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Physical and functional interactions between p53 and cell cycle co-operating transcription factors, E2F1 and DP1

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One way in which wild-type p53 is able to regulate cell cycle progression is thought to be via the induction of its downstream target gene Waf1/CIP1, thus indirectly regulating the transcriptional activity of E2F. The E2F transcription factors are known to be key effectors of the cell cycle. We report here that there is a physical and functional interaction between p53 and two of the components of the E2F transcription factors, E2F1 and DP1. The expression of wild-type p53 can inhibit the transcriptional activity of E2F, and the expression of both E2F1 and DP1 can also downregulate p53-dependent transcription. The transcriptional activity of p53 is known to be inhibited by the direct binding of mdm2, but we demonstrate here that both E2F1 and DP1 can inhibit p53 transcriptional activity independently of mdm2. Detailed studies of protein–protein interactions have provided evidence that E2F1 and its co-operating factor DP1 can complex with p53 both *in vitro* and *in vivo*.

Keywords: cell cycle regulation/p53/protein–protein interactions/transcriptional activity

Introduction

The E2F transcription factor is a key regulator of cell cycle progression. Increasing E2F transcriptional activity by introducing exogenous E2F1 protein or expression plasmid is sufficient to drive quiescent cells into the S phase of the cell cycle (Johnson *et al.*, 1993). E2F transcription factor activity consists of a heterodimer between a member of the E2F family and a member of the DP family. Five members of the E2F family have been identified (E2F1, E2F2, E2F3, E2F4 and E2F5; Lam and La Thangue, 1994; Hijmans *et al.*, 1995; Sardet *et al.*, 1995) and two members of the DP family have also been isolated (DP1 and DP2; Lam and La Thangue, 1994; Zhang and Chellappan, 1995). It is generally believed that E2F family proteins, but not DP, can bind to DNA with sequence specificity as homodimers. However, a significant increase in DNA binding activity can be achieved when an E2F family member heterodimerizes with a DP family member (Lam and La Thangue, 1994). This stimulation of DNA binding activity is reflected in the increase in E2F-dependent transcription when E2F is cotransfected with DP1 (Lam and La Thangue, 1994; Wu *et al.*, 1995). In addition to the association with DP family proteins, the transcriptional activity of E2F can be regulated *in vivo* by members of a family of pocket binding proteins including Rb and Rb-related proteins p107 and p130. Direct protein–protein interactions between members of the E2F family and the Rb or Rb-related proteins result in downregulation of the E2F-dependent transcription. Again, the heterodimerization between E2F1 and DP1 can result in synergistic binding to Rb. Binding to E2F, resulting in the repression of E2F-dependent transcription, is thought to be the pathway through which Rb and the other Rb-related proteins negatively regulate cell cycle progression in mammalian cells.

Another important tumour suppressor involved in cell cycle regulation is p53, and this is the most frequently mutated gene found in a large variety of human tumours. One of the most important functions of wild-type p53 is its ability to cause G_1 arrest in response to DNA damage. This is caused by the increase in p53 protein level as well as its transcriptional activity (Kastan *et al.*, 1991; Lu and Lane, 1993). Like E2F, p53 is a sequence-specific DNA binding protein. p53 transactivates a set of target genes including mdm2 (Barak *et al.*, 1993) and Waf1/CIP1 (El-Deiry *et al.*, 1993). Interestingly, upregulation of Waf1/CIP1 expression in response to γ-radiation is dependent on p53 (Dulic *et al.*, 1994). Waf1/CIP1 is a universal inhibitor of cyclin-dependent protein kinases (inhibitor for CDK2, CDK4; Xiong *et al.*, 1993), and cyclin-dependent protein kinases are known to be engaged in phosphorylating the retinoblastoma protein Rb and in positively regulating the transcriptional activity of E2F by releasing it from its association with Rb. So, increasing p53 in irradiated cells is thought to trigger G_1 arrest via a Waf1/CIP1- and Rb-dependent inhibition of E2F activity (Harper *et al.*, 1993; Dulic *et al.*, 1994). However, very little is known about the function of the tumour suppressor p53 in the control of normal cell cycle progression. Nevertheless, p53 has been shown to be able to repress the transcriptional activity of the promoters of thymidine kinase, c-*myc* and DNA polymerase α (Lin *et al.*, 1992; Moberg *et al.*, 1992; Yuan *et al.*, 1993) *in vitro*. Interestingly, these are also the genes which can be transactivated by E2F. This suggests that p53 may be able to interfere with E2F function in the normal cell cycle. The balance between the activities of E2F and tumour suppressors such as Rb and p53 is crucial to ensure the normal progression of the cell cycle. In normal cells, the transcriptional activity of p53 is believed to be regulated mainly at the protein level through its short half-life (Oren *et al.*, 1981). However, other cell factors might also regulate the transcriptional activity of p53. To allow cells to enter the S phase, it

might be necessary to overcome any residual inhibitory effects of the normal levels of p53 on DNA replication and cell cycle progression. E2F would be an obvious candidate for this type of activity because it becomes active at the correct time in the cell cycle.

We have investigated the transcriptional activities of E2F and p53 in different cells and provide direct evidence that p53 can suppress E2F transcriptional activity. This inhibition can also be observed in cells lacking functional Rb, indicating that E2F activity may be regulated by Waf1/CIP1 through Rb-related proteins, or it may even be regulated through an alternative pathway which is independent of Waf1/CIP1. Interestingly, an inhibition of p53 transcriptional activity by two components of the E2F transcription factors, E2F1 or DP1, was also observed. Because mdm2 can inhibit the transcriptional activity of p53 by directly binding to the conserved domain I of p53 (Marston *et al.*, 1994), the involvement of mdm2 in this inhibition was also investigated. In addition, the molecular basis for the inhibition was studied by investigating the physical associations between p53 and E2F1 or DP1.

Results

p53 can inhibit E2F-dependent transcriptional activity and this inhibition is independent of Rb

To test the effects of p53 on E2F-dependent transcription, we transfected fibroblasts of p53(–/–) cells (Donehower *et al.*, 1992), derived from p53 knockout mice, with an E2F reporter plasmid (3xwt–CAT; Bandara *et al.*, 1993) and a p53 reporter plasmid (mdm2–Luc; Juven *et al.*, 1993). When a wild-type p53 expression plasmid but not the control plasmid was cotransfected with both the E2F and p53 reporter plasmids, chloramphenicol acetyl transferase (CAT) activity from the 3xwt–CAT reporter was inhibited greatly by p53, demonstrating that the introduced wild-type p53 can inhibit endogenous E2F-dependent transcription (Figure 1A, labelled p53 or vector). In contrast, the transcriptional activity of a p53 reporter plasmid was significantly stimulated in the same transfected cells (Figure 1B, labelled wtp53). Therefore, the repression of E2F-dependent transcription was not the result of cell death. In addition, when the E2F1 expression plasmid or E2F1 and DP1 expression plasmids were cotransfected with wild-type p53 expression plasmid in the same transfection assay, the repression of endogenous E2F-dependent transcription by wild-type p53 was abolished (Figure 1A, labelled E2F1+p53, E2F1+DP1+p53). In Figure 1C, it can be seen that wild-type p53 also inhibits activated transcription by exogenous E2F1, and that such inhibition can be partly titrated away by increasing the amount of E2F1 expression plasmid used in the cotransfection (10–100 ng pCMV–E2F1, Figure 1C). Using a control reporter plasmid J4–β-galactosidase (gal), in which the *lacZ* gene is under a Mo-MuLVLTR promoter, it is also clear that the introduced wild-type p53 has very little effect on the J4–β-gal control reporter activity compared with that of the E2F reporter plasmid 3xwt–Luc, demonstrating that the inhibition of E2F transcriptional activity by wild-type p53 is rather specific (Figure 1D). Cotransfecting DP1 expression plasmid pGAD–DP1 with wild-type p53 in this experiment did not overcome the repression of endogenous E2F-dependent transcriptional activity by p53 (Figure 1A, labelled DP1+p53). When the effects of exogenous E2F1 and DP1 expression on p53 reporter activity were investigated, an interesting observation was obtained (see below).

The repression activity of wild-type p53 on E2F-dependent transcription was investigated further using the B-*myb* and DHFR promoters, whose activities are regulated by the E2F binding sites which they contain. As shown in Figure 2A, the activities of the B-*myb* and DHFR promoters and the reporter 3xwt–Luc were inhibited significantly by wild-type p53. In contrast, the ability of mutant p53 (His175) to repress the E2F-dependent transcription is much lower than that of wild-type p53 (Figure 2A and B), although the same amounts of p53 were expressed for both wild-type and mutant p53 (data not shown). The inhibition by wild-type p53 was dependent on the E2F binding site because the transcriptional activity of a mutant B-*myb* promoter, in which the E2F site had been mutated, or a synthetic mutant E2F reporter construct (3xmt–Luc) was much less inhibited by p53 (Figure 2A). Finally, because mdm2 has been shown to be able to bind to both p53 and E2F1, the involvement of mdm2 in the p53-mediated inhibition of E2F transcriptional activity was also investigated using a p53 mutant ΔIp53, which is defective for mdm2 binding. As shown in Figure 2C, it is clear that the inhibition of E2F reporter activity is independent of mdm2.

One way in which p53 might inhibit E2F transcriptional activity is through its ability to induce the expression of Waf1/CIP1, which can prevent the phosphorylation of Rb, resulting in the inactivation of E2F transcriptional activity (Dulic *et al.*, 1994). To test whether p53 can also inhibit E2F-dependent transcription in an Rb-independent manner, we performed similar transient transfection studies on Saos-2 cells in which there was no functional p53 or Rb (Shew *et al.*, 1990). As with that seen in the p53(–/–) cells, transfected wild-type p53 also inhibited the transcriptional activity of E2F in Saos-2 cells (Figure 2B). Therefore, p53 can inhibit E2F transcriptional activity through a pathway which is independent of Rb, at least in Saos-2 cells.

Inhibition of p53 transcriptional activity by E2F1 or DP1 is independent of mdm2

The inhibition of E2F by p53 could be explained by the Waf1/CIP1 pathway, but a reciprocal effect of E2F on the transcriptional activity of p53 cannot. To investigate whether E2F1 or DP1 could repress p53-dependent transcription, p53(–/–) cells were transfected with the p53 reporter plasmid mdm2–Luc and either an E2F1 or a DP1 expression plasmid or control vector. As shown in Figure 1B, the p53-dependent activation of the reporter plasmid mdm2–Luc was reduced greatly by exogenous E2F1. DP1 also reduced p53-dependent transcription, although to a lesser extent. The DP1 expression plasmid used in this experiment was pGAD–DP1. When the plasmid pCMV–DP1 was used (see below), the inhibition of the transcriptional activity of p53 by DP1 was greater. This was because of the higher expression level of DP1 from pCMV–DP1 than that from the pGAD–DP1 plasmid (data not shown). When both E2F1 and DP1 were co-expressed, their ability to inhibit p53-dependent transcription was

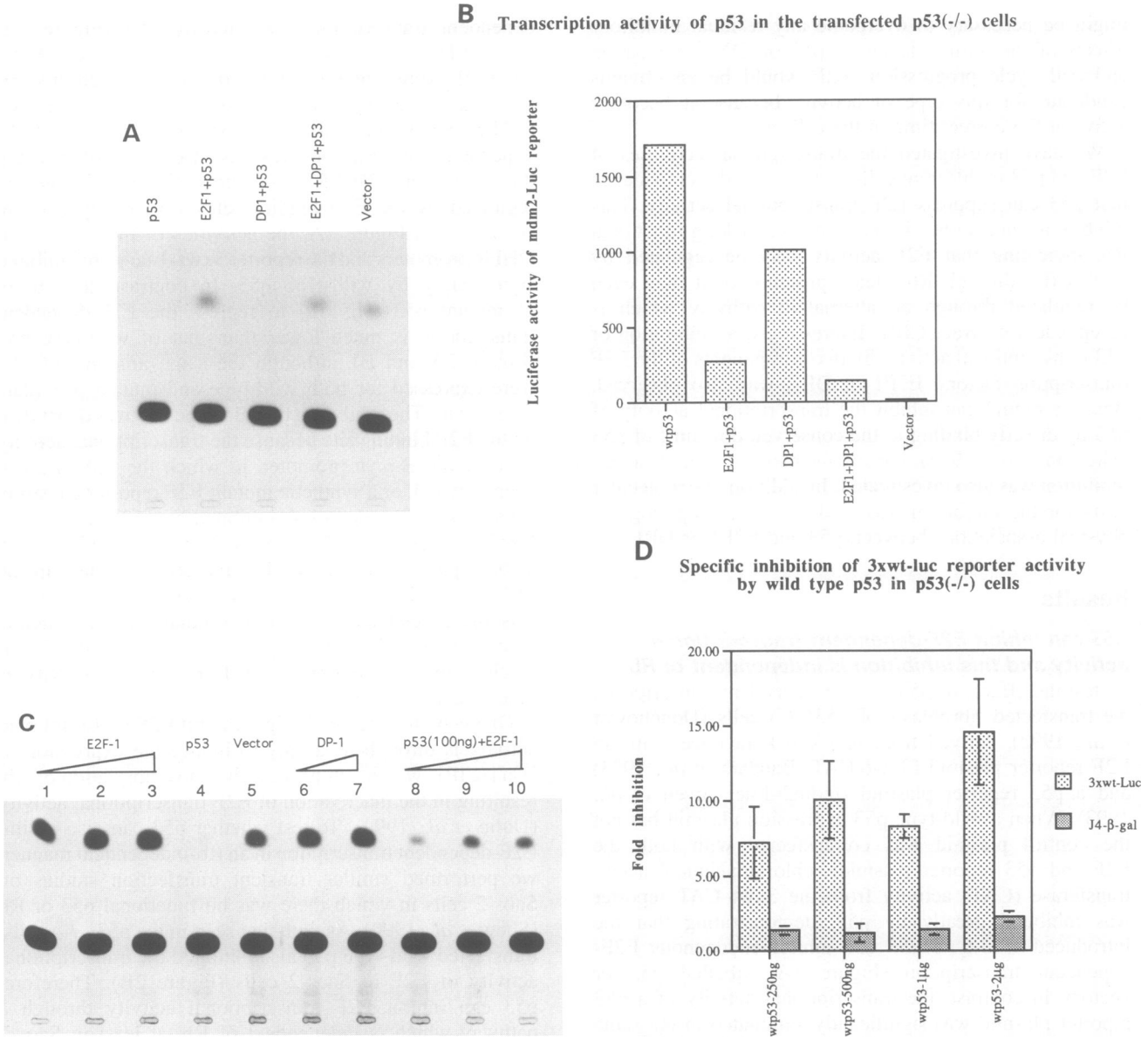

Fig. 1. CAT (**A**) and luciferase (**B**) assays to measure the transcriptional activities of E2F and p53, respectively, in transfected p53(–/–) cells. In all, 10^5 cells were cotransfected with both E2F and p53 reporter plasmids (3xwt–CAT and mdm2–Luc, 1 μg of each) plus 100 ng E2F1 (pCMV–E2F1), 100 ng wild-type p53 (pCMV–SN3) and 1 μg DP1 (pGAD–DP1) or vector in the indicated combinations. (**C**) CAT assay to demonstrate the ability of p53 to repress E2F1 activated transcription in p53(–/–) cells. Cells were transfected under the same conditions as that shown in (A) and (B). Increasing amounts of CMV–E2F1 plasmid (10, 50 and 100 ng) or pGAD–DP1 plasmid (100 ng and 1 μg) were transfected to the cells to transactivate the E2F reporter plasmid 3xwt–CAT activity (lanes 1–3 for CMV–E2F1 and lanes 6 and 7 for pGAD–DP1, respectively). 100 ng of p53 plasmid (pCMV–SN3) were transfected alone (lane 4) or in conjunction with increasing amounts of E2F1 plasmid CMV–E2F1 (10, 50 and 100 ng, lanes 8–10, respectively). The endogenous E2F transcriptional activity is shown in lane 5, in which the control vector was transfected (labelled as vector). (**D**) Effect of wild-type p53 on the activity of E2F and the control reporter plasmids 3xwt–Luc and J4–β-gal, respectively. The amount of wild-type p53 containing plasmid pCMV–SN3 used in the transfection assays is indicated as wtp53–250 ng, wtp53–500 ng, wtp53–1 μg or wtp53–2 μg.

greater than that of E2F1 or DP1 alone, but the difference was not that significant (Figure 1B).

The effect of E2F1 on the transcriptional activity of endogenous p53 was also measured in MCF7 cells which contain wild-type p53 (Casey *et al.*, 1991). To demonstrate the effect of E2F1 on p53, our study was not limited to a single reporter construct; rather a second p53 reporter plasmid p53CON–Luc and its control plasmid Δp53–Luc, which lacks a p53 binding site, were used (Chen *et al.*, 1993). The transcriptional activity of p53CON–Luc, but not Δp53-Luc, was inhibited greatly by the introduction of exogenous E2F1 (Figure 3A). The inhibition of p53 transcriptional activity by E2F1 in MCF7 cells was not caused by simple squelching because the basal transcriptional activity of the mutated p53 reporter, Δp53–Luc, was similar in the cells with and without the exogenous E2F1 expression.

The ability of E2F1 and its co-operating factor DP1 to inhibit endogenous p53-dependent transcription was also studied in NIH 3T3 cells using two different p53 reporter plasmids (mdm2–Luc and Waf1–Luc) derived from two different p53 target genes (mdm2 and Waf1/CIP1 respectively). Again, the activity of either p53 reporter plasmid was inhibited by either E2F1 or DP1 (Figure 3B).

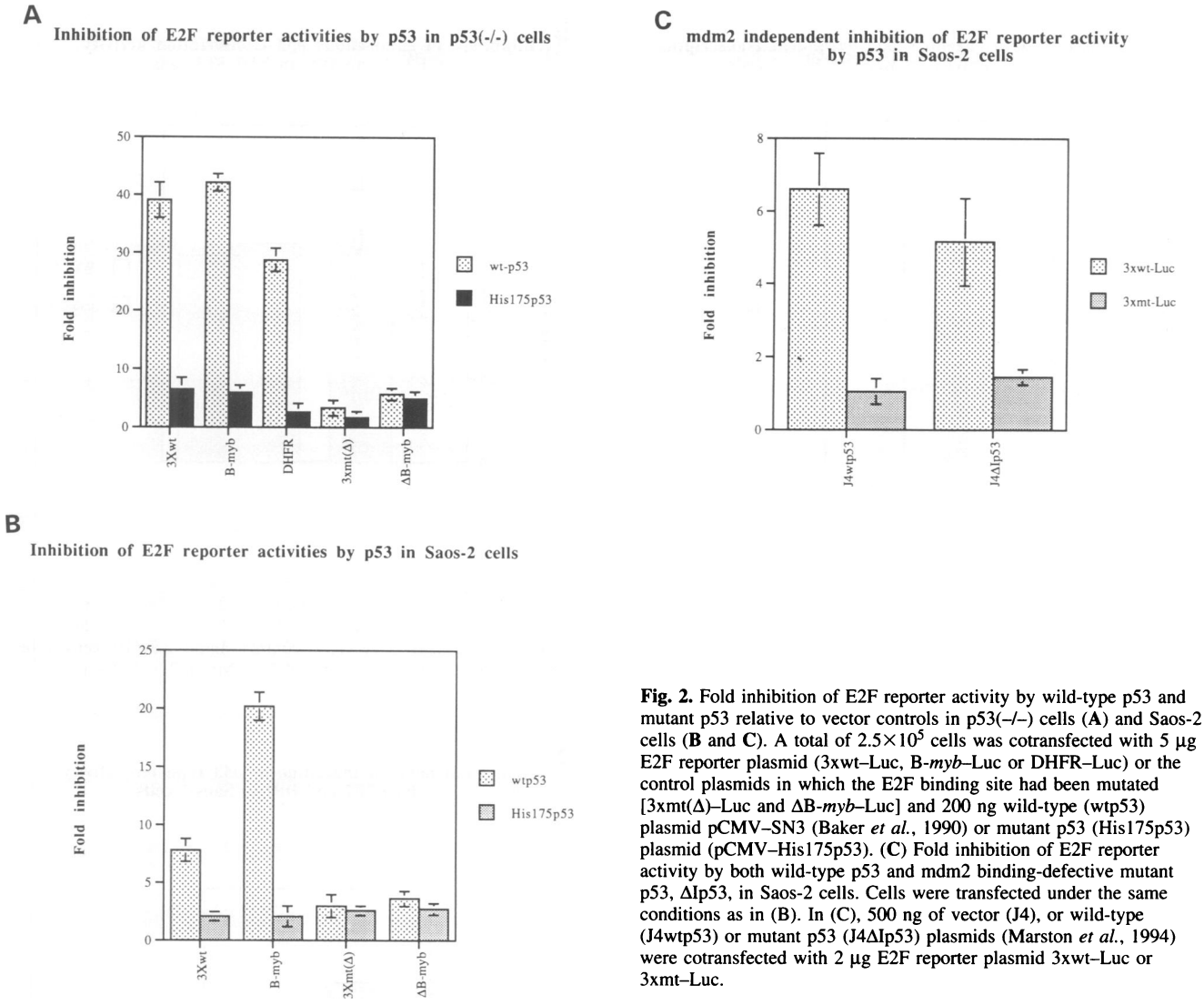

Fig. 2. Fold inhibition of E2F reporter activity by wild-type p53 and mutant p53 relative to vector controls in p53(–/–) cells (**A**) and Saos-2 cells (**B** and **C**). A total of 2.5×10^5 cells was cotransfected with 5 μg E2F reporter plasmid (3xwt–Luc, B-*myb*–Luc or DHFR–Luc) or the control plasmids in which the E2F binding site had been mutated [3xmt(Δ)–Luc and ΔB-*myb*–Luc] and 200 ng wild-type (wtp53) plasmid pCMV–SN3 (Baker *et al.*, 1990) or mutant p53 (His175p53) plasmid (pCMV–His175p53). (**C**) Fold inhibition of E2F reporter activity by both wild-type p53 and mdm2 binding-defective mutant p53, ΔIp53, in Saos-2 cells. Cells were transfected under the same conditions as in (B). In (C), 500 ng of vector (J4), or wild-type (J4wtp53) or mutant p53 (J4ΔIp53) plasmids (Marston *et al.*, 1994) were cotransfected with 2 μg E2F reporter plasmid 3xwt–Luc or 3xmt–Luc.

The transcriptional activity of p53 can be inhibited by associating it with mdm2 protein (Momand *et al.*, 1992; Oliner *et al.*, 1993). Therefore we tested whether the association with mdm2 contributed to the suppression of p53 transcription by E2F1 or DP1. We transfected Saos-2 and p53(–/–) cells with the p53-dependent reporter, mdm2–Luc, an E2F1 or DP1 expression plasmid, and wild-type p53, ΔIp53 or control plasmids. The p53 mutant ΔIp53 was deleted for amino acids 13–19 and was transcriptionally active but defective for mdm2 binding (Marston *et al.*, 1994). As in wild-type p53, the transcriptional activity of the ΔIp53 mutant was still inhibited by E2F1 or DP1 in Saos-2 and p53(–/–) cells (Figure 4). The inhibition of p53 transcriptional activity by E2F1 in both p53(–/–) and Saos-2 cells is not a result of the reduction of exogenous p53 expression because the p53 level is not altered by exogenous E2F1 expression (Figure 5). Thus it is clear that E2F1 and DP1 can inhibit p53 transcriptional activity independently of mdm2.

Physical association between p53 and E2F1 or DP1 in vitro and in vivo

Because there was a reciprocal inhibition between the transcriptional activities of E2F and p53, we tested whether p53 and E2F1 or DP1 can interact physically. First we investigated the potential interactions using purified wild-type p53 from baculovirus with glutathione S-transferase (GST) fusion proteins GST–E2F1 (amino acids 89–437) and GST–DP1 (amino acids 1–410) from *Escherichia coli*. Wild-type p53 (10 ng) produced from baculovirus was incubated with GST (100 ng), GST–E2F1(50 ng) or GST–DP1 (50 ng) protein. The GST-containing proteins were pulled down specifically by precipitation with glutathione-conjugated agarose beads. The presence of p53 in the pulled down precipitates was detected with a p53-specific antibody in an immunoblotting assay. As shown in Figure 6A, the wild-type p53 was pulled down specifically with GST–E2F1 or GST–DP1 but not GST by glutathione–agarose beads, and was detected with a p53-specific monoclonal antibody (mAb) DO-1 (Vojtesek *et al.*, 1992; Figure 6A). This result indicates that there is a direct and specific physical association between p53 and GST–E2F1 or GST–DP1 *in vitro*, and it seems that more p53 was pulled down by GST–E2F1 than by GST–DP1. The interactions between p53 and E2F1 or DP1 were confirmed further using *in vitro*-translated E2F1 and DP1. As shown in Figure 6B, when purified p53 protein was incubated with the *in vitro*-translated E2F1 or DP1 in reticulocyte lysate, both E2F1 and DP1 were specifically coimmunoprecipitated with p53 by a p53-specific mAb

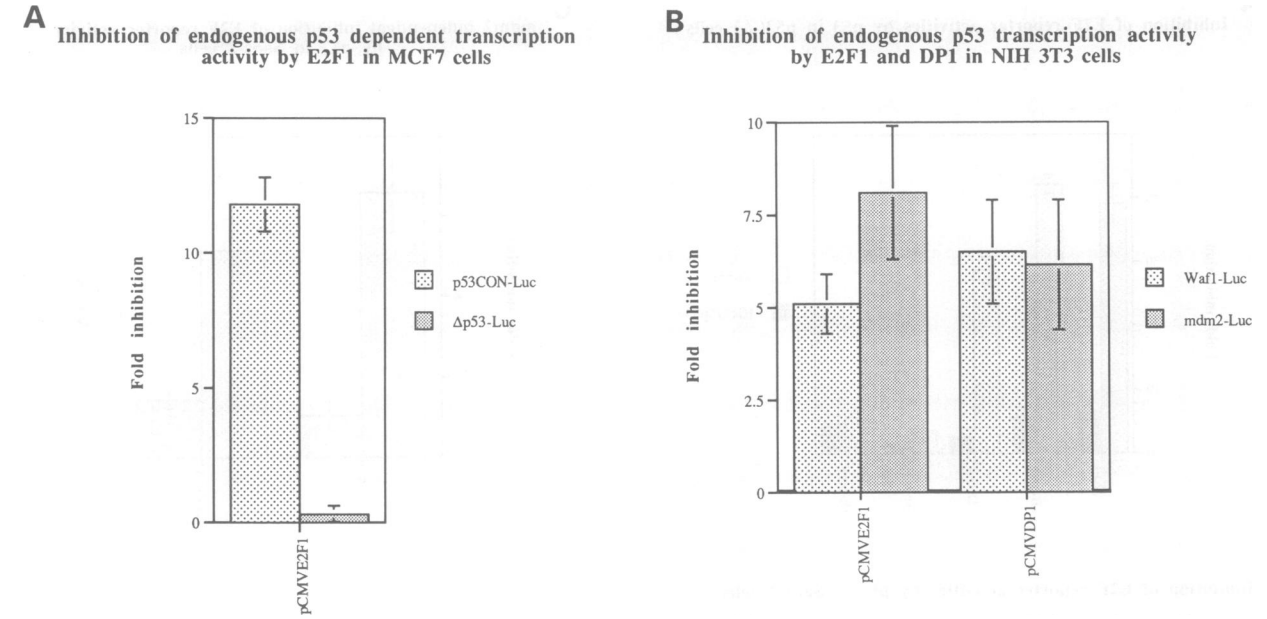

Fig. 3. (**A**) Inhibition of endogenous p53 transcriptional activity by E2F1 in MCF7 cells relative to vector controls. In all, 2.5×10^5 cells were cotransfected with 5 μg p53 reporter plasmid p53CON–Luc or its control plasmid Δp53–Luc plus 200 ng E2F1 (pCMV–E2F1) expression plasmid. (**B**) Inhibition of endogenous p53 transcriptional activity by E2F1 or DP1 in NIH 3T3 cells relative to the vector controls. In all, 2.5×10^5 cells were cotransfected with 2 μg p53 reporter plasmid mdm2–Luc or Waf–Luc plus 200 ng E2F1 (pCMV–E2F1) or 1 μg DP1 (pCMV–DP1) (Helin and Harlow, 1994).

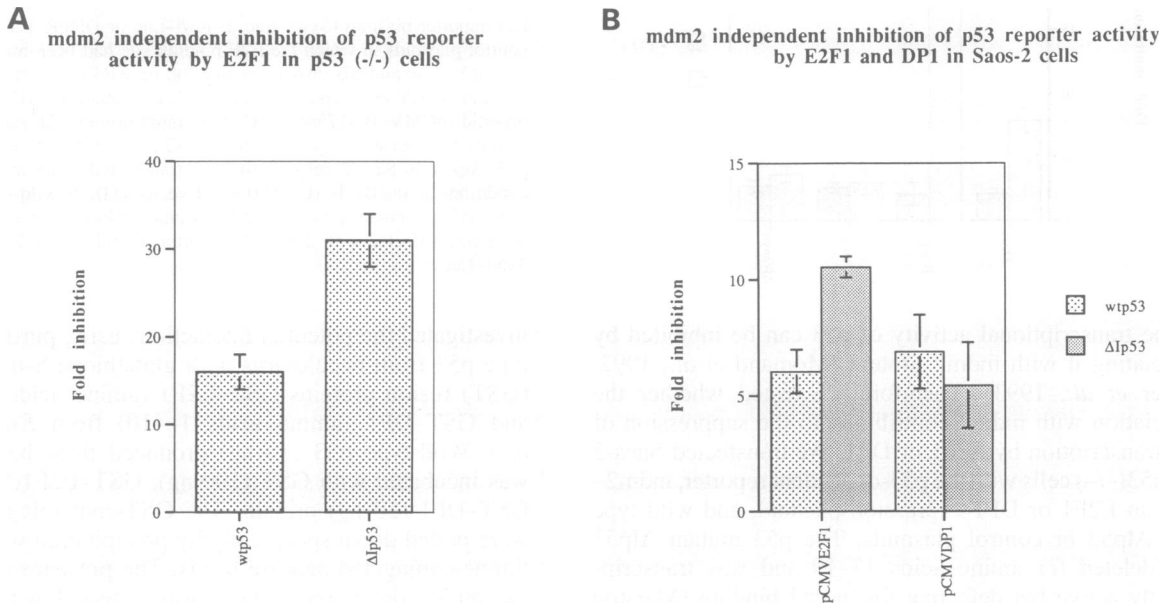

Fig. 4. mdm2-independent inhibition of p53 transcriptional activity by E2F1 and DP1 in p53(–/–) cells (**A**) and Saos-2 cells (**B**), respectively. In all, 2.5×10^5 cells were cotransfected with 2 μg p53 reporter plasmid mdm2–Luc plus 200 ng E2F1 (pCMV–E2F1) or 1 μg DP1 (pCMV–DP1) and 500 ng of either wild-type p53 (J4Ωp53) or 500 ng ΔIp53 (J4ΩΔIp53) (Marston *et al.*, 1994).

pAb421, but not by the control antibody pAb423 which recognizes the large T antigen of SV40 virus. In the absence of p53, the *in vitro*-translated E2F1 or DP1 does not bind to pAb421 under the same conditions (data not shown).

Next, we tested for interactions between p53 and E2F1 or DP1 *in vivo*. Lysates from Saos-2 cells transfected with wild-type p53 were immunoprecipitated using either an E2F1-specific mAb KH95 (Helin *et al.*, 1993) or a DP1-specific polyclonal antibody (Bandara *et al.*, 1993). The presence of p53 in the immunoprecipitates was detected using p53-specific antibodies CM1 (Midgley *et al.*, 1992; to detect p53 from KH95 immunoprecipitates) or DO-1 (to detect p53 from poly-DP1 immunoprecipitates). The exogenous p53 was specifically coimmunoprecipitated down with endogenous E2F1 or DP1 by KH95 or DP1 polyclonal antibody, respectively (Figure 7A and B, labelled p53). When exogenous E2F1 or DP1 was cotransfected with wild-type p53, more introduced p53 was coimmunoprecipitated down with both endogenous and exogenous E2F1 or DP1 using the same E2F1- or DP1-specific antibodies (Figure 7A and B, labelled p53+E2F1

and p53+DP1, respectively), indicating that the interactions between p53 and E2F1 or DP1 are dependent on the expression levels of the interacting proteins. Control antibody pAb419 (specific to SV40 large T antigen) did not immunoprecipitate down p53 under the same conditions (data not shown). Finally, physical interactions between endogenous p53 and E2F1 or DP1 were studied using cell extracts derived from C6 cells, the cell line in which the p53–mdm2 association was identified originally (Momand *et al.*, 1992). Either an E2F1-specific mAb KH95 (Helin *et al.*, 1993) or a DP1-specific polyclonal antibody (Bandara *et al.*, 1993) was used to immunoprecipitate E2F1 or DP1, respectively, from the cell extracts. As shown in Figure 8, p53 was readily detected in E2F1 or DP1 immune complexes in C6 cells (Figure 8B and C, respectively). KH95 does not crossreact with p53 because it does not detect equivalent amounts of purified p53 protein (Figure 8A and B).

Discussion

Here, we have demonstrated that E2F and p53 can reciprocally antagonize transcriptional activity. Because both proteins are key regulators of cell cycle progression, these functional interactions are of potential importance in the overall control of the cell cycle. Furthermore, we have demonstrated that p53 and E2F1 or DP1 associate *in vitro* and *in vivo*. Thus, these physical associations may contribute to the cross-inhibition of transcriptional function of p53 and E2F observed in cells.

One pathway hypothesized to mediate the p53-dependent inhibition of E2F function is Waf1/CIP1 (Dulic *et al.*, 1994). The retinoblastoma gene product is the key component in this pathway. However, the inhibition of the transcriptional activity of E2F in Saos-2 cells, which lack functional Rb, strongly argues that p53 can inhibit E2F transcriptional activity independently of Rb. Although it is possible that Rb-related proteins such as p107 or p130 may substitute for Rb, it is also possible that Rb family-independent pathways do exist and may be mediated by direct physical associations or the inhibition of E2F DNA binding activity.

When investigating the effects of E2F1 or DP1 on p53, it was particularly interesting to observe that the transcriptional activity of p53 was inhibited by either E2F1 or DP1, and that this inhibition was independent of mdm2 or Rb. This is the first demonstration that the two cell cycle regulating proteins E2F1 and DP1 are able to inhibit the transcriptional activity of p53. One possible explanation for the reciprocal inhibition of the transcriptional activity observed between p53 and E2F is that the two transcription factors may form a protein–protein complex, therefore preventing DNA binding. To be able to detect p53 and E2F1 or DP1 protein complexes *in vitro* strongly suggests that the interactions between p53 and E2F1 or DP1 can be direct. However, the interactions between p53 and E2F1 or DP1 *in vivo* could be more complicated because mdm2 has now been shown to be able to bind to E2F1, DP1, Rb and p53 (Martin *et al.*, 1995; Xiao *et al.*, 1995). To be able to inhibit the p53-dependent transcriptional activity by E2F1 or DP1 and also in an mdm2-independent manner argues that the direct interactions between p53 and E2F1 or DP1 may be at least partly responsible for such inhibition. Finally, it is known that p53, E2F1 and DP1 are all phosphoproteins (Meek *et al.*, 1990; Bandara *et al.*, 1994; Fagan *et al.*,

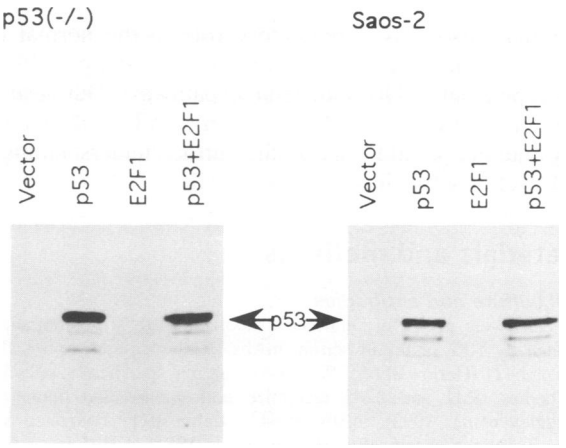

Fig. 5. p53 expression in p53(–/–) and Saos-2 cells with or without E2F1 cotransfection. The plasmids transfected into the cells are indicated as the following: Vector, CMV vector; p53, pCMV–SN3; E2F1, pCMV–E2F1; p53+E2F1, pCMV–SN3 + pCMV–E2F1.

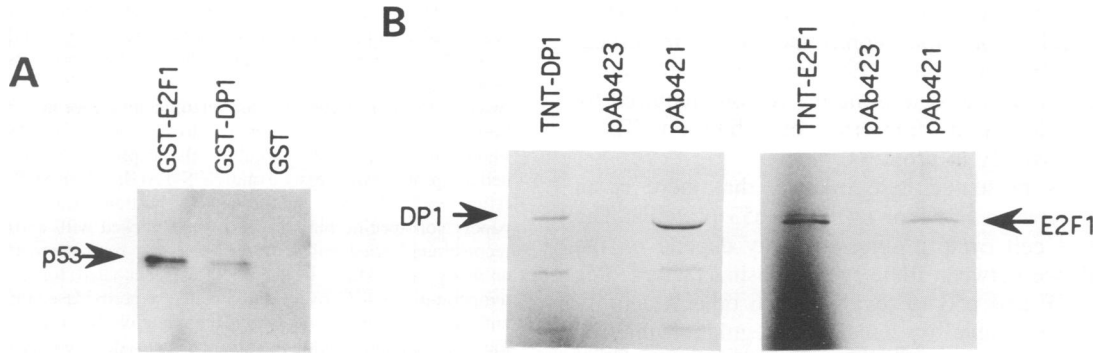

Fig. 6. (**A**) Physical association between p53 and E2F1 or DP1 *in vitro*. Purified wild-type p53 (10 ng) from baculovirus was incubated with purified GST–E2F1 (50 ng), GST–DP1 (50 ng) or GST (100 ng) from *E.coli*. The complexes were pulled down with glutathione–agarose beads and the p53 protein was detected by p53-specific antibody DO-1. (**B**) Physical association between p53 and E2F1 or DP1 *in vitro*. Purified wild-type p53 (400 ng) from *E.coli* was incubated with *in vitro*-translated E2F1 or DP1, which were both labelled with [^{35}S]methionine. After incubation, the complexes were pulled down by the p53-specific mAb pAb421 (labelled pAb421) or the p53 non-crossreactive antibody pAb423 (labelled pAb423). The *in vitro*-translated E2F1 and DP1 lysates alone are labelled TNT–E2F1 or TNT–DP1 respectively. The amount of TNT–DP1 or TNT–E2F1 loaded directly on the gel is 1/10 of the amount used in the immunoprecipitation assay.

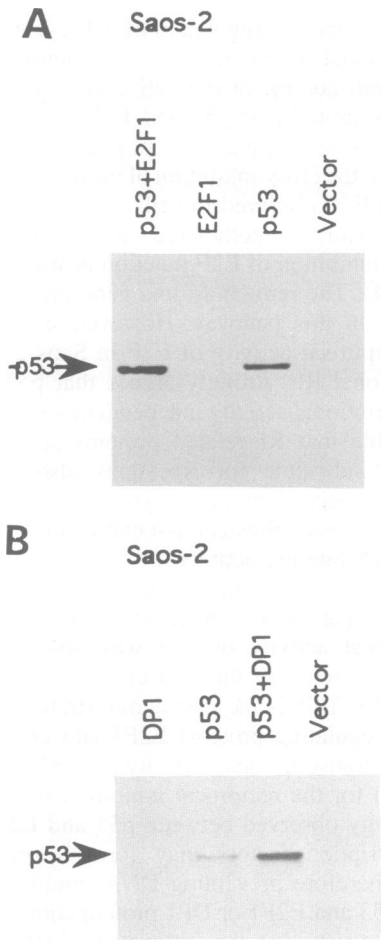

Fig. 7. Co-immunoprecipitation of E2F–p53 by the E2F-specific mAb KH95 (**A**) and DP1–p53 by the DP1-specific polyclonal antibody (**B**) from cell extracts derived from transfected Saos-2 cells. The presence of p53 was detected with p53-specific polyclonal antibody CM1 (A) or DO-1 (B). The plasmids transfected into the cells are indicated as the following: Vector, CMV vector; p53, pCMV–SN3; E2F1, pCMV–E2F1; DP1, pCMV–DP1; p53+E2F1, pCMV–SN3 + pCMV–E2F1; p53+DP1, pCMV–SN3 + pCMV–DP1.

1994), and that the involvement of phosphorylation of these three proteins in the regulation of their protein–protein interactions has yet to be elucidated. How the protein–protein interaction is regulated, and to what extent these physical associations contribute to the reciprocal inhibition of transcription of E2F and p53, will require mutagenesis studies to determine the regions required by physical associations and transcriptional inhibitions. These studies are currently in progress.

The results presented here indicate that there is an interaction between E2F1 or DP1 and p53 proteins. The regulation of cell cycle progression may depend on the precise balance between these two opposing factors. The ability of E2F and p53 to regulate each other's activity provides an extra level by which this regulation may be achieved. Commitment to S phase entry in a normal cell cycle may depend on circumventing the inhibitory effect of p53 activity on cell cycle progression. This may normally be achieved by the rapid degradation of the protein and the blockage of any residual p53 activity by cellular factors such as mdm2 and co-operating transcription factors, E2F1 and DP1, as shown here. Conversely,

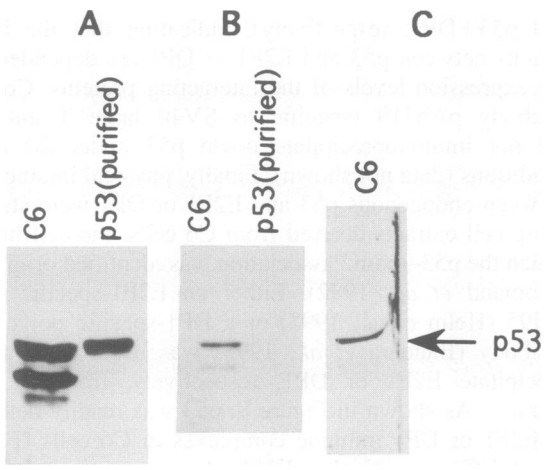

Fig. 8. Physical association between the endogenous p53 and the co-operating transcription factors E2F and DP1 in C6 cells. The protein complexes p53–E2F (**B**, antibody KH95) and p53–DP1 (**C**, anti-DP1 antiserum) can be readily detected in C6 cells. (**A**) Western blot to show that similar amounts of p53 were used in the immunoprecipitations in (B). The purified p53 used as a control against crossreactivity between p53 and the antibody KH95 is indicated as p53 (purified).

p53 may also play a regulatory role in the normal cell cycle by limiting E2F transcriptional activity, possibly by Rb-dependent and Rb-independent pathways. Our observation that p53 and E2F1/DP1 can physically and functionally interact should lead to the further understanding of cell cycle control in mammalian cells.

Materials and methods

Cell culture and antibodies

All cells were grown in Dulbecco's modified Eagle's medium supplemented by 10% fetal calf serum. mAbs DO-1 (Vojtesek *et al.*, 1992) and pAb421 (Harlow *et al.*, 1981) recognize p53 specifically; polyclonal antibodies CM1 and CM5 recognize both mouse and human p53 (Midgley *et al.*, 1992). mAbs pAb423 and pAb419 recognize SV40 large T antigen specifically (Harlow *et al.*, 1981). KH95 is an E2F1-specific mAb (Helin *et al.*, 1993; purchased from Santa Cruz Biotechnology Inc.). Rabbit serum of DP1-specific polyclonal antibody was a gift from Dr N.La Thangue (Bandara *et al.*, 1993).

Immunoprecipitation and immunoblotting

Cells were lysed with NET buffer containing 1% NP-40 (Harlow and Lane, 1988) on ice for 30 min before centrifugation. The lysates were precleared with protein G beads and then incubated with the appropriate antibodies, as indicated. The antibody complexes were pulled down by protein G beads, washed three times with 1% NP-40/NET buffer and twice with NET buffer. The immunoprecipitate–protein G beads were boiled in SDS sample buffer and loaded onto SDS–PAGE gels. For immunoblotting, 20 μl of soluble cellular proteins (5–8 mg/ml of total cellular protein) were loaded onto SDS–PAGE gels in SDS sample buffer. After electrophoresis, the proteins were transferred to nitrocellulose paper. Non-specific binding sites were blocked with a 10% solution of reconstituted dried milk powder for 1 h at room temperature. Primary antibody was added to the blot and incubated for 2–3 h at room temperature or 4°C overnight. Finally, a peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulin was incubated with the blot. Bound immunocomplexes were detected by the enhanced chemiluminescence (ECL) method, as described by the manufacturer (Amersham).

DNA transfection, CAT and luciferase assays

DNA was transfected into cells using calcium phosphate precipitation. At 24 h after the transfection, cells were lysed in 100–150 μl lysis buffer (Reporter lysis buffer from Promega) at room temperature for 5–15 min. The cell debris was removed by centrifugation at 14 000 r.p.m. for

2 min. In all, 20 µl supernatant were used for assaying the luciferase activity in a Luminometer using the luciferase kit from Promega. The mean values were derived from at least two independent experiments, and two measurements were made for each experiment. For CAT assays, the cell lysate supernatants were incubated at 68°C for a further 30 min and centrifuged at 14 000 r.p.m. for 5 min. A total of 30 µl of this supernatant were incubated with 0.1 µCi [^{14}C]chloramphenicol and acetyl-CoA, as described previously (Sambrook *et al.*, 1989). The acetylated chloramphenicol was separated by TLC.

Production of GST, GST–E2F1 and GST–DP1

To generate GST–DP1, a full-length mouse DP1 (amino acids 1–410) cDNA sequence was amplified by PCR and cloned into pGEX3X (Pharmacia). Subsequently, the amplified sequence was verified by direct sequencing. Its expression in *Escherichia coli* was tested using Western blotting. GST–E2F1 (amino acids 89–437) has been described previously (Bandara *et al.*, 1994).

GST fusion proteins were expressed in *E.coli* and induced with 1 mM IPTG (Sigma). The GST fusion proteins were purified on glutathione-conjugated agarose beads, as described previously (Bandara *et al.*, 1994). The immobilized fusion proteins were eluted from the beads with 20 mM reduced glutathione in 50 mM Tris–HCl (pH 8.0). The purity of the GST fusion proteins was examined by SDS–PAGE, followed by staining with Coomassie Blue; their concentrations were determined by a Bradford protein assay (Bio-Rad).

p53 pull-down assays using GST–DP1 and GST–E2F1

50 ng of the appropriate GST fusion proteins were incubated with 10 ng of p53 protein (produced in baculovirus) in 50 µl of DNA binding buffer at 4°C for 1 h. Glutathione-conjugated agarose beads, which had been incubated previously with 10 mg/ml bovine serum albumin in NET buffer, were added to the binding reaction to bind the GST fusion proteins. After incubating at 4°C for 10 min, the beads were washed extensively with NET buffer containing 0.5% NP-40. The bound proteins were released in SDS gel sample buffer, resolved on a 10% SDS–PAGE gel and immunoblotted. The blots were probed using the anti-p53 mAb DO-1 and detected using an ECL Western blotting detection kit (Amersham).

In vitro translation and immunoprecipitation

Full-length DP1 and E2F1 coding sequences were transcribed and translated separately *in vitro* in the presence of [^{35}S]methionine using the Promega TNT Coupled Reticulocyte Lysate System, according to the manufacturer's instructions (Bandara *et al.*, 1994). 10 µl of the lysate containing the appropriate *in vitro*-translated protein product were mixed with 400 ng p53 protein (produced in bacteria) and allowed to interact in 50 µl DNA binding buffer at 4°C for 1 h, before diluting to 500 µl with NET buffer containing 0.5% NP-40. Anti-p53 antibody pAb421 or the control antibody pAb423 (anti-SV40 large T antigen) immobilized on protein G–agarose beads was added to the binding reactions and incubated with mixing at 4°C for 1 h. The agarose beads were then washed extensively with NET buffer containing 0.5% NP-40. The bound proteins were released in SDS gel sample buffer and resolved by SDS–PAGE.

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