The alternative product from the human CDKN2A locus, p14ARF, participates in a regulatory feedback loop with p53 and MDM2

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The two distinct proteins encoded by the CDKN2A locus are specified by translating the common second exon in alternative reading frames. The product of the α transcript, p16INK4a, is a recognized tumour suppressor that induces a G1 cell cycle arrest by inhibiting the phosphorylation of the retinoblastoma protein by the cyclin-dependent kinases, CDK4 and CDK6. In contrast, the product of the human CDKN2A β transcript, p14ARF, activates a p53 response manifest in elevated levels of MDM2 and p21CIP1 and cell cycle arrest in both G1 and G2/M. As a consequence, p14ARF-induced cell cycle arrest is p53 dependent and can be abrogated by the co-expression of human papilloma virus E6 protein. p14ARF acts by binding directly to MDM2, resulting in the stabilization of both p53 and MDM2. Conversely, p53 negatively regulates p14ARF expression and there is an inverse correlation between p14ARF expression and p53 function in human tumour cell lines. However, p14ARF expression is not involved in the response to DNA damage. These results place p14ARF in an independent pathway upstream of p53 and imply that CDKN2A encodes two proteins that are involved in tumour suppression.

Keywords: cell cycle/MDM2/p53 response/replicative senescence/tumour suppression

Introduction

The CDKN2A locus on human chromosome 9p21 (Kamb et al., 1994; Nobori et al., 1994) and the cognate loci on mouse chromosome 4 (Jiang et al., 1995; Quelle et al., 1995a) and rat chromosome 5 (Swafford et al., 1997) encode two distinct proteins translated from alternatively spliced mRNAs (see diagram in Figure 1A). The cyclin-dependent kinase inhibitor from which the locus takes its name (also known as p16INK4a) is specified by an RNA comprising exons 1α, 2 and 3 (Serrano et al., 1993; Kamb et al., 1994; Nobori et al., 1994) referred to as the α transcript. The alternative product, designated ARF for ‘alternative reading frame’, is encoded by the slightly smaller β transcript that comprises exons 1β, 2 and 3 (Duro et al., 1995; Jiang et al., 1995; Mao et al., 1995; Quelle et al., 1995b; Stone et al., 1995b). The primary amino acid sequences of ARF and p16INK4a are completely unrelated since they are produced by translating the common exon 2 sequences in different reading frames. Exon 1β bears no homology to exon 1α and, therefore, has the features of a distinct gene that has become inserted between the tandemly linked genes encoding p16INK4a and its close relative p15INK4b (reviewed in Larsen, 1996; Sidransky, 1996).

Most of the current information about ARF relates to the mouse homologue. The mouse β transcript was first noted during attempts to isolate cDNAs encoding mouse p16INK4a (Quelle et al., 1995a) and subsequently shown to specify a protein of 169 amino acids, designated p19ARF, that has no obvious relatives in the current databases (Quelle et al., 1995b). Quelle et al. went on to demonstrate that although mouse p19ARF does not interfere directly with the function of cyclin-dependent kinases, it nevertheless invokes a cell cycle arrest when ectopically expressed in rodent cells (Quelle et al., 1995b). The most striking facet of the cell cycle arrest is the accumulation of cells with both a G1 and G2/M DNA content, with apparent exclusion of cells in S-phase. It was also noted that the levels of p19ARF were generally higher in cells that had sustained mutations in the p53 tumour suppressor gene or in which p53 had been functionally compromised by overexpression of MDM2 (Quelle et al., 1995b).

Much less is known about the human equivalent of p19ARF. The sequence of the human CDKN2A exon 1β was originally deduced from a combination of genomic DNA analysis, cDNA cloning and PCR-based approaches (Duro et al., 1995; Mao et al., 1995; Stone et al., 1995b). In the genomic DNA, the open reading frame continues for some distance upstream of the ATG that aligns with the start of the mouse p19ARF-coding sequence. However, as this ATG is in a favourable context for translation initiation and is the first ATG in the presumptive transcript (Mao et al., 1995; Stone et al., 1995b), it is generally assumed that the human protein starts at this point. The encoded protein would therefore be predicted to comprise 132 amino acids with a molecular weight of 13 902 Da (see Figure 1B) and, by analogy to the corresponding mouse protein (p19ARF), we refer to the human homologue as p14ARF (Duro et al., 1995; Jiang et al., 1995; Mao et al., 1995; Quelle et al., 1995b; Stone et al., 1995b). The mouse and human proteins show only 50% identity over the region of overlap (Figure 1B), but transfection experiments have indicated that a cDNA representing the human β transcript can also induce a cell cycle arrest (Liggett et al., 1996; Arap et al., 1997).
The functional characterization of ARF has understandably been influenced by the approaches taken to study p16INK4a and its relatives which bind directly and specifically to the cyclin-dependent kinases CDK4 and CDK6, thereby inhibiting their ability to promote cell cycle progression via the phosphorylation of the retinoblastoma gene product, pRb (Sherr and Roberts, 1995; Weinberg, 1995; Sherr, 1996; Ruas and Peters, 1998). Thus, ectopic expression of p16INK4a or p15INK4b causes cells to accumulate with a G₀/G₁ DNA content, but cells that lack functional pRb are resistant to these effects (Guan et al., 1994; Okamoto et al., 1994; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995; Stone et al., 1995a). Conversely, cells that lack INK4 function are likely to have a proliferative advantage, and p16INK4a is now recognized as a major tumour suppressor implicated in a wide variety of tumour types (reviewed in Sherr, 1996; Ruas and Peters, 1998). Significantly, both the α transcript and p16INK4a accumulate when primary cells are propagated in tissue culture, suggesting that p16INK4a may participate in the G₁ arrest associated with replicative senescence (Alcorta et al., 1996; Hará et al., 1996; Loughran et al., 1996; Reznikoff et al., 1996; Wong and Riabowol, 1996; Palmero et al., 1997; Zindy et al., 1997). As well as explaining why p16INK4a, rather than other members of the INK4 family, acts as a tumour suppressor, a role in senescence would account for the higher frequency of p16INK4a alterations noted in tumour cell lines as opposed to primary tumours (Cairns et al., 1994; Spruck et al., 1994), since there would be a strong selection against the expression of p16INK4a during the establishment of immortal clones. 

Immortalization of human cells is facilitated by disruption of both pRb- and p53-dependent mechanisms (Shay and Wright, 1989; Wright et al., 1989; Hará et al., 1991; Shay et al., 1991a). Whereas the accumulation of p16INK4a with population doublings probably accounts for the pRb-linked mechanism, the p53 dependence is presumably associated with the accumulation of the p21CIP1 CDK inhibitor (Noda et al., 1994; Alcorta et al., 1996). Through its ability to inhibit G₁-specific cyclin-dependent kinase complexes and to bind to proliferating cell nuclear antigen (PCNA), p21CIP1 is believed to be a major executor of the p53-dependent cell cycle arrest that occurs in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995; reviewed in Sherr and Roberts, 1995; Levine, 1997). It has also been implicated directly in senescence by experiments showing that targeted disruption of p21CIP1 can extend the lifespan of human diploid fibroblasts (Brown et al., 1997) and that activation of the RAS–RAF signalling pathway can elicit a cell cycle arrest via the up-regulation of p21CIP1 (Lloyd et al., 1997; Sewing et al., 1997; Woods et al., 1997). However, the relative contributions of p21CIP1 and p16INK4a in senescence have yet to be evaluated fully, and fibroblasts derived from mice with targeted disruptions of either gene fail to undergo senescence (Deng et al., 1995; Serrano et al., 1996). Interestingly, recent evidence suggests that this is also true for mouse cells with a specific disruption of exon 1β, further complicating the interpretation (Kamijo et al., 1997). Since there are inherent differences in the immortalization frequencies of mouse and human cells and presumably in the underlying mechanisms, we considered it important to characterize the expression patterns and biological properties of the human homologue of ARF.
Elevated expression of p14ARF therefore leads to the accumulation of both MDM2 and p53 by affecting the balance between transcriptional activation and protein turnover. In turn, the expression of endogenous p14ARF is negatively regulated by p53, and we show that in a panel of human tumour cell lines there is an inverse correlation between p14ARF expression and p53 status that has parallels with the feedback loop observed between p16INK4a expression and pRb status in human cells (Li et al., 1994b; Hara et al., 1996; Palmero et al., 1997). However, there is no evidence that the β transcript is involved in a DNA damage response. We therefore favour a model in which p14ARF is a component of a separate pathway upstream of p53 such that targeted disruption of exon 1β is likely to reproduce some but not all of the phenotypic characteristics of p53-nullizygous mice. These results are discussed in relation to the possible role of ARF as a tumour suppressor.

**Results**

**Detection of human p14ARF**

Using the known sequence to predict suitable primers, a cDNA corresponding to the human CDKN2A β transcript was generated by reverse transcription and PCR using RNA obtained from TIG3 human diploid fibroblasts. The DNA sequence predicts a protein of 132 amino acids that terminates prematurely compared with the mouse homologue (Figure 1B). Polyclonal antisera were generated against a synthetic peptide corresponding to the C-terminal 15 amino acids of human p14ARF and validated in a number ways. For example, the antisera were capable of immunoprecipitating [35S]methionine-labelled p14ARF generated by coupled in vitro transcription and translation (not shown). In Western blots, the antisera detected a 14 kDa protein in cells that had been engineered to express the β cDNA under the control of an isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible promoter (see below). Although the mouse protein migrates anomalously in SDS–PAGE, possibly due to the high arginine content (Quelle et al., 1995b), the shorter human protein migrates as expected at 14 kDa and is clearly distinguishable from p16INK4a.

Following affinity purification on immobilized peptide, the antisera were able to detect endogenous p14ARF by immunofluorescence. As illustrated in Figure 2, staining of the 5637 bladder carcinoma cell line revealed a distinctive punctate pattern as well as staining of larger more diffuse bodies (Figure 2A). By direct comparison with phase contrast images, it is clear that these correspond to nucleoli (Figure 2C and D). The staining could be competed by excess peptide (Figure 2B) and was present in almost all cells (Figure 2E and F). Less distinct staining was noted in mitotic figures. A similar staining pattern was detected in U2OS cells that expressed inducible p14ARF, and in these cells positive staining was dependent on the addition of IPTG (Figure 2G and H). This pattern of staining is virtually identical to that seen with mouse p19ARF (Quelle et al., 1995b) and has recently been described for the human protein in HeLa and HS27 cells (Della Valle et al., 1997).

**Cell cycle arrest by inducible expression of p14ARF**

Mouse p19ARF has previously been shown to arrest cells in both G1 and G2/M phases when ectopically expressed using retroviral vectors (Quelle et al., 1995b), but the nature of the arrest elicited by human ARF has not been defined (Liggett et al., 1996; Arap et al., 1997). To address this issue and to compare directly the properties of p14ARF and p16INK4a, the U2OS osteosarcoma cell line, which is wild-type for both p53 and pRb (Diller et al., 1990), was

**Fig. 2.** Localization of p14ARF by immunofluorescence. Logarithmically growing monolayer cultures were fixed and stained with an affinity-purified polyclonal antiserum against the C-terminal 15 residues of human p14ARF. (A) 5637 bladder carcinoma cells; (B) the same cells with the primary antibody pre-adsorbed against excess peptide antigen; (C and D) matching immunofluorescence and phase contrast images of 5637 cells; (E and F) the same cells at lower magnification; (G) U2OS cells expressing IPTG-inducible p14ARF; (H) the same cells without IPTG induction. (A–D) and (G–H) were photographed at 100× magnification, whereas (E) and (F) were at 20× magnification.
Figure 3. Cell cycle arrest by inducible expression of p14ARF and p16INK4a. (A) EH1 and NARF1 cells were treated with increasing amounts of IPTG as indicated, and the expression of p16INK4a and p14ARF monitored by immunoblotting. The same samples were also immunoblotted with an antibody to pRb to determine the extent of phosphorylation. (B) FACS profiles of EH1 and NARF1 cells either untreated or treated for 48 h with 1 mM IPTG.

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used to establish clones expressing regulatable forms of the corresponding cDNAs, based on the LAC SWITCH™ expression system (Stratagene). With the β cDNA, all six of the clonal lines tested (NARF1–NARF6) were found to express p14ARF upon addition of IPTG, whereas only two of the 30 lines transfected with the α cDNA (EH1 and EH2) showed inducible expression of p16INK4a. In EH1 cells, the expression of p16INK4a was activated by as little as 0.01 mM IPTG and was maximal at between 0.3 and 1.0 mM IPTG, whereas the expression of p14ARF in NARF2 cells showed a steeper dose responsiveness, with maximal levels achieved with 0.1 mM IPTG (Figure 3A). Similar results were obtained with different clones, and two clones of p14ARF-expressing cells, NARF1 and NARF2, were used interchangeably in subsequent experiments. When the same cell lysates were immunoblotted for pRb protein (Figure 3A), it was clear that the phosphorylation of pRb, as judged by the presence of the more slowly migrating forms, was inhibited by the induction of either p16INK4a or p14ARF. The degree of inhibition paralleled the dose responsiveness of p16INK4a and p14ARF expression (Figure 3A).

Since the inhibition of pRb phosphorylation is generally equated with a G1-phase arrest, we also checked the cell cycle distribution of the inducible cell clones by propidium iodide staining and flow cytometry. As shown in Figure 3B, asynchronously growing EH1 and NARF2 cells had a normal cell cycle profile, with cells distributed in the G1, S and G2/M fractions. Upon addition of 1 mM IPTG, the EH1 cells, expressing inducible p16INK4a, arrested with a G1 DNA content as expected. In contrast, the NARF2 cells expressing inducible p14ARF accumulated in both the G1 and G2/M phases (Figure 3B).

More detailed analyses were performed to determine the time scale, dose responsiveness and reversibility of the arrests imposed by either p14ARF or p16INK4a. Changes in cell cycle profiles were apparent as early as 6 h after IPTG addition and were essentially maximal after 24 h in the case of p14ARF, whereas the proportion of p16INK4a-expressing cells in G1 continued to increase up to 48 h (not shown). The effects were more pronounced at higher concentrations of IPTG, reflecting the dose responsiveness of p14ARF or p16INK4a induction, and in both cases cells treated with 1 mM IPTG for 48 h were able to resume normal cycling upon removal of the inducer (not shown).

**Activation of p53 and p21 by ectopic expression of p14ARF**

The ability of p14ARF to arrest cells in the G1 and G2/M phases of the cycle suggested that the effects might be mediated via p53 (Agarwal et al., 1995). Lysates prepared from NARF2 and EH1 cells treated with increasing concentrations of IPTG (as in Figure 3A) were immunoblotted with antibodies against p53, p21CIP1, and MDM2, as indicated. The results for p16INK4a and p14ARF are reproduced from Figure 3A.

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Fig. 4. Up-regulation of p53, p21CIP1 and MDM2 by p14ARF. Lysates prepared from EH1 and NARF1 cells treated with increasing doses of IPTG (as in Figure 3A) were immunoblotted with antibodies against p53, p21CIP1 and MDM2, as indicated. The results for p16INK4a and p14ARF are reproduced from Figure 3A.
in vitro using labelled proteins expressed by coupled IPTG (Figure 5A). This interaction was investigated further obtained, specifically in cells that had been treated with against MDM2, clear evidence for co-precipitation was cells were immunoblotted with a monoclonal antibody lacking the C-terminal 51 amino acids (1–440) were direct binding assay, wild-type MDM2 and a mutant form of p14ARF and MDM2. The left hand panel shows the individual [35S]methionine-labelled translation products and the right hand panel shows the co-immunoprecipitation of labelled products with p14 ARF antiserum. Wild-type MDM2 and a form lacking the C-terminal 51 residues (1–440) associated with p14 ARF in this assay, whereas the Δ222–437 mutant did not. (C) Co-precipitation of p14ARF, p53 and MDM2 from transiently transfected SAOS2 cells with antiserum against p14ARF. The efficient immunoprecipitation of p53 depended on the co-expression of MDM2 and was not apparent with a deleted form of p53 (ΔI) that lacks the MDM2-binding site.

**Fig. 5.** Physical interaction of p14 ARF with MDM2 and p53. (A) Lysates prepared from NARF1 cells with and without addition of 1 mM IPTG were either analysed directly (Lysate) or immuno-precipitated with p14ARF antiserum or the pre-immune control (IgG) and immunoblotted for MDM2. (B) In vitro binding of p14ARF and MDM2. The left hand panel shows the individual [35S]methionine-labelled translation products and the right hand panel shows the co-immunoprecipitation of labelled products with p14ARF antiserum. Wild-type MDM2 and a form lacking the C-terminal 51 residues (1–440) associated with p14ARF in this assay, whereas the Δ222–437 mutant did not. (C) Co-precipitation of p14ARF, p53 and MDM2 from transiently transfected SAOS2 cells with antiserum against p14ARF. The efficient immunoprecipitation of p53 depended on the co-expression of MDM2 and was not apparent with a deleted form of p53 (ΔI) that lacks the MDM2-binding site.

**Direct binding of p14ARF and MDM2**

In other settings, such as the response to DNA damage, a rapid increase in p53 levels is brought about through stabilization of the protein. In undamaged cells, the p53 protein is turned over rapidly at least in part through interaction with MDM2 which targets p53 for ubiquitination and proteasome-mediated degradation (Haupt et al., 1997; Kubbutat et al., 1997). We therefore asked whether the ability of p14ARF to activate a p53 response in NARF cells occurred via a physical interaction with either MDM2 or p53. When p14ARF immunoprecipitates from NARF cells were immunoblotted with a monoclonal antibody against MDM2, clear evidence for co-precipitation was obtained, specifically in cells that had been treated with IPTG (Figure 5A). This interaction was investigated further in vitro using labelled proteins expressed by coupled transcription and translation in reticulocyte lysates. In this direct binding assay, wild-type MDM2 and a mutant form lacking the C-terminal 51 amino acids (1–440) were co-precipitated efficiently with p14ARF (Figure 5B). In contrast, the cyclin D1 control and a mutant form of MDM2 lacking residues 222–437 did not bind efficiently to p14ARF under these conditions. Note that the Δ222–437 mutant did show some residual degree of binding when the corresponding plasmid constructs were co-expressed in U2OS cells (data not shown). However, attempts to map the p14ARF-binding domain on MDM2 in this way have so far produced equivocal results.

The transient co-transfection assay was also used to determine whether p14ARF could form ternary complexes with p53 and MDM2. SAOS2 cells, which lack endogenous p53, were transfected with various combinations of plasmids encoding p14ARF, MDM2, p53 or a form of p53 (ΔI) lacking conserved box I that is incapable of binding MDM2 (Marston et al., 1994; Kubbutat et al., 1997). To avoid potential apoptotic effects, both forms of p53 used in this experiment were also deleted for conserved box II (kindly provided by M. Ashcroft). The p14ARF immunoprecipitates were then immunoblotted for p53 and MDM2. From these data, it was clear that p14ARF was capable of forming a three-way complex with p53 and MDM2, and that the co-precipitation of p53 was absolutely dependent on the presence of MDM2 (Figure 5C). The ΔI mutant of p53 did not enter a ternary complex with p14ARF presumably because of its inability to bind MDM2.

**Stabilization of p53 by expression of p14ARF**

We next asked whether the association of p14ARF with MDM2 had any effect on its ability to promote the degradation of p53. As shown previously (Kubbutat et al., 1997), co-transfection of MDM2 leads to a dose-dependent reduction in the amount of p53 protein detectable after 24 h (Figure 6A). This MDM2-induced turnover of p53 was completely inhibited by co-transfection of p14ARF. Similar effects were observed with mouse and human MDM2 and in U2OS and SAOS2 cells (data not shown). Significantly, there was also a marked accumulation of MDM2 in the p14ARF-transfected cells, analogous to the effects seen with inducible p14ARF in NARF cells. In the transiently transfected cells, the increased expression of MDM2 cannot be attributed to transcriptional activation by p53 since it is being expressed from a heterologous promoter. Moreover, the LLN L proteasome inhibitor, which can protect p53 from MDM2-mediated degradation (Kubbutat et al., 1997), had no additional effect on p53 levels over those attributable to co-expression of p14ARF (Figure 6B). Thus, p14ARF appears to stabilize both p53 and MDM2.

To explore further the effects of p14ARF on MDM2, co-transfections were carried out with two mutant forms of MDM2 (1–440 and 6–339) that lack the C-terminal domain and are therefore impaired in their ability to target p53 for ubiquitin-mediated degradation (M. Kubbutat and K. H. Vousden, in preparation). Both mutants were stabilized by p14ARF in the absence of co-transfected p53 (Figure 6C). Moreover, co-expression of the E6 protein of HPV16, which independently targets p53 for degradation (Scheffner et al., 1990), did not diminish the effects of p14ARF on MDM2 levels (Figure 6D).

**p53 dependence of the p14ARF-mediated arrest**

The ability of p14ARF to activate the p53-dependent expression of p21CIP1 suggested that the cell cycle arrest
imposed by p14ARF would depend on the presence of functional p53, in the same way that the arrest imposed by p16INK4a is dependent on functional pRb (Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). We therefore tested this prediction in a number of different ways. The most direct assay was to transfect established human tumour cell lines of known p53 and pRb status with pcDNA3-based plasmids capable of expressing the CDKN2A α and β RNAs from a cytomegalovirus (CMV) promoter. The cells were co-transfected with a plasmid encoding the CD20 cell surface marker and, 48–60 h post-transfection, the proportions of the CD20-expressing cells in different phases of the cell cycle were determined by dual-colour flow cytometry (van den Heuvel and Harlow, 1993). In this transient assay, the G2 arrest associated with p14ARF-encoding virus showed a markedly reduced proliferative capacity compared with the vector-only controls (Figure 8A). In the continued presence of serum, the p14ARF-infected cells adopted phenotypic characteristics typical of replicative senescence, such as increased size and the expression of senescence-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995). We have previously shown that ectopic expression of either p21cip1 or p16INK4a can elicit similar effects in early passage TIG3 cells (McConnell et al., 1998). Co-infection with a virus encoding E6 markedly reduced the proportion of cells staining positively for SA-β-gal activity (Figure 8B).

**Relationship of p14ARF expression to p53 status**

Previously we have shown that TIG3 cells transformed by SV40 T-Ag (SVts8) express elevated levels of the
ARF participates in a feedback loop with p53 and MDM2

Fig. 7. p53 dependence of the p14ARF-induced cell cycle arrest. (A) MCF7, H1299 and SAOS2 cells were co-transfected with vectors encoding the cell surface marker CD20 plus p16INK4a, p21CIP1 or p14ARF as indicated. After 48 h, the relative DNA contents of the CD20-positive cells were assessed by dual-colour flow cytometry. The results are plotted as the percentage change in the G1 fraction relative to cells transfected with vector alone. Only the p53-positive MCF7 cells were arrested by p14ARF whereas p21CIP1 affected all cells and the p16INK4a arrest was pRb dependent. (B) Immunoblotting of the transiently transfected cell lysates to confirm expression of p14ARF. Note the concomitant up-regulation of p21CIP1 and p53 only occurred in the p53-positive MCF7 cells. (C) MCF7 cells stably expressing HPV16 E6 protein were shown to be partially resistant to the cell cycle arrest imposed by transiently transfected p14ARF but still responded to p16INK4a. (D) Confirmation that the cells in (C) were expressing equivalent levels of p14ARF but that p21CIP1 and p53 were not induced in the presence of E6. (E) Transfection of exon 1β sequences alone (N65ARF) induced a G1 arrest in MCF7 cells that was indistinguishable from the arrest imposed by full-length p14ARF.

Fig. 8. Abrogation of p14ARF-induced growth arrest by HPV16 E6. (A) TIG3 primary human fibroblasts and the same cells infected with a retrovirus encoding E6 (puromycin resistant) were infected with recombinant retroviruses encoding p14ARF (hygromycin resistant) and pools of infected cells were selected over 7 days. Equal numbers of cells were then seeded in 24-well dishes and cell proliferation was monitored over the following 7 days. (B) At 15 days post-infection, cells were stained for SA-β-gal activity (Dimri et al., 1995). The proportion of SA-β-gal-positive cells in pools infected with p14ARF virus was markedly reduced by co-infection of E6.

CDKN2A β transcript, suggesting a link with the status of either the p53 or pRb genes (Hara et al., 1996). Indeed, in nuclear run-on experiments, it was possible to demonstrate modulation of exon 1β transcription by a temperature-sensitive form of SV40 T-Ag (Hara et al., 1996). These data did not distinguish between effects related to pRb or p53, both of which are targeted by SV40 T-Ag, but the availability of TIG3 cells infected with an E6 retrovirus enabled us to clarify this issue. In line with the previous findings, SVts8 cells grown at the permissive temperature for T-Ag function contained readily detectable levels of p14ARF, and the same was true for TIG3 cells expressing HPV16 E6 (Figure 9A). In contrast, little if any p14ARF could be observed in the control TIG3 cells (Figure 9A). Conversely, the TIG3 cells expressing SV40T-Ag and HPV16 E6 showed the expected down-regulation of endogenous p21CIP1 levels.

Up-regulation of p14ARF expression by E6 would be consistent with a negative feedback loop in which p53 represses the expression of the CDKN2A β transcript. To explore this possibility further, we examined p14ARF levels in SAOS2 cells that had been engineered to express wild-type p53 under the control of an inducible promoter or that had been stably transfected with a plasmid encoding a temperature-sensitive form p53 (V143A). In both settings, activation of p53 resulted in markedly decreased expres-
The existence of this feedback loop, together with previous observations on mouse ARF expression, suggested that there should be an inverse correlation between p14ARF and p53 status (see Figure 9). No p14ARF was detected in the 5637 bladder carcinoma line (p53–/pRb–) grown at the permissive temperature. The same lysates were used to confirm the effects of E6 and T-Ag on endogenous p21CIP1 levels. This table summarizes the expression patterns of p16INK4a and p14ARF in the indicated tumour cell lines and primary fibroblast strains. The relative intensities of the signals are indicated as +++, + and +/−. The latter signifies a situation in which low levels of β RNA have been observed but the protein is below the level of detection with the available antiserum. del refers to situations in which the genomic DNA has been deleted and meth where exon 1β has been transcriptionally silenced by methylation. Apart from Kit225 (see text), the status of pRb and p53 in these cell lines is based on published information (for example, Horowitz et al., 1990; Bates et al., 1994b; Hainaut et al., 1997).
The patterns of p14ARF expression were quite distinct from those of p16INK4a which, as expected from previous work, showed an inverse correlation with the status of pRb (Figure 9C and Table I). Whereas all pRb-negative cells expressed high levels of p16INK4a, no expression was detected in pRb-positive cell lines such as Kit225, T24 and U2OS, in which exon 1α is methylated (unpublished observations) or in CEM and MCF7 where the gene has been homozygously deleted.

**p14ARF expression is not linked to DNA damage**

Having established that p14ARF is capable of activating p53 and may itself be regulated by p53, we were interested in determining what other signals might impact on the expression of p14ARF. A variety of different signals have been shown to activate p53-mediated responses, including DNA damage, hypoxia, oncogenic stimulation and senescence (Bates and Vousden, 1996). Several of the cell lines surveyed in Table I as well as primary diploid fibroblasts were therefore tested for their responsiveness to actinomycin D, a chemotherapeutic drug which at low doses elicits a strong p53 response. We were unable to detect any p14ARF in fibroblasts before or after treatment with actinomycin D (data not shown). Conversely, the MCF7 and A375 cell lines showed the expected up-regulation of p53 upon treatment with actinomycin D despite the absence of p14ARF, whereas the Kit225 and H1299 cell lines, which express relatively high levels of p14ARF, showed no discernible p53 response (Figure 10). Although the low levels of actinomycin D used in these experiments should not block transcription, there was a noticeable decrease in p14ARF levels in Kit225 and H1299 cells. The origin of this effect remains unclear and is the subject of ongoing research.

**Discussion**

The data we present here reaffirm the existence and importance of the alternative product encoded by the mammalian CDKN2A locus by providing unequivocal evidence for the presence of the human homologue, p14ARF, in the nuclei of human tumour cells and by linking its function to that of the p53 tumour suppressor. Like its mouse counterpart, human p14ARF has the ability to elicit its function to that of the p53 tumour suppressor. Like its
discernible p53 response (Figure 10). Although the low levels of actinomycin D used in these experiments should not block transcription, there was a noticeable decrease in p14ARF levels in Kit225 and H1299 cells. The origin of this effect remains unclear and is the subject of ongoing research.

In seeking a mechanism for the effects of p14ARF on p53, we showed that p14ARF can interact directly with the MDM2 protein, both in vivo and in vitro (Figure 5), consistent with recently published data from other groups (Pomerantz et al., 1998; Zhang et al., 1998). As well as being a transcriptional target for p53, MDM2 can interfere with the transcriptional activity of p53 by direct binding via its N-terminal region (Chen et al., 1993; Oliner et al., 1993) and can target p53 for rapid degradation by ubiquitin-mediated proteolysis (Haupt et al., 1997; Kubbutat et al., 1997). Although the domains on MDM2 that bind p14ARF have not been defined unambiguously (data not shown and Pomerantz et al., 1998; Zhang et al., 1998), they are likely to be distinct from the p53-binding site since it is possible to form ternary complexes containing p14ARF, MDM2 and p53 (Figure 5C). Indeed, co-precipitation of p53 and p14ARF was dependent on MDM2, whereas the latter two proteins can associate in the absence of p53.

Functionally, the binding of p14ARF to MDM2 results in the stabilization of both p53 and MDM2 (Figure 6). These effects were apparent with mouse and human MDM2 and in different cell types. Significantly, there was also an increase in the steady-state levels of MDM2 in cells co-transfected with p14ARF whether or not the cells expressed exogenous p53. Moreover, truncated forms of MDM2 that are impaired in their ability to invoke degradation of p53 (M.Kubbutat and K.H. Vousden, in preparation) were clearly stabilized by the co-expression of p14ARF (Figure 6C). While our data broadly agree with recent reports as regards the stabilization of p53 by p14ARF (Pomerantz et al., 1998; Zhang et al., 1998), they are the first to demonstrate stabilization of MDM2 and are clearly at odds with the findings of Zhang et al. (1998). These authors proposed the destabilization of MDM2 by p14ARF as a mechanism for releasing free p53. However, the marked accumulation of endogenous MDM2 in NARF cells expressing inducible p14ARF, the accumulation of exogenous MDM2 in the co-transfection assays and the detection of ternary p14ARF–p53–MDM2 complexes seem incompatible with this interpretation. In seeking an explanation for these discrepancies, we considered the possibility that different results might prevail in cell lines expressing viral oncoproteins that interfere with p53 function (Zhang et al., 1998). In our hands, the co-expression of HPV16 E6 did not affect the ability of p14ARF to stabilize MDM2 (Figure 6D).

Because of these functional links between p14ARF and p53, the cell cycle arrest elicited by p14ARF is dependent on the status of p53 (Figure 7; Kamijo et al., 1997; Pomerantz et al., 1998), in striking contrast to the pRb-dependent G1-phase arrest imposed by p16INK4a (Guan et al., 1994; Okamoto et al., 1994; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995; Stone et al., 1995a). It is also clear that the effects of p14ARF can be negated by the presence of HPV E6 protein, which targets p53 for degradation. However, there is another important facet to the regulatory connections between p53 and p14ARF. As demonstrated in Figure 9, the expression of p14ARF appears to be negatively controlled by p53, invoking intriguing parallels with the feedback loop through
which pRb influences the expression of the CDKN2A α transcript in human cells (Li et al., 1994b; Hara et al., 1996; Palmero et al., 1997). We have reported previously that in human diploid fibroblasts the transcription of exon 1β is inversely related to the function of SV40 T-antigen, although we did not draw a distinction between the effects of T-Ag on pRb or p53 in this earlier work (Hara et al., 1996). We now show that introduction of the HPV16 E6 protein into these cells dramatically increases the expression of p14ARF (Figure 9A). Moreover, direct modulation of p53 activity, using a tetracycline-regulatable expression system or with a temperature-sensitive mutant of p53, was clearly able to down-regulate the expression of endogenous p14ARF in SAOS2 cells (Figure 9B and C). There are as yet no definitive data to indicate whether mouse p19ARF expression can be modulated directly by p53 (Quelle et al., 1995b; 1997; Kamijo et al., 1997).

In our survey of human tumour cell lines, there was complete concordance between the expression of the protein and the detection of the β transcript, although a recent report showed that some haemopoietic cells express the RNA but not the protein (Della Valle et al., 1996). This report showed that some haemopoietic cells express the RNA but not the protein (Della Valle et al., 1996). We now show that introduction of the HPV16 E6 protein into these cells dramatically increases the expression of p14ARF (Figure 9A). Moreover, direct modulation of p53 activity, using a tetracycline-regulatable expression system or with a temperature-sensitive mutant of p53, was clearly able to down-regulate the expression of endogenous p14ARF in SAOS2 cells (Figure 9B and C). There are as yet no definitive data to indicate whether mouse p19ARF expression can be modulated directly by p53 (Quelle et al., 1995b; 1997; Kamijo et al., 1997).

In our survey of human tumour cell lines, there was complete concordance between the expression of the protein and the detection of the β transcript, although a recent report showed that some haemopoietic cells express the RNA but not the protein (Della Valle et al., 1997). Moreover, a consistent staining pattern has been observed in different human cell lines that is similar to the nuclear speckles and nucleolar staining previously reported for p53 in different human cell lines that is similar to the nuclear speckles and nucleolar staining previously reported for mouse p19ARF (Figure 2; Quelle et al., 1995b; Della Valle et al., 1997). Although provocative, the significance of this staining pattern has not been explored fully. One possibility is that ARF is a component of nuclear structures that are important for some facet of DNA/RNA synthesis or processing such that excessive levels interfere with the normal function of these structures and trigger a p53-dependent growth arrest.

However, it is clear that ARF plays no part in the p53-mediated response to DNA damage. Actinomycin D treatment (Figure 10) stabilizes p53 without detectable increases in p14ARF expression, and ARF-negative cell lines, such as A375, and the MEFs from ARF nullizygous mice (Kamijo et al., 1997) both show normal DNA damage-induced p53 responses. Curiously, low levels of actinomycin D caused an appreciable down-regulation of p14ARF in cells lacking p53. Whether this reflects the effect of actinomycin D on transcription of exon 1β or p53-independent effects on p14ARF stability is currently under investigation.

Taken together, our data imply that p14ARF is both regulated by and can regulate p53, adding a new dimension to the known feedback loops between p53 and MDM2 (Figure 11). The burning question, therefore, is what activates ARF expression? Unfortunately, the low levels of RNA and protein in primary cells have so far precluded drawing any connection with population doublings, but senescence remains an attractive possibility. For example, up-regulation of ARF as cells approach their finite lifespan could in part explain the accumulation of p21CIP1, although the currently available data suggest that p21CIP1 may be under p53-independent as well as p53-dependent regulation in this setting (Tahara et al., 1995). A strong argument in favour of this idea has come from the recent report on mice that have a targeted disruption of CDKN2A exon 1β (Kamijo et al., 1997). The mice develop normally but have a high incidence of spontaneous tumours, and the derived embryo fibroblasts do not undergo the growth arrest indicative of the M1 phase of replicative senescence.

Since these properties resemble the phenotypes reported for mice with a targeted disruption of the shared CDKN2A exon 2 sequences (Serrano et al., 1996), it has been argued that the phenotype of the latter mice may be entirely attributable to the loss of ARF function, implicating ARF in both senescence and tumour suppression (Kamijo et al., 1997).

Although the data we present here are consistent with such a role for ARF, we propose a different interpretation in which parallels can be drawn between loss of ARF and loss of p53 function, rather than between ARF and p16INK4a. In the first place, p53−/− mice sustain spontaneous tumours that are broadly similar in pathology and incidence to those reported for the CDKN2A exon 1β and exon 2 knockouts (Donehower et al., 1992; Serrano et al., 1996; Kamijo et al., 1997). In all three cases, the nullizygous embryo fibroblasts immortalize spontaneously and are sensitive to transformation by RAS (Harvey et al., 1993; Tsukuda et al., 1993; Serrano et al., 1996; Kamijo et al., 1997). Secondly, Kamijo et al. noted mutually exclusive loss of ARF or p53 during immortalization of mouse fibroblasts (Kamijo et al., 1997) which would be consistent with genes that function within a common pathway. A key point in their argument was that immortalization occurred without any deleterious effects on the expression or function of the p16INK4a protein. However, it is well known that rodent cells undergo spontaneous immortalization at an unusually high frequency compared with cells from other higher eukaryotic species, and there is a large body of evidence to indicate that dysregulation of either p53- or pRb-dependent mechanisms is sufficient to bypass senescence in cultured rodent cells (Shay et al., 1991b). Thus, the majority of established mouse cell lines have been shown to be defective for p53 function, whereas deletions affecting the mouse CDKN2A locus are comparatively rare (Harvey and Levine, 1991; Rittling and Denhardt, 1992; Linardopoulos et al., 1995).

In contrast, lifespan extension of human cells generally involves abrogation of both p53- and pRb-dependent pathways (Shay and Wright, 1989; Shay et al., 1991a), and p53 and p16INK4a feature prominently among the most frequently altered genes in human cancers. It has been estimated that up to 75% of established human tumour cell lines have sustained alterations that incapacitate p16INK4a (Ruas and Peters, 1998), confirming the strong selection of ARF for immortalization.
against this gene as cells escape senescence. Indeed, even cells derived from Li–Fraumeni patients, which are p53 defective, have been shown to sustain p16INK4a deletions during escape from senescence (Rogan et al., 1995; Noble et al., 1996). Although many of the deletions that affect p16INK4a also encompass p14ARF, only a minority of the tumour-associated mutations in CDKN2A affect the sequence of p14ARF (in exon 2), and no mutations have yet been recorded in exon 1β (Ruas and Peters, 1998). Moreover, mutagenesis of exon 2 has been shown to have no effect on the known properties of ARF, and the protein encoded by exon 1β alone is sufficient to induce a cell cycle arrest (Figure 7E; Arap et al., 1997; Quelle et al., 1997). These data argue strongly against the notion that targeted disruption of exon 2 produces the same phenotype as the disruption of exon 1β.

Our data suggest an inverse correlation between p53 and p14ARF such that ARF is essentially below the current levels of detection in p53-positive cells. Coupled with the idea that ARF and p53 are functionally linked, we would argue that all human tumour cell lines that retain and express ARF are de facto p53 negative (see Table I). However, not all p53-negative cells express ARF because many of them have sustained 9p21 deletions that target p16INK4a and coincidentally remove exon 1β. The pattern is likely to depend on the order of events. If the deletion of 9p21 is an early event, and ARF is co-deleted, then the tumour cell line can remain wild-type for p53. Conversely, if mutation of p53 is an early event, then there would be little selection against ARF. However, the strong selection against p16INK4a would frequently result in co-deletion of ARF.

It is interesting to speculate about the evolutionary pressure for locating exon 1β next to p16INK4a and the likelihood that this occurred after the duplication that presumably gave rise to the tandem arrangement of INK4a and INK4b. One possibility is that it was driven by the fact that both genes are involved in facets of senescence. Although the current data suggest that the genes are not regulated coordinately by the p53 and pRb pathways, there may well be a common mechanism that regulates their expression to population doublings or other signals. This is clearly an interesting concept that requires further investigation. Conversely, the close proximity of the two genes would also make them vulnerable to co-deletion, a curious state of affairs if both are involved in the mechanisms underlying tumour suppression.

Materials and methods

Mammalian cell culture
The human tumour cell lines MCF7, BT549, MDA MB468, MDA MB231, A375, T24, 5637, U2OS, SAOS2, HaCat, C33A, NCI H596 and H292 were maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). The primary diploid fibroblast strains, WI38 and TIG3, and their SV40-transformed derivatives, VA13 and SVts8, respectively, were grown under the same conditions except that the SVts8 cells were kept routinely at 34°C which is permissive for the function of the temperature-sensitive form of SV40 T-Ag. The CEM and Kit225 lymphocyte cell lines were maintained in RPMI medium + 10% FCS. Kit225 cultures were supplemented with 20 ng/ml of IL-2 (from EuroCetus).

Construction of expression vectors
A cDNA encoding p14ARF was isolated by RT–PCR using mRNA extracted from TIG3 cells. Following first strand cDNA synthesis (Boehringer Mannheim kit), the relevant sequences were amplified by PCR using the forward and reverse primers: 5’-AGGCGGCGGATCCATGGTGCGCAGGTTC3’ and 5’-CCGAAATCTACAGCAGCAGCCACG-3’. The PCR product was 5’ digested with BamHI and EcoRI and subcloned into the corresponding sites in the Bluescript KS+ and pcDNA3 vectors. The IPTG-regulatable constructs in the pOPRSVI vector (LAC SWITCH™ from Stratagene) were engineered in a two-step process. The DNA fragments encoding p14ARF and p16INK4a were excised from the pdNA3-based plasmids using XbaI and BamHI and subcloned into Bluescript to exploit the NotI sites in the polylinker. The inserts were then recovered by digestion with NotI and subcloned into the NotI site in pOPRSVICAT. The orientation of the inserts was determined by DNA sequencing using the primers described above for PCR. Recombinant retroviruses were constructed by transfecting the relevant BamHI-EcoRI fragments into the pBABE-puro and pBabe-hygro vectors (Morgenstern and Land, 1990). The expression constructs for p53, mouse Mdm2 and human MDM2 have been described previously (Marston et al., 1994; Chen et al., 1995; Elenbaas et al., 1996; Kubbutat et al., 1997).

Transient and stable transfections
For transient transfection assays, cells were plated at a density of 2 × 10⁵ cells per 10 cm dish. Twenty μg of pcDNA3-based plasmids were co-transfected with 7 μg of plasmid encoding the CD20 cell surface marker, using Lipofectamine (Gibco-BRL). For co-transfections with HPV E6, the amount of p14ARF-encoding plasmid was reduced to either 5 or 10 μg to allow addition of 10 μg of the pcB6+Q169 HPV16 E6 construct. The transiently transfected cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD20 antibody (Becton Dickinson) to detect surface expression of CD20 and with propidium iodide to determine their relative DNA content. The proportion of CD20-positive cells in different phases of the cell cycle was then determined by fluorescence-activated cell sorting (FACS; van den Heuvel and Harlow, 1993).

IPTG-inducible expression was established in the U2OS human osteosarcoma cells using the LAC SWITCH™ vector system (Stratagene). The p16INK4a and p14ARF cDNAs in the pOPRSVI-Lac operator vector were co-transfected with the p3′SS plasmid containing the LAC repressor in a 1:1 ratio. Stable transfectants were selected with 150 μg/ml hygromycin (Sigma) and 300 μg/ml genetin (Gibco-BRL), and after 48 h the cells were replated at a low density to allow the isolation of single colonies. The clonal cell lines derived from the transfectants, EH1 and 2 and NARF1–6, were maintained in DMEM + 10% FCS containing 150 μg/ml hygromycin and 300 μg/ml genetin. To ensure that the cells were not metabolically challenged during various assays, the antibiotics were removed from the medium 24 h prior to assay.

Immunofluorescence
Between 5 × 10³ and 1 × 10⁵ cells seeded on coverslips in a 24-well plate were fixed in 50% methanol/50% acetone for 5 min, and blocked in 10% bovine serum albumin (BSA) for 15 min. The anti-human p14ARF antibody (IP1) was affinity purified against the peptide antigen using the Sulfolink coupling kit (Pierce) to immobilize the antigen. Affinity-purified antibody (IP1) was affinity purified against the peptide antigen using the Affinity Purification Kit (Affinity Purification). The peptide antigen purified serum (IP1) was incubated undiluted with the fixed cells for 45 min. The coverslips were washed twice for 3 min in phosphate-buffered saline (PBS) and incubated for 45 min with TRITC (Texas Red)-conjugated anti-rabbit secondary antibody (DAKO) diluted 1:20 in PBS. Cells were again washed twice for 3 min in PBS before mounting onto slides.

Protein analysis
Cell lysates for immunoblotting were prepared by scraping cells into 1× Laemmli buffer lacking bromophenol blue and mercaptoethanol. These lysates were boiled and the protein concentration was determined using the BCA protein assay reagent (Pierce). Balanced amounts of cell proteins (20–40 μg) were then boiled again after addition of mercaptoethanol and bromophenol blue and fractionated by SDS–PAGE in 15% acrylamide gels. After transferring the proteins onto Immobilon-P (Millipore), the membranes were blocked in 5% milk powder, 0.2% Tween-20 in PBS for 30 min and incubated with the primary antibody for 1 h. Membranes were then washed in 0.2% Tween-20 in PBS once for 15 min and three times for 5 min at room temperature. Depending on the experiment, sheep anti-mouse horseradish peroxidase (HRP) (1:1000 dilution) and donkey anti-rabbit HRP (1:2000 dilution) were used as secondary antibodies (Amersham). Antibody binding was visualized using Amersham ECL reagents. The primary antibodies used in these studies were against human p16INK4a (DCS50; Lukas et al.,
In vitro binding assay
The sequences encoding cyclin D1, Flag-tagged p14ARF, human MDM2 and the Δ222–437 mutant were cloned into the pcDNA3 vector and expressed by coupled transcription and translation in the Promega TnT system. Selected proteins were labelled with [35S]methionine and in vitro binding assays were performed as described previously (Parry et al., 1995).

p53 stability assay
MDM2-mediated degradation of p53 was analysed in transiently transfected cells as previously described (Kubbutat et al., 1997). Briefly, U2OS or SAOS2 cells were transfected using calcium phosphate co-precipitation with 3 μg of pCB6+53 or pCB6+53D1, 0–9 μg of pCOCmd2 or pCHDM, and 0–15 μg of pcDNA3ARF (Kubbutat et al., 1997). Cells were harvested 24 h post-transfection and p53 expression analysed by Western blotting with the monoclonal antibody PAβ1801. Equal transfection efficiency was confirmed by co-transfection of 1 μg of pEGFP-N1, and equal expression of green fluorescent protein (GFP) confirmed by Western blotting with a monoclonal antibody (Clontech). In some experiments, the LHNl proteasome inhibitor (Boehringer) was added to the culture medium at a final concentration of 50 μM.

Retroviral infection of primary fibroblasts
The TK6 strain of human diploid fibroblasts were infected at 40–45 population doublings with an amphotropic virus encoding the mouse cationic amino acid transporter (Serrano et al., 1995) and screened for characteristic LNCi morphology. Where appropriate, vector-only controls were set up using both pBABE-puro and pBABE-hygro with dual selection. Cell proliferation and SA-β-gal assays were performed as previously described (Dimitri et al., 1995; Serrano et al., 1997; McConnell et al., 1998).

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