed whether DP-1 and E2F-1 exist as a complex in HeLa cell extracts using antibodies that specifically recognize each protein. Initially, we determined by gel retardation whether DP-1 is a component of HeLa cell DRTF1/E2F. Thus, as in F9 embryonal carcinoma (EC) cell extracts, anti-DP-1 peptide antiserum disrupted HeLa cell DRTF1/E2F in a specific fashion since its effects were competed by including in the binding reaction the homologous, but not an unrelated, peptide (Figure 1a, compare tracks 2−5 with 6−9). Anti-DP-1 antiserum was used to immunoprecipitate DRTF1/E2F from HeLa cell extracts, the immunoprecipitate subsequently being released and then immunoblotted with an anti-E2F-1 monoclonal antibody. The DRTF1/E2F DNA binding activity immunoprecipitated by anti-DP-1 (Figure 1b, compare tracks 4 and 7) contained the E2F-1 protein because immunoblotting the immunoprecipitates with an anti-E2F-1 monoclonal antibody revealed a polypeptide with the molecular weight expected for E2F-1 (Figure 1c, track 4, indicated by arrow). The presence of E2F-1 was dependent upon the anti-DP-1 activity since it was not present when the immunoprecipitation was performed in the presence of the homologous peptide (Figure 1c, compare tracks 3 and 4). Thus, DP-1 and E2F-1 exist as a complex in HeLa cell extracts.

DP-1 and E2F-1 interact in vitro in a DNA binding heterodimer

Both DP-1 and E2F-1 contain sequence-specific DNA binding domains, located in similar positions of each protein (between amino acid residues 84 and 204 in DP-1, and 89 and 191 in E2F-1; Girling *et al.*, 1993), which contain a region of similarity that extends outside of the DNA binding domain, to amino acid residue 249 in DP-1. In agreement with previous studies (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Girling *et al.*, 1993) both DP-1 and E2F-1 alone were able to bind to the E2F site, either in the context of the adenovirus E2A promoter (Figure 2a, tracks 2 and 3) or as a single E2F site (which was apparent on increased exposure of Figure 2a, track 6; data not shown). The DNA binding activity of DP-1 was somewhat less than that of E2F-1, the reasons for which are currently unclear. However, when both proteins were

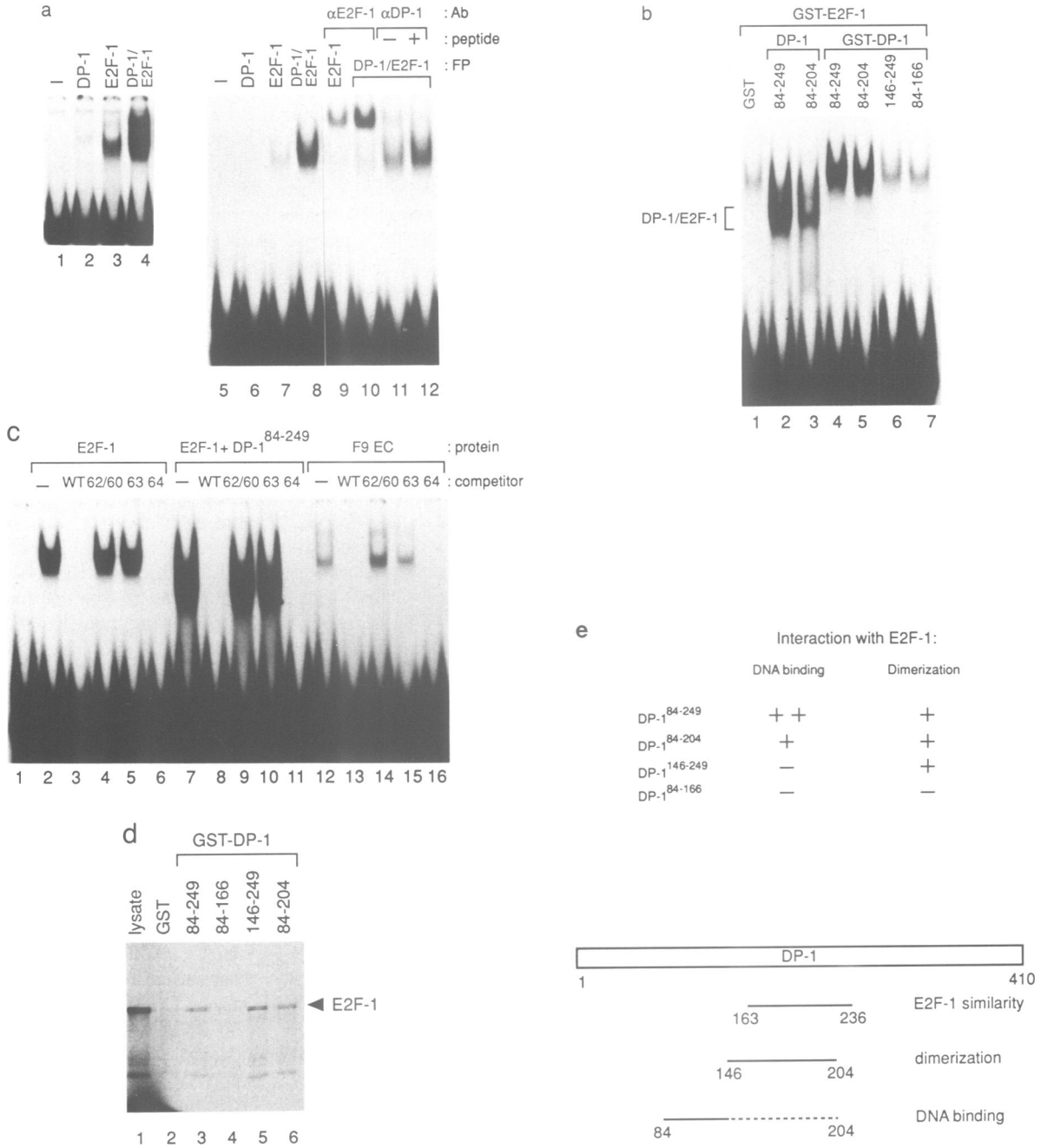

Fig. 2. DP-1 and E2F-1 bind to the E2F site as a complex. (**a**) DP-1 and E2F-1 interact synergistically in DNA binding to the E2F site: GST−DP-1^{59-410} (~25 ng) or GST−E2F-1^{89-437} (~50 ng) were assayed either alone (tracks 2, 3, 6 and 7) or together (tracks 4 and 8) for binding to the adenovirus E2A promoter (tracks 1−4) or the distal E2F site taken from the E2A promoter (tracks 5−8); tracks 1 and 5 show the binding sites alone. Note that a DNA binding complex was apparent in track 6 upon increased exposure (data not shown). The E2F site specificity of the complexes was confirmed by performing the appropriate competition experiments (data not shown). The effect of anti-E2F-1 (tracks 9 and 10) or anti-DP-1 (tracks 11 and 12; anti-peptide 18; Girling *et al.*, 1993) was assessed on GST−E2F-1^{89-437} alone (track 9) or GST−E2F-1^{89-437} and GST−DP-1^{59-410} together (tracks 10, 11 and 12). In addition, the reactions in tracks 11 and 12 contain either an unrelated (track 11) or the homologous (peptide 18; track 12) peptides. (**b**) DP-1 and E2F-1 form DNA binding heteromers: GST−E2F-1^{89-437} (~50 ng) was incubated with a control GST fusion protein (~300 ng; track 1) or DP-1^{84-249} or DP-1^{84-204} (~150 ng, released after cleavage with thrombin; tracks 2 and 3), GST−DP-1^{84-249}, GST−DP-1^{84-204}, GST−DP-$1^{146-249}$ or GST−DP-1^{84-166} (~300 ng, without cleavage; tracks 4, 5, 6 and 7). (**c**) Sequence specificity of the E2F-1^{89-437}/DP-1^{84-249} heteromer: the DNA sequence specificity of complexes formed by either GST−E2F-1^{89-437} (50 ng; tracks 2−6) or GST−E2F-1^{89-437} with DP-1^{84-249} (50 ng and 150 ng respectively; tracks 7−11) was determined by competing with the wild-type or mutated derivatives of the distal E2F site from the adenovirus E2A promoter (~100-fold molar excess of the binding sites indicated). For comparison, a similar experiment is shown in an F9 EC cell extract (tracks 12−16). Both mono- and heteromeric DNA binding complexes had very similar sequence specificities to F9 EC cell DRTF1/E2F. Track 1 shows the probe alone. Details of the competing binding sites are given in Materials and methods. (**d**) DP-1 contains a dimerization domain: the indicated regions of DP-1 were expressed as GST fusion proteins (tracks 3−6) and ~2 μg incubated with 5 μl of a reticulocyte lysate containing translated wild-type E2F-1^{1-437}. GST fusion proteins, or GST protein alone (track 2), were collected with glutathione−agarose beads and bound E2F-1 polypeptide released. Track 1 shows the lysate with the E2F-1 polypeptide. Note that DP-$1^{146-249}$ binds to E2F-1, and that usually between 10 and 20% of the input E2F-1 was specifically retained. (**e**) Summary of the data and molecular properties of DP-1. The C-terminal border of the DNA binding domain, which is known to lie within the region indicated by the broken line, has not been defined.

present in the same binding reaction, increased E2F site DNA binding activity was apparent (Figure 2a, compare tracks 2 and 3 with 4, and 6 and 7 with 8). The DNA binding activity was much greater than that expected from an additive effect of the two DNA binding activities, indicating that together DP-1 and E2F-1 recognize the E2F site synergistically.

The presence of both DP-1 and E2F-1 in the DNA binding complex was confirmed using antisera specific for either protein. An anti-E2F-1 peptide antiserum supershifted the DNA binding complex (Figure 2a, compare track 8 with track 10), whereas the anti-DP-1 peptide antiserum inhibited the DNA binding activity (Figure 2a, compare tracks 11 and 12). However, the effect of the anti-DP-1 antiserum was less dramatic, the reasons for which are unclear, but may be related to the availability of the epitope which, for this antibody, is located close to the DNA binding domain of DP-1 (Girling *et al.*, 1993).

We used this assay to determine the regions in DP-1 which are necessary to produce a DNA binding complex with E2F-1. Thus, various derivatives of DP-1 were expressed as GST fusion proteins, cleaved with thrombin, and then assessed for any interaction with E2F-1. Since these derivatives of DP-1 were truncated versions of the wild-type protein, any of them which was able to interact with E2F-1 to produce functional DNA binding activity should result in a smaller and hence faster migrating DNA binding complex. Moreover, if only one faster migrating complex were apparent, a heterodimer of the two proteins would be the most likely explanation. Indeed, when either DP-1^{84-249} or DP-1^{84-204} were mixed with E2F-1 (GST−E2F-1^{89-437}), a faster migrating DNA binding complex was formed relative to E2F-1 alone (Figure 2b, compare track 1 with 2 and 3) or E2F-1/DP-1 (Figure 2a) indicating that these two derivatives of DP-1 were able to interact with E2F-1 and that they were likely to form a heterodimer. Again, the DNA binding activity of the E2F-1/DP-1^{84-249} complex was greater than that for E2F-1 alone (Figure 2b, compare track 1 with 2 and 3) or DP-1^{84-249} which had low DNA binding activity in the conditions employed in this assay (data not shown) but nevertheless can specifically recognize the E2F site (Girling *et al.*, 1993). The DNA binding activity of the E2F-1/DP-1^{84-204} reaction was less than E2F-1/DP-1^{84-249} indicating that the region of DP-1 between amino acid residues 204 and 249, which shows significant similarity to E2F-1 (Girling *et al.*, 1993), also influences DNA binding activity. The synergistic DNA binding effects of DP-1^{84-249} and DP-1^{84-204} were also apparent when the uncleaved GST fusion proteins were mixed with E2F-1 although, because of their increased size, a faster migrating DNA binding complex did not occur (Figure 2b, compare track 1 with 4 and 5). Further deletion of this region, either from the N- or C-terminus (DP-$1^{146-249}$ and DP-1^{84-166} respectively) yielded derivatives of DP-1 that failed to form a DNA binding complex with E2F-1 either as GST fusion proteins (Figure 2b, compare track 1 with 6 and 7) or after cleavage (data not shown), indicating that DP-1^{84-204} is the minimal region so far defined which is capable of producing a DNA binding complex with E2F-1.

Analysis of the DNA binding specificity of the E2F-1/DP-1^{84-249} complex with a panel of binding sites derived from the adenovirus E2A promoter distal E2F site (La Thangue *et al.*, 1990; Shivji and La Thangue, 1991) indicated that it was very similar to that for E2F-1 alone (Figure 2c, compare tracks 3−6 with 8−11) and furthermore, the DRTF1/E2F site DNA binding activity defined in F9 EC cell extracts (Figure 2c, compare tracks 13−16).

To characterize further the interaction between DP-1 and E2F-1 we employed an assay in which *in vitro* transcribed and translated E2F-1 polypeptide could bind to DP-1−GST fusion proteins. The ability of E2F-1 to interact with DP-1 was assessed after collecting the GST fusion protein with glutathione−agarose beads and subsequently releasing the bound E2F-1 polypeptide. Both DP-1^{84-249} and DP-1^{84-20} could interact with E2F-1 since the amount of E2F-1 bound to GST−DP-1^{84-249} and GST−DP-1^{84-204} was significantly greater than that bound by the GST beads alone (Figure 2d compare track 2 with 3 and 6), consistent with their ability to form a DNA binding heteromer (Figure 2b). DP-1^{146-24} also bound to E2F-1 whereas DP-1^{84-166} failed to do so (Figure 2d, compare track 2 with 4 and 5). DP-1^{146-24} therefore contains a domain, which based on the earlier results is likely to be a dimerization domain, that allows it to interact with E2F-1 but lacks sufficient amino acid sequence for the heteromer to bind to DNA. The additional information in DP-1^{84-249} is necessary for the complex to bind to DNA. These data therefore suggest that the region of DP-1 which is similar to E2F-1 (amino acids 163−236) contains a dimerization domain, and that additional N-terminal sequence is necessary for DNA binding activity. A summary of these data is presented in Figure 2e.

DP-1 and E2F-1 interact in yeast cells

To determine if DP-1 and E2F-1 interact directly *in vivo* we adapted a previously described assay system in yeast cells (Fields and Song, 1989) which utilized expression vectors that synthesize two hybrid proteins, one derived from DP-1 and the other from E2F-1. In the first, the DP-1 coding sequence was fused to the DNA binding domain of the bacterial LexA protein, to make pLEX.DP-1 and in the second, pGAD.E2F-1, the E2F-1 coding sequence was fused with the acidic transcriptioal activation domain (AAD) of the yeast Gal4 protein. pLEX.DP-1 failed to activate a reporter construct driven by a LexA binding site, whereas a hybrid protein that contained the *trans*-activation domain taken from the p53 protein could (Figure 3). However, when pLEX.DP-1 and pGAD.E2F-1 were expressed together, the transcriptional activity of the LexA reporter construct was increased considerably (~75-fold) relative to its activity when either pLEX.DP-1 or pGAD.E2F-1 were expressed alone (Figure 3). This result, combined with the earlier studies presented in this paper, strongly suggest that DP-1 and E2F-1 interact directly *in vivo*. Using the same experimental strategy, we have failed to obtain evidence for an interaction between pLEX.DP-1 and pGAD.DP-1 (V.M.Buck, L.H.Johnston and N.B.La Thangue, data not shown).

DP-1 regulates E2F site-dependent transcription in vivo

Increasing the levels of the DP-1 protein in a variety of mammalian cells (for example, F9 EC, SAOS-2 and 3T3) and growth conditions failed to stimulate significantly the transcriptional activity of an E2F site-dependent reporter (data not shown). In order to assess if DP-1 and E2F-1 functionally interact we therefore had to take an alternative approach which involved developing the appropriate assay

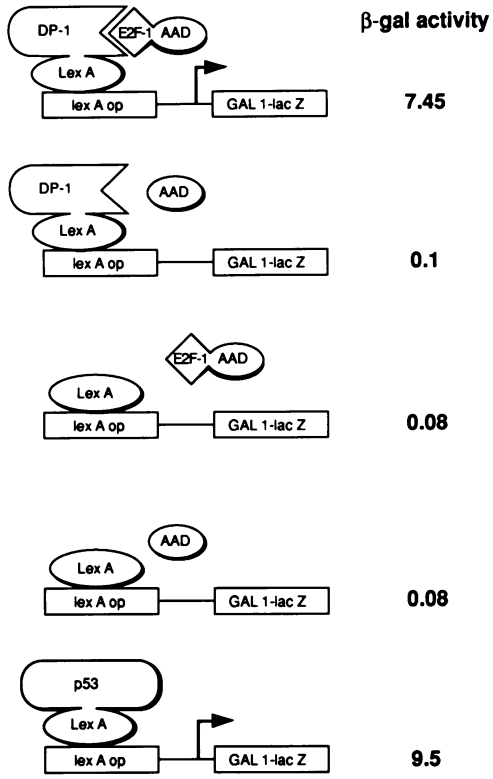

Fig. 3. DP-1 and E2F-1 interact in yeast cells: summary of results. Details of the expression vectors and reporter construct are described in Materials and methods.

in *Drosophila* SL2 cells, a cell system which has been used previously to study the activity of mammalian transcription factors (Courey and Tjian, 1988). These cells were particularly appropriate for this analysis because the endogenous E2F site DNA binding activity is very low when assayed by gel retardation (data not shown). In order to assess the functional interaction of DP-1 and E2F-1, we determined the effects of each protein alone and when expressed together on the transcriptional activity of p3×WT, a reporter construct driven by three E2F sites (Figure 4a; Zamanian and La Thangue, 1991). Thus, E2F-1 was able to activate p3×WT in a dose-dependent fashion (Figure 4b and c, compare lanes 1 and 2) whereas DP-1 failed to do so (Figure 4b and c, compare lanes 3 and 4), results which are similar to the behaviour of E2F-1 and DP-1 in mammalian cells (Helin *et al.*, 1992; Kaelin *et al.*, 1993; and data not shown). However, when DP-1 and E2F-1 were expressed together much greater E2F site-dependent transcriptional activation was apparent relative to either alone (Figure 4b and c, compare lanes 1, 3 and 5). Moreover, this synergistic effect was titratable because increasing the level of DP-1 produced more E2F site-dependent transcription (Figure 4b and c, compare lanes 1, 5 and 6) and specific since co-expression of an unrelated DNA binding, derived from the Gal4 protein, did not produce any significant effects (Figure 4b and c, compare lanes 5 and 6 with 7 and 8). Moreover, similar expreriments performed with p3×MT indicated that this activation was specific for the wild-type E2F site (data not shown). We conclude therefore that DP-1 and E2F-1 functionally interact in E2F site-dependent transcription and that this interaction is synergistic.

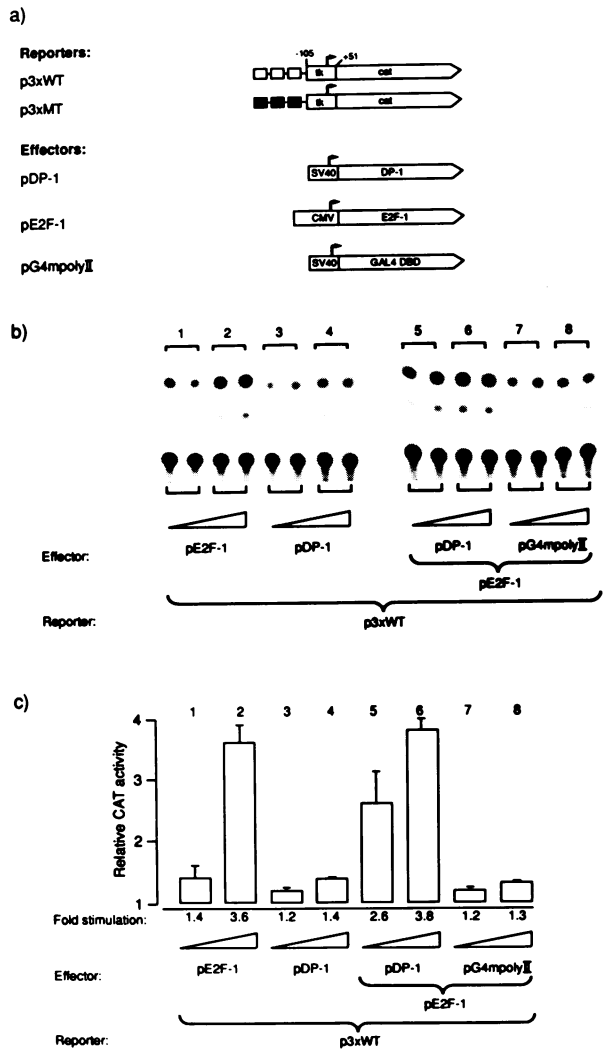

Fig. 4. Functional synergy between DP-1 and E2F-1 in *Drosophila* SL2 cells. (**a**) Summary of constructs: p3×WT and p3×MT have been described previously (Zamanaian and La Thangue, 1992). pDP-1 and pE2F-1 contain full length proteins, and pG4MpolyII the Gal4 DNA binding domain. (**b**) and (**c**) SL2 cells were transfected with p3×WT and the indicated expression vectors. The amounts of expression vector in each treatment were as follows: 50 ng (lanes 1, 5, 6, 7 and 8) or 500 ng (lane 2) for E2F-1, 5 μg (lanes 3 and 5) or 10 μg (lanes 4 and 6) for DP-1, and 3.7 μg (lane 7) or 7.0 μg (lane 8) for pG4Mpoly II. All values are expressed relative to p3×WT alone which was given an arbitrary value of 1.0, and are representative of at least three separate experiments. (b) shows an example of the crude data which is quantitatively represented in (c).

DP-1 and E2F-1 activate E2F site-dependent transcription yeast cells

We next assessed if DP-1 and E2F-1 can functionally interact in E2F site-dependent transcription in yeast cells. For this, we used constructs in which the yeast *cyc1* promoter was driven by E2F binding sites taken from the adenovirus E2A promoter. In p4×WT CYC1, four E2F binding sites drive the *cyc1* promoter (Figure 5a), activating transcription ~12-fold above the activity of p4×MT CYC1 (data not shown). This transcriptional activity could be stimulated further upon introduction of the E2F-1 expression vector, pGAD.E2F-1. Thus, pGAD.E2F-1 increased the transcriptional activity of p4×WT CYC1 ~10-fold, compared with the small effect that the DP-1 expression

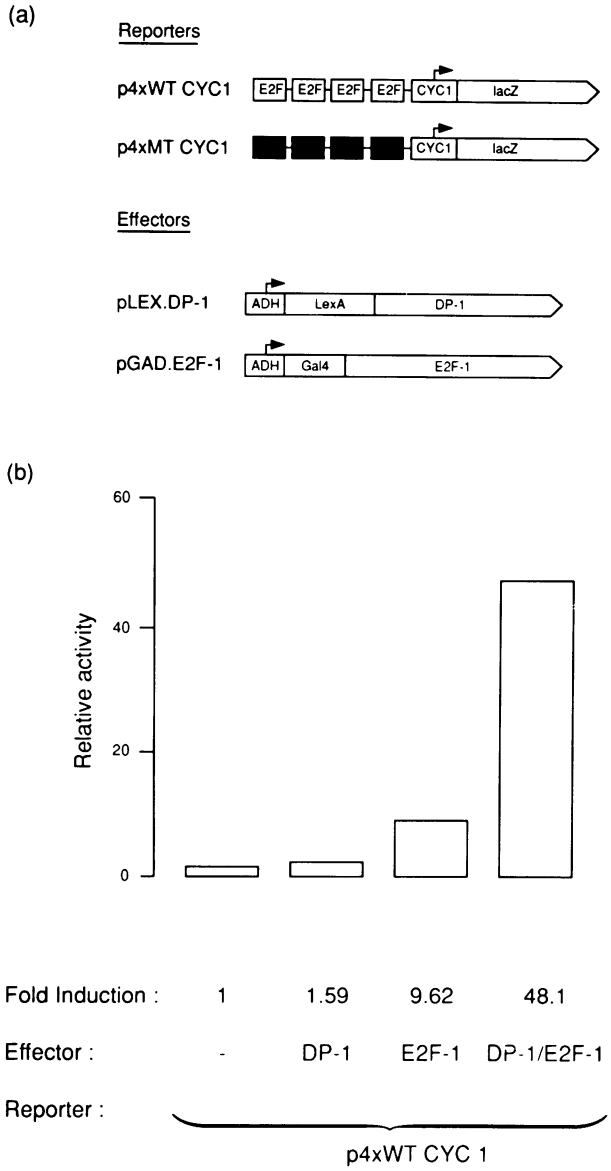

Fig. 5. DP-1 and E2F-1 activate E2F site-dependent transcription in yeast cells. (**a**) Summary of constructs. (**b**) β-galactosidase activity was measured in *S.cerevisiae* strain W3031a carrying p4×WT CYC1 and the indicated effector expression vector. All values are expressed relative to the activity of p4×WT CYC1 which was given an arbitrary value of 1.0 and are representative of at least three separate experiments.

vector, pLEX.DP-1, had on the same reporter construct (Figure 5b). However, when E2F-1 and DP-1 were expressed together, the activity of p4×WT CYC1 was even greater, and usually ~50-fold above basal p4×WT CYC1 activity (Figure 5b); the activity of p4×MT CYC1 was not significantly affected by either the E2F-1 or DP-1 expression vector (data not shown). We conclude that DP-1 and E2F-1 activate E2F site-dependent transcription more efficiently when present together than either does alone, suggesting again that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation.

Discussion

DP-1 and E2F-1 interact in mammalian cells

Previous studies have indicated that DP-1 is a universal component of DRTF1/E2F DNA binding activity in F9 EC cells because all the DNA binding complexes that occur on the E2F site are disrupted by anti-DP-1 antibodies (Girling *et al.*, 1993). The same situation exists in HeLa cell extracts where all the DRTF1/E2F DNA binding complexes are affected by anti-DP-1 antibodies (Figure 1a). Based on these observations, and combined with studies performed in other cell types (L.R.Bandara, T.S.Sørensen, M.Zamanian and N.B.La Thangue, in preparation), we believe that DP-1 is a frequent component of transcription factor DRTF1/E2F.

In the light of these observations, we were interested to determine if DP-1 can interact with the other E2F site DNA binding protein, E2F-1 (Helin *et al.*, 1992; Kaelin *et al.*, 1992) and, furthermore, establish whether such an interaction occurs in physiological conditions. Our results indicate that DP-1 and E2F-1 exist as a complex in HeLa cell extracts, and thus imply that at least a proportion of the total DRTF1/E2F DNA binding activity is likely to be a heteromeric complex involving DP-1 and E2F-1. It is unclear, at the moment, just how much of the DRTF1/E2F DNA binding activity is a complex of DP-1 and E2F-1 because our attempts to use anti-E2F-1 antibodies to affect the DNA binding activity in gel retardation assays have been unsuccessful (data not shown). Also, we cannot rule out that other proteins bind to DP-1, in the place of E2F-1. In fact, this would seem a likely possibility because several polypeptides in affinity purified DRTF1/E2F with distinct molecular weights (from 45 to 55 000) are capable of specifically binding to the E2F site (Shivji and La Thangue, 1991; Girling *et al.*, 1993).

A physical interaction between DP-1 and E2F-1 in vitro and in yeast cells

We established that DP-1 and E2F-1 can interact directly by studying their DNA binding properties in gel retardation assays. DP-1 and E2F-1 formed a heteromeric DNA binding complex with exactly the same DNA binding specificity as that possessed by DRTF1/E2F in crude cell extracts (Figure 2 and La Thangue *et al.*, 1990). Moreover, it was apparent that the DNA binding activity of the heteromer was considerably greater than for E2F-1 or DP-1 alone, suggesting that DP-1 and E2F-1 interact synergistically. A molecular analysis of the region in DP-1 which was necessary to form a DNA binding complex with E2F-1 indicated that the region of similarity between the two proteins, together with an additional N-terminal domain, was required (summarized in Figure 2e). The region of similarity allowed DP-1 and E2F-1 to bind to each other and thus is likely to constitute a dimerization domain.

We confirmed these observations in yeast cells using an assay which makes use of the modular organization of transcription factors (Fields and Song, 1989). Thus, DP-1 was fused to the bacterial LexA DNA binding domain and, in a separate molecule, E2F-1 to the acidic transcriptional activation domain of the yeast Gal4 protein. In this assay, a functional activation domain is recruited to the LexA-dependent promoter only if there is a physical interaction between the two hybrid proteins. When the two hybrid proteins were expressed together there was strong activation of the LexA-dependent reporter. Thus, DP-1 and E2F-1 are able to interact physically in yeast cells. Moreover, this result indicates that they are able to do so in the absence of DNA binding since the DNA binding specificity was provided by

LexA and thus took place independently of the E2F binding site.

Transcriptional synergy by DP-1 and E2F-1 in vivo

We addressed the functional consequences of the interaction between DP-1 and E2F-1 for E2F site-dependent transcription in both *Drosophila* and yeast cells. We took this approach because our attemps to activate transcription by introducing wild-type DP-1 into mammalian cells have met with limited success, the reasons for which are unclear but may be related to the levels of endogenous DP-1 protein.

Both types of assay, whether performed in *Drosophila* or yeast cells, indicated that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcription since when both proteins were expressed together transcriptional activation was more efficient than for either protein alone. A likely explanation for such an effect is that the DNA binding activity of the DP-1/E2F-1 heterodimer is more stable than either homodimer and thus transcriptional activation is more efficient. This idea would be entirely consistent with the *in vitro* DNA binding data presented earlier in this study which suggested that DP-1 and E2F-1 interact synergistically. We cannot, however, rule out other potential influences, such as activation of a cryptic transcriptional activation domain in the DP-1/E2F-1 heterodimer and, in fact, recent experiments have suggested that such a possibility is likely to be correct (M.Zamanian and N.B.La Thangue, unpublished data).

In conclusion, we have demonstrated that DP-1 and E2F-1 interact in transcription factor DRTF1/E2F, to produce a DNA binding complex which is the preferred state over either homodimer. Since E2F-1 can bind to pRb (Helin *et al.*, 1992; Kaelin *et al.*, 1992) in such a complex it is likely that E2F-1 will provide an interface recognized by pRb, thus enabling the transcriptional activity of this particular E2F site DNA binding activity to be regulated by pRb. It is possible that other molecules heterodimerize with DP-1, in the place of (and perhaps related to) E2F-1, providing an interface recognized by other proteins which are known to interact with DRTF1/E2F, such as p107 (Zamanian and La Thangue, 1993), thus allowing these molecules also to regulate E2F site-dependent transcription. We suggest therefore that distinct heterodimers recognize the E2F site, with DP-1 as a common component, enabling different molecules, such as pRb and p107, to integrate their biological activities with the transcription apparatus and hence to regulate genes driven by DRTF1/E2F.

Materials and methods

Preparation of cell extracts, gel retardation and immunochemical techniques

Cell extracts were prepared as previously described (La Thangue *et al.*, 1990). Gel retardation in F9 EC and HeLa cell extracts (~6.0 μg) in the presence of anti-DP-1 was performed as previously described (Girling *et al.*, 1993), and immunoprecipitation with anti-DP-1 from HeLa cell extracts was performed by standard procedures. The immunoprecipitates were treated with 1% DOC and 1.5% NP40 and the detergent released material assayed for DRTF1/E2F by gel retardation and the presence of E2F-1 by immunoblotting with the anti-E2F-1 monoclonal antibody SQ41 (Kaelin *et al.*, 1992). The anti-DP-1 antibodies, anti-peptide A and anti-peptide 18, have been previously described (Girling *et al.*, 1993). Rabbit anti-E2F-1 antiserum (antiserum 134) was raised against a peptide which represents E2F-1 amino acid sequence 426–437. The sequences of the binding sites used to assess DNA binding specificity were derived from the adenovirus E2A promoter (−71 to −50) and were as follows: WT; TAGTTTTCGCGCTTAAATTTGA; 62/60, TAGTTTTCGATATTAAATTTGA; 63, TAGTTTTCTCGCTTAAATTTGA; 64, TAGTTTTAGCGCTTAAATTTGA. In Figure 2a (tracks 1–4), the adenovirus E2A promoter (−96 to + 68) was used; in all other cases, the distal E2F site in the E2A promoter (sequences −71 to −50) was used. About 100-fold excess of competing binding sites were used in the gel retardation assays.

Fusion proteins and in vitro translation

DP-1 and E2F-1 were expressed as, and released from, GST fusion proteins as previously described (Girling *et al.*, 1993). About 100-fold excess of the competing binding sites were used in gel retardation assays, with the binding site taken from the adenovirus E2A promoter (−71 to −50). The wild-type E2F-1 coding sequence was transcribed and further translated using reticulocyte lysate (Promega) and radiolabelled with [^{35}S]methionine. In the dimerization assay (Figure 2d), GST–DP-1 fusion protein was incubated with E2F-1 polypeptide for 30 min at 30°C, collected with glutathione–agarose (Sigma), and washed repeatedly with 0.1% NP40 in PBSA. Bound E2F-1 polypeptide was released by denaturation in SDS sample buffer and resolved in a 10% polyacrylamide gel.

Yeast assays

pBTM116 contains the complete LexA coding sequence (1–202) under the control of the yeast *ADH1* promoter. pLEX.DP-1 carries the coding sequence for DP-1 (from amino acid 59 to the C-terminus) downstream of the LexA coding sequence in pBTM116. pBTM126 carries the wild-type murine p53 coding sequence (amino acids 1–346) downstream of the LexA DNA binding domain. pGAD.L6 is a derivative of pGAD2F (Chien *et al.*, 1991) containing the Gal4 transcription activating domain (from amino acid residue 768 to 881) under the control of yeast *ADH1* promoter. pGAD.E2F-1 contains the entire E2F-1 coding sequence (from amino acid 1 to 437) downstream of the Gal4 activation domain. p4×WT CYC1 and p4×MT CYC1 were derived from pLGΔ178 (Guarente and Mason, 1983). The wild-type E2F site was taken from the −71 to −50 region of the adenovirus E2A promoter and the mutant site was mutated in nucleotides −62 to −60 (La Thangue *et al.*, 1990). For the yeast interaction assay (Figure 3), the indicated expression vectors were transformed into the yeast strain CTY10-5d (*MATa ade2 trp1-901 leu2-3, 112 his3-200 gal4 gal80 URA3::lexAop-lacZ*) which contains an integrated plasmid which carries two copies of a 78 bp oligonucleotide, each copy containing two colE1 operators or four binding sites for LexA dimers upstream of the transcription start site of GAL1–lacZ. For the yeast E2F site-dependent transcription assay (Figure 5), the yeast strain W3031a (*MATa ade 2-100 tryp1-1 leu2-3,112 his3-11 ura3*) was used carrying either p4×WT CYC1 or p4×MT CYC1 and was transformed with the indicated expression vectors. β-galactosidase activity of mid-log phase cultures was quantitated as described previously (Johnson *et al.*, 1986). β-galactosidase activity was measured for at least three independent transformants.

Transfection of Drosophila tissue culture cells

Reporter constructs were all derived from pBLcat2 and have been described previously (Zamanian and La Thangue, 1992). Open and solid boxes denote wild-type and mutant E2F binding sites, respectively. pDP-1 encodes a complete DP-1 protein, and pG4mpolyII the Gal4 DNA binding domain (Webster *et al.*, 1989). pE2F-1 has been previously described as pCMV RBAP-1 (Kaelin *et al.*, 1982). Cells were transfected by the calcium phosphate procedure and harvested 40–45 h later and for each transfection, pBluescript KS was included to maintain the final DNA concentration constant. All transfections included an internal control pCMV β-gal. The assay for CAT activity, correction for transfection efficiency and quantitation of TLC plates have been described previously (Zamanian and La Thangue, 1992).

Acknowledgements

We thank Paul Bartel, Stan Fields, Cheng-Tien Chen and Rolf Sternglanz for reagents used in the yeast assay, Bill Kaelin for SQ41 and pCMVE2F-1 and Kristian Helin for E2F-1$^{89-437}$. L.R.B. is supported by an MRC Collaborative Studentship with Roche Products Ltd, UK. We thank Peter Rigby for helpful comments and the MRC for supporting this research.

References

Bandara,L.R. and La Thangue,N.B. (1991) *Nature*, **351**, 494–497.
Bandara,L.R., Adamczewski,J.P., Hunt,T. and La Thangue,N.B. (1991) *Nature*, **352**, 249–251.
Bandara,L.R., Adamczewski,J.P., Poon,R.C.Y., Zamanian,M., Hunt,T. and La Thangue,N.B. (1992) *J. Cell Sci.*, **16**, 77–85.
Blake,M.C. and Azizkhan,J.C. (1989) *Mol. Cell. Biol.*, **9**, 4994–5002.

Chellappan,S.P., Hiebert,S., Mudryj,M., Horowitz,J.M. and Nevins,J.R. (1991) *Cell*, **65**, 1053–1061.
Chien,C.-T., Bartel,P.L., Sternglanz,R. and Fields,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9578–9582.
Courey,A.J. and Tjian,R. (1988) *Cell*, **55**, 887–898.
Dalton,S. (1992) *EMBO J.*, **11**, 1797–1808.
Devoto,S.H., Mudryj.M., Pines,J., Hunter,T. and Nevins,J.R. (1992) *Cell*, **68**, 167–176.
Fields,S. and Song,O. (1989) *Nature*, **340**, 245–246.
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.
Guarente,L. and Mason,T. (1983) *Cell*, **32**, 1279–1286.
Helin,K., Lees,J.A., Vidal,M., Dyson,N., Harlow,E. and Fattaey,A. (1992) *Cell*, **70**, 337–350.
Hiebert,S.W., Chellappan,S.P., Horowitz,J.M. and Nevins,J.R. (1992) *Genes Dev.*, **6**, 177–185.
Johnson,A.L., Barker,D.G. and Johnston,L.H. (1986) *Curr. Genet.*, **11**, 107–112.
Kaelin,W.G. *et al.* (1992) *Cell*, **70**, 351–364.
La Thangue,N.B. and Rigby,P.W.J. (1987) *Cell*, **49**, 507–513.
La Thangue,N.B. and Taylor,W. (1993) *Trends Cell Biol.*, **3**, 75–76.
La Thangue,N.B., Thimmappaya,B. and Rigby,P.W.J. (1990) *Nucleic Acids Res.*, **18**, 2929–2938.
Lees,E., Faha,B., Dulic,V., Reed,S.I. and Harlow,E. (1992) *Genes Dev.*, **6**, 1874–1885.
Means,A.L., Slansky,J.E., McMahon,S.L., Knuth,M.W. and Farnham,P.J. (1992) *Mol. Cell. Biol.*, **12**, 1054–1063.
Mudryj,M., Devoto,S.H., Hiebert,S.W., Hunter,T., Pines,J. and Nevins,J.R. (1991) *Cell*, **65**, 1243–1253.
Shirodkar,S., Ewen,M., DeCaprio,J.A., Morgan,J., Livingston,D.M. and Chittenden,T. (1992) *Cell*, **68**, 157–166.
Shivji,M.K. and La Thangue,N.B. (1991) *Mol. Cell. Biol.*, **11**, 1686–1695.
Webster,N.J.G., Green,S., Tasset,D., Ponglikitmongkol,M. and Chambon,P. (1989) *EMBO J.*, **8**, 1441–1446.
Zamanian,M. and La Thangue,N.B. (1992) *EMBO J.*, **11**, 2603–2610.
Zamanian,M. and La Thangue,N.B. (1993) *Mol. Biol. Cell*, **4**, 389–396.

Received on July 5, 1993; revised on August 17, 1993