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Distinct mechanisms of nuclear accumulation regulate the functional consequence of E2F transcription factors

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SUMMARY

Transcription factor E2F plays an important role in co-ordinating and integrating early cell cycle progression with the transcription apparatus. It is known that physiological E2F arises when a member of two families of proteins, E2F and DP, interact as E2F/DP heterodimers and that transcriptional activity is regulated through the physical association of pocket proteins such as pRb. However, little information is available regarding the mechanisms which control the levels of functional E2F. In this study, we have characterised one such mechanism which regulates the nuclear accumulation and activity of E2F. Specifically, we show that E2F proteins fall into two distinct categories according to their ability to accumulate in nuclei, one being exemplified by E2F-1 and the other by E2F-4 and -5. Thus, E2F-1 possesses an intrinsic nuclear localization signal whereas E2F-4 and -5 are devoid of such a signal. Furthermore, we find for E2F-4 and -5 that two distinct processes govern their nuclear accumulation whereby the nuclear localization signal is supplied in trans from either a DP heterodimer partner or a physically associated pocket protein. It is consistent with the role of pocket proteins in regulating nuclear accumulation that we find E2F-5 to be nuclear during early cell cycle progression with an increased cytoplasmic concentration in cycling cells. Our data show that the mechanism of nuclear accumulation determines the functional consequence of E2F on cell cycle progression: pocket protein-mediated accumulation impedes cell cycle progression, whereas DP-regulated nuclear accumulation promotes cell cycle progression. Moreover, the inactivation of pocket proteins by the adenovirus E1a protein, and subsequent release of E2F, failed to displace nuclear E2F. Our study identifies a new level of regulation in the control of E2F activity exerted at the level of nuclear accumulation where subunit composition and interaction with pocket proteins dictates the functional consequence on cell cycle progression.

Key words: E2F, DP, Transcription factor, Cell cycle, Nuclear localization, Pocket protein

INTRODUCTION

Progression through the mammalian cell cycle requires that gene expression is co-ordinated with the activity of cell cycle control proteins. A critical period when this occurs is during the transition from G_1 into S phase, as cells become committed to the division cycle. The E2F transcription factor, together with its afferent regulators which includes the retinoblastoma tumour suppressor protein pRb, play a critical role in regulating this transition process (La Thangue, 1994; Lam and La Thangue, 1994; Nevins, 1992). Indeed, a considerable body of evidence supports the idea that E2F transcription factors are pre-eminent regulators in growth control, allowing early cell cycle progression to be integrated and co-ordinated with transcription (La Thangue, 1994; Lam and La Thangue, 1994; Nevins, 1992).

The pathway regulated by pRb receives signals from members of the cyclin-dependent kinase (cdk) family which control cell cycle progression through the phosphorylation of critical substrates (Sherr, 1993; Taya, 1997; Weinberg, 1995). The pRb protein becomes progressively phosphorylated as cells move through G_1 into S phase (Sherr, 1993; Taya, 1997; Weinberg, 1995), a modification which correlates with the loss of its negative growth control (Sherr, 1993; Taya, 1997; Weinberg, 1995). Cyclins A, D and E together with an appropriate catalytic subunit are believed to be primarily responsible for phosphorylating pRb (Sherr, 1993; Weinberg, 1995), where cyclin D-dependent phosphorylation is followed by cyclin E-dependent phosphorylation (Weinberg, 1995).

Two other members of the group of proteins exemplified by pRb have been identified. Like pRb, both p107 and p130 associate with E2F although the temporal characteristics of their association differ. For example, the p130-E2F complex is predominant in G_0 cells, the complex disappearing at an early stage as cells progress through G_1 (Cobrinik et al., 1993; Moberg et al., 1996; Vairo et al., 1995). In contrast, p107-E2F is observed later in G_1 and peaks during early S phase (Cobrinik et al., 1993; Lees et al., 1992; Moberg et al., 1996;

Schwarz et al., 1993; Vairo et al., 1995). However, clear evidence for different roles of the pocket proteins in regulating the cell cycle remains enigmatic. It is known that *Rb* is the only member of the family which is frequently mutated in human tumour cells (Ewen et al., 1991; Horowitz et al., 1990; Li et al., 1993; Zhu et al., 1993), although the biological explanation for why it is *Rb* and not *p107* or *p130* is not clear. All three pocket proteins can have negative effects on cell cycle progression (Claudio et al., 1994; Ewen et al., 1993; Zamanian and La Thangue, 1993; Zhu et al., 1993) and, consistent with this, viral oncoproteins which subvert the E2F pathway, such as adenovirus E1a, bind all three proteins (Bandara and La Thangue, 1991; Li et al., 1993; Zamanian and La Thangue, 1992, 1993). The phenotype observed in knock-out mice carrying targeted disruptions in *pRb, p107* or *p130* has revealed certain differences. The $Rb^{-/-}$ mice suffer increased levels of apoptosis in some tissues (Clarke et al., 1992; Jacks et al., 1992) whereas either $p107^{-/-}$ or $p130^{-/-}$ knock-out mice lack an obvious phenotype although double knock-out $p107^{-/-}/p130^{-/-}$ embryos die in utero due to cartilage and bone malfunction (Cobrinik et al., 1996; Lee et al., 1996).

Progress in uncovering the molecular composition of E2F has defined a heterodimeric transcription factor composed of an E2F and DP family member (Girling et al., 1993, 1994; La Thangue, 1994; Lam and La Thangue, 1994; Wu et al., 1995). Five E2F and at least three DP family members have been isolated and characterised (La Thangue, 1994; Lam and La Thangue, 1994). Each E2F protein can interact combinatorially with a DP protein, thus generating an array of sequence specific heterodimers (La Thangue, 1994; Lam and La Thangue, 1994). Regulation of E2F transcription factor activity is exerted through the physical association of a pocket protein and the C-terminal domain within the E2F partner, which also harbours a trans activation domain (Flemington et al., 1993; Helin et al., 1993), and binding of the pocket protein to the heterodimer is believed to prevent the activation domain from contacting the transcription machinery.

Several studies have suggested that different E2F/DP heterodimers have preferred DNA sequence specificities (Li et al., 1994; Slansky and Farnham, 1996; Tommas and Pfeifer, 1995; Wells et al., 1996). Thus, although each heterodimer has generic specificity for the E2F sequence, preferred binding sites are likely. Potentially, therefore, this property provides a mechanism that enables the activity of different pocket proteins to be integrated with distinct banks of genes through the regulation of different E2F heterodimers. Related to this idea are studies which have addressed the functional importance of E2F sites within physiological cellular promoters. For example, the E2F site in the *B-myb*, *E2F-1* and *cdc2* promoters plays a repressive role in regulating promoter activity during the early cell cycle since mutation of the site increases transcription up to wild-type levels (Hsiao et al., 1994; Johnson et al., 1994; Lam and Watson, 1993; Neuman et al., 1994; Zwicker et al., 1995; Zwicker and Muller, 1997). In other cases, for example the *cyclin E* promoter, the E2F site plays a positive role in regulating the periodic induction of transcription (Botz et al., 1996).

The mechanism involved in negative transcriptional regulation through the E2F site has been suggested from studies on pocket proteins. When delivered to a promoter context as, for example, a DNA binding hybrid protein both pRb and p107 can inactivate in cis promoter activity (Bremner et al., 1995; Weintraub et al., 1992, 1995). Some evidence suggests that this can occur by impeding contact between the activation domains of DNA bound transcription factors and the transcription machinery (Bremner et al., 1995; Weintraub et al., 1992, 1995).

The five members of the E2F family fall into two functionally and structurally distinct groups. Members of the first group, E2F-1, -2 and -3, are principally regulated by pRb and can overcome cell cycle arrest imposed by pRb (Zhu et al., 1993) or $p16^{INk4a}$ (Lukas et al., 1996; Mann and Jones, 1996). They are related at the structural level, each possessing within the N-terminal region a domain dedicated to cyclin A binding (Krek et al., 1994) which together with cdk2 is involved in the phosphorylation control of DP-1 (Krek et al., 1994, 1995). The second group includes E2F-4 and E2F-5, which can associate with either p107 or p130 (Beijersbergen et al., 1994; Buck et al., 1995; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995; Vairo et al., 1995). In addition they have a short N-terminal region and do not contain the cyclin A binding domain (Krek et al., 1994).

Each E2F protein requires a DP protein as an obligate heterodimeric partner (La Thangue, 1994; Lam and La Thangue, 1994). The DP-1 protein is a widespread component of physiological E2F, appearing as a constitutive E2F component in many different cell-types (Bandara et al., 1993, 1994; Girling et al., 1993; Wu et al., 1995). In contrast to other DP family members, DP-3 is unique in that its RNA undergoes extensive processing due to alternative splicing (Ormondroyd et al., 1995). Processing events in the 5′ untranslated and coding region give rise to spliced variants which are restricted both in cells and tissues (Ormondroyd et al., 1995). Four distinct protein isoforms of DP-3, known as α, β, γ and δ, have been identified in which α and δ share a common alternatively spliced exon, encoding 16 amino acid residues known as the E region, which is absent in the β and γ isoforms (Ormondroyd et al., 1995; de la Luna et al., 1996). Previously, we have defined a role for the E region by demonstrating that it functions as a nuclear localization signal (NLS; de la Luna et al., 1996). Other DP family members which contain the E region also can localize efficiently to the nucleus (Magae et al., 1996; Lindeman et al., 1997). Furthermore, DP family members which lack the E region, referred to as E^- and including DP-1, can be recruited to nuclei through an interaction with E2F-1, which harbours an intrinsic and dedicated NLS (de la Luna et al., 1996; Magae et al., 1996).

In this study, we have investigated the mechanisms which regulate nuclear accumulation within the E2F family. We show that the E2F proteins fall into two distinct categories according to their ability to accumulate in nuclei, each class being exemplified by E2F-1 or E2F-4 and E2F-5. Thus, E2F-1 possesses an intrinsic NLS whereas E2F-4 is devoid of such a signal. We find for E2F-4 and E2F-5 that two distinct mechanisms govern its nuclear accumulation in which the NLS is supplied in trans from an associated protein. Specifically, either pocket proteins or an appropriate DP heterodimeric partner can provide NLS activity. Furthermore, the mechanism of nuclear accumulation determines the functional consequence of E2F on cell cycle progression since pocket protein-mediated nuclear uptake impedes cell cycle progression whereas DP-regulated nuclear uptake promotes cell cycle progression. Consistent with the role of pocket proteins in nuclear uptake, we find E2F-5 to be

nuclear during early cell cycle progression with an increased cytoplasmic concentration in cycling cells. Importantly, the inactivation of pocket proteins by adenovirus E1a, and the subsequent release from E2F, failed to displace nuclear E2F. These data define a level of regulation in the control of E2F activity exerted by regulated nuclear accumulation in which subunit composition and interaction with pocket proteins dictates the functional consequence of E2F on cell cycle progression.

MATERIALS AND METHODS

Cell culture and DNA transfection

Monkey kidney COS7, human osteosarcoma SAOS2 and U2OS and human glioblastoma T98G cell lines were cultured in a 5% CO_2 atmosphere at 37°C using Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% foetal calf serum (FCS). NIH3T3 cells were cultured under the same conditions using D-MEM supplemented with 10% newborn calf serum (NBCS). Human T98G cells were synchronized by serum starvation as described by Mayol et al., 1995. For calcium phosphate transfections in U2OS cells, cells were plated at 2×10^5 cells per 60 mm dish 24 hours before transfection. Fresh medium was added to cells and 4 hours later cells were transfected with a total of 20 μg DNA as described previously (Zamanian and La Thangue, 1992). Then 16 hours after transfection cells were washed and fresh medium replaced and 4 hours later cells were trypsinized, plated onto coverslips and cultured for a further 24 hours. For lipofectamine transfection of U2OS cells, cells were plated at 1×10^5 cells on coverslips in 35 mm dishes and transfected 24 hours later with 2 μg DNA and 4 μl lipofectamine (Gibco BRL) following the manufacturer's instructions. At 16 hours after transfection, fresh medium was replaced and cells cultured for a further 24 hours. COS7 cells were transfected with lipofectin (Gibco BRL) as described previously (de la Luna et al., 1996). For SAOS2 transfections, 5×10^5 cells were plated in 100 mm dishes 24 hours before transfection. Medium was changed 2 hours before transfection and a total of 40 μg DNA was transfected by the calcium phosphate precipitation method. At 16 hours after transfection the cells were washed with D-MEM, changed into D-MEM supplemented with 0.2% FCS and cultured for a further 24 hours. To generate the E2F-5 expressing NIH3T3 cells HA-tagged human E2F-5 was cloned into pBabe-puro (Morgenstern and Land, 1990) and transfected into BOSC packaging cells (ATCC). The supernatant was harvested 48 hours after transfection and used undiluted on NIH3T3 cells at 20% confluency. After 48 hours cells were cultured in the presence of puromycin, and puromycin resistant pools grown on coverslips under different conditions. For high serum conditions, cells were grown in 10% NBCS and for low serum conditions in 0.1% NBCS for 48 hours

Antibodies

Rabbit polyclonal antibodies to DP-1 and DP-3 have been described previously (de la Luna et al., 1996). Other primary antibodies used were the anti-p107 mouse monoclonal antibody SD9 (Santa Cruz), the anti-E1a monoclonal antibody M73 (Oncogene Science), the anti-E2F-5 monoclonal antibody MH5 (Hijmans et al., 1995), the anti-HA mouse polyclonal antibody HA11 (BabCO) and the FITC-conjugated anti-CD20 antibody (Becton Dickinson).

Immunostaining

Cells on coverslips were washed in PBS, fixed in 4% paraformaldehyde in PBS for 15 minutes, permeabilized in 1% Triton X-100 in PBS for 10 minutes and incubated in 5% FCS in PBS for 15 minutes. Cells were then incubated with primary antibodies for 30 minutes at room temperature. Antibodies were diluted in 1% FCS in PBS. Rabbit polyclonal antibodies were used at a 1:200 dilution, affinity purified mouse monoclonal antibodies were used at a 1:100 dilution and mouse polyclonal antibodies were used at a 1:1,000 dilution. Coverslips were then washed in 1% FCS in PBS and incubated with anti-mouse or anti-rabbit FITC- or TRITC-conjugated secondary antibodies (Southern Biotechnology Associates, Inc.) at a 1:200 dilution in 10% FCS in PBS for 15 minutes. Coverslips were then washed in 1% FCS in PBS and mounted using Citifluor (Citifluor, Ltd). Cells were photographed with an Olympus BX60 microscope. All photographs were taken at ×630 magnification. For staining the E2F-5 expressing NIH3T3 cells, a modification of the above procedure was used. Cells were fixed in 2% paraformaldehyde in PBS for 10 minutes at room temperature, permeabilized in 0.5% Triton X-100 for 5 minutes and thereafter treated with 5% milk powder in PBS containing 0.05% Tween-20. Cells were further treated with anti-E2F-5 monoclonal antibody MH5 as described above.

Plasmid constructs

Plasmid pBS1.1 which contains mouse E2F-5 cDNA sequence from amino acid residue 2 in the plasmid pBluescript-SK (Stratagene) was digested with *Eco*RI and *Xho*I and the resulting insert cloned into pCMV-HA1 digested with *Eco*RI and *Xho*I to construct pCMVHA-E2F-5 which encodes the mouse E2F-5 ORF from codons 2-338 preceded by the 3×HA epitope. Plasmid pHA-E2F-5ΔTAD was made by digesting pCMVHA-E2F-5 with *Eco*0109I which cuts at nucleotide 635 within the E2F-5 cDNA and within the backbone vector and religating, introducing a stop codon after codon 211 of the mouse E2F-5 ORF. The fusion sequences were verified by sequence analysis. The plasmid pCMVE2F-4 which encodes human E2F-4 has been described previously (Beijersbergen et al., 1994). The plasmid pRcCMVHAE2F-1wt encoding human E2F-1 was a kind gift from Dr W. Krek (Krek et al., 1994). Plasmids pG4-DP-1 encoding mouse DP-1 and pG4DP-3α, β, γ and δ encoding the corresponding mouse DP-3 genes have been described previously (Bandara et al., 1993; de la Luna et al., 1996). Plasmid pCMV107 encoding human p107 was a kind gift from Dr L. Zhu (Zhu et al., 1993). Plasmid pC53-SN3 expressing wild-type p53 was a gift from Professor D. Lane (Baker et al., 1990). Plasmid pT7E1A expressing T7 tagged 13S E1a was constructed by subcloning the 13S E1a coding sequence into plasmid pET28a (Novagen), introducing a 6×His and T7 tag at the N terminus of the E1a coding sequence. The tagged E1a coding region was then subcloned into pcDNA3 (Invitrogen).

Cell extract preparation and immunoblotting

Cell extracts were prepared and immunoblotting carried out as previously described (de la Luna et al., 1996). E2F-5 proteins were detected with either the rabbit polyclonal anti-E2F-5 antibody 2197 (Buck et al., 1995) or the rabbit polyclonal anti-HA antibody HA11. For the immunoprecipitation/immunoblotting procedure, U2OS cells were transfected by the calcium phosphate method using 40 μg of total expression plasmid per 100 mm tissue culture dish as described. Approximately 3×10^6 cells were lysed in 50 μl microextract buffer as previously described (Zamanian and La Thangue, 1992). Extracts were diluted to 250 μl in TNN buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP40, 0.2 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 20 mg/ml aprotinin) and precleared by incubation with Protein A-agarose at 4°C for 30 minutes. Then 75 μl precleared extract was incubated with 50 μl of a 50% slurry (w/v) of Protein A-agarose beads preincubated with either SD9 or HA11 monoclonal antibodies and washed extensively in TNN buffer. Incubations were carried out at 4°C overnight. Immune complexes were collected by centrifugation and washed four times in TNN buffer. Proteins were released in sodium dodecyl sulphate (SDS) sample buffer, electrophoresed and immunoblotted using standard techniques. HA-tagged proteins were detected by immunoblotting with the anti-HA monoclonal antibody 12CA5 directly conjugated to horseradish peroxidase (Boehringer Mannheim), used at a 1:1,000 dilution, and an ECL detection system (Amersham), used according to the manufac-

turer's instructions. Approximately 15 μl of transfected cell extract was also electrophoresed and immunoblotted to allow detection of HA-tagged E2F-5 protein.

FACS analysis

Cells were fixed and stained for the CD20 cell surface marker and DNA content essentially as described previously (Zhu et al., 1993). Samples were analysed on a Becton Dickinson fluorescence activated cell sorter using the Consort 30 software package. About 1×10^4 events were collected for each sample.

RESULTS

E2F family members exhibit distinct cellular distributions

Previously we found that alternatively spliced forms of DP-3 RNA encode protein isoforms which differ in their cellular distribution (de la Luna et al., 1996). To further this analysis and gain information on the importance of these observations, we undertook to investigate the intracellular distribution of individual members of the E2F family. Thus, haemagglutinin (HA)-tagged versions of the E2F-1, 4 and 5 proteins (Fig. 1A) were expressed by transient transfection in both COS7 and U2OS cells and their intracellular location determined by immunostaining with an anti-HA monoclonal antibody. As observed previously, E2F-1 is exclusively nuclear both in COS7 and in U2OS cells (Fig. 1B; de la Luna et al., 1996; Magae et al., 1996; Lindeman et al., 1997). In contrast, E2F-4 and E2F-5 were distributed throughout the cytoplasm in both cell types (Fig. 1C and D, and data not shown; Magae et al., 1996; Lindeman et al., 1997). In these experiments, whilst the majority of cells expressing HA-E2F-4 or HA-E2F-5 showed nuclear exclusion, a small percentage (<5%) of expressing cells exhibited some nuclear staining, an observation which may be explained by the experiments discussed below. These results indicate that whereas E2F-1 can be efficiently localised to the nucleus, E2F-4 and E2F-5 are predominantly cytoplasmic. Whilst suggesting a novel level of regulation, they also strengthen the argument that the E2F family can be divided into two subgroups, as suggested from their primary sequence and functional properties (Buck et al., 1995; Krek et al., 1994; Lukas et al., 1996; Mann and Jones, 1996).

NLS containing variants of DP-3 can recruit E2F family members to the nucleus

Following on from the above observations, we reasoned that a DP protein which is able to efficiently accumulate in nuclei may mediate the nuclear import of a cytoplasmic E2F partner. To test this hypothesis, we expressed HA-tagged E2F-4 or E2F-5 together with NLS-containing forms of DP-3, either DP-3α or δ, in U2OS cells and assessed their intracellular distribution. As expected, and in contrast to E2F-4 and E2F-5, DP-3α was predominantly nuclear (Fig. 2B,C,F,G). Co-expression of the DP-3α or δ with either E2F-4 or E2F-5 led to a striking alteration in the distribution of the E2F partner: over 90% of cells expressing both E2F and DP proteins contained nuclear E2F (Fig. 2D,E,H,I). Further, co-expression of the β and γ forms of DP-3, which lack the alternatively spliced region encoding the NLS (de la Luna et al., 1996), did not alter the distribution of E2F-4 or E2F-5 (data not shown). These data indicate that the cytoplasmic location of E2F-4 and -5 is likely to be passive since it can be overcome upon interacting with an NLS containing DP partner, defining a dominant role for the DP partner in the context of this heterodimer in regulating the nuclear accumulation of the heterodimer.

Nuclear accumulation of E2F containing heterodimers mediated by pocket proteins

It has been found in many different types of cells that DP-1 is a constitutive component of the E2F heterodimer (Bandara et al., 1991, 1994; Girling et al., 1993; Wu et al., 1995), whereas other members of the DP family appear to be less frequent (Wu et al., 1995; data not shown). Thus, we were interested to under-

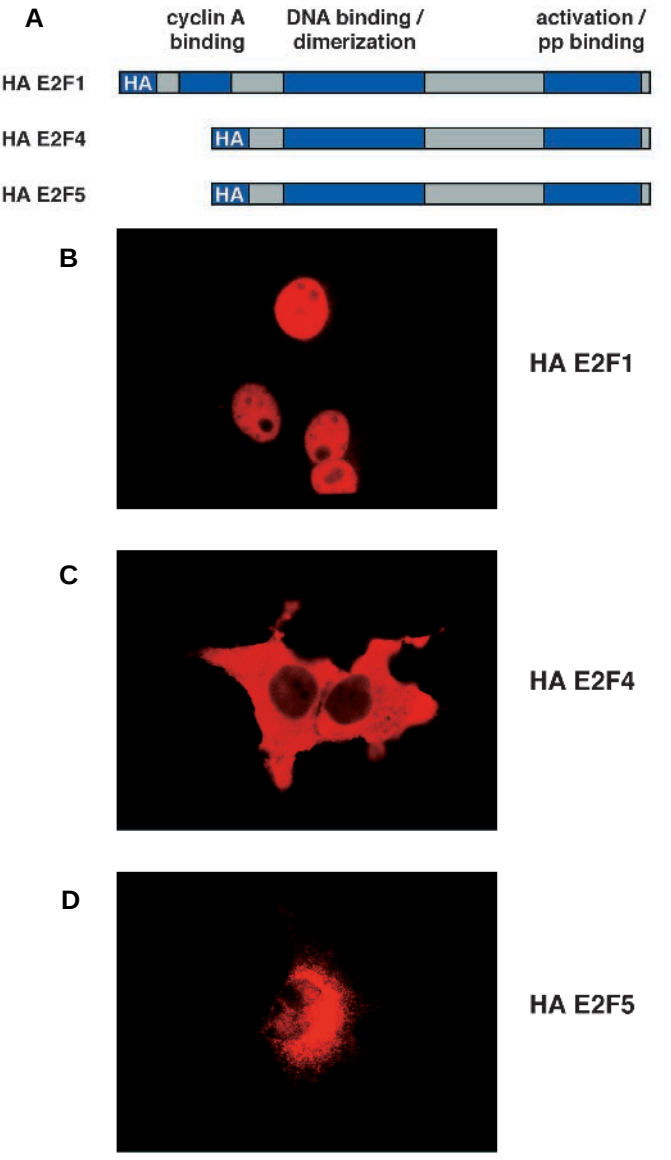

Fig. 1. The E2F proteins differ in their intracellular distribution properties. (A) Diagrammatic representation of the HA-tagged E2F family members E2F-1, 4 and 5, illustrating the conserved domains mediating cyclin A binding, DNA binding/dimerization and trans activation/pocket protein binding. COS7 cells were transfected with 2 μg of the relevant expression plasmid and the intracellular location of exogenously expressed HA-tagged E2F-1 (B), E2F-4 (C) and E2F-5 (D) was assessed by immunofluorescence with an anti-HA monoclonal antibody.

stand how a DP-1/E2F-4 or DP-1/E2F-5 heterodimer might undergo nuclear accumulation since either component, when expressed alone, is predominantly cytoplasmic. When E2F-4 or E2F-5 were expressed together with their heterodimeric partner DP-1, the majority (80-90%) of cells expressing E2F/DP heterodimers exhibited cytoplasmic staining for both proteins (see Fig. 3A,B,E,F) consistent with the absence of NLS activity in each of these proteins (Fig. 1; de la Luna et al., 1996). One potential mechanism through which nuclear accumulation of the E2F heterodimer may occur could be through an interaction with a pocket protein. Therefore we expressed either E2F-4 or E2F-5 together with DP-1 and the pocket protein p107 which can physically associate with both E2F-4/DP-1 and E2F-5/DP-1 heterodimers (Beijersbergen et al., 1994; Buck et al., 1995; Hijmans et al., 1995; Vairo et al., 1995). In these conditions, the expression of p107 led to a dramatic change in the cellular distribution of both the E2F and DP partner from a cytoplasmic to nuclear location (Fig. 3, compare A and B with C and D, and E and F with G and H). In successive experiments we reproducibly observed that co-expression of p107 altered the proportion of transfected cells exhibiting strong nuclear localization of DP-1 and E2F-4 or -5 from below 5% to greater than 90% (see Fig. 3C,D,G,H). The nuclear accumulation of DP-1 was dependent on the presence of an E2F partner as the expression of p107 with DP-1 without an E2F partner failed to affect the cellular distribution of DP-1 (data not shown).

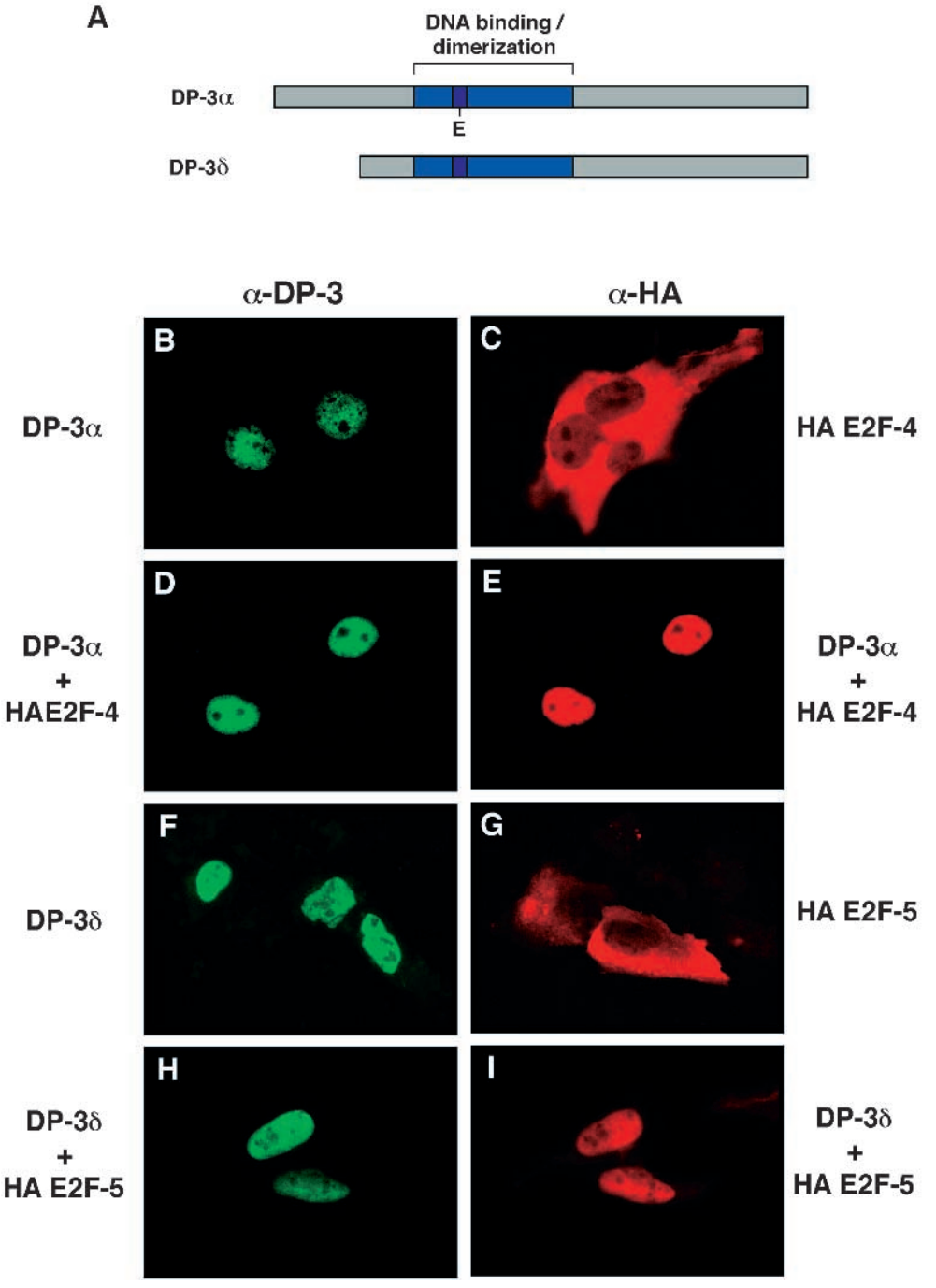

Fig. 2. An NLS-containing form of DP-3 mediates the nuclear accumulation of a cytoplasmic E2F partner. (A) Diagrammatic representation of the two NLS-containing forms of DP-3 (de la Luna et al., 1996). The DNA binding/dimerization and E region of DP-3 are indicated in light and dark blue, respectively. U20S cells were transfected with 1 μg of each of the relevant DP-3 and HA-tagged E2F expression plasmids using lipofectamine as described in Materials and Methods. The intracellular distribution of the DP-3 subunit and the relevant HA-tagged E2F partner were detected by immunofluorescence with a rabbit polyclonal anti-DP-3 peptide antibody (left hand panel) and an anti-HA monoclonal antibody (right hand panel). The intracellular distribution of exogenous DP-3α (B) and E2F-4 (C), exogenous DP-3α (D) and E2F-4 (E) in cells expressing both proteins, exogenous DP-3δ (F) and E2F-5 (G), and exogenous DP-3δ (H) and E2F-5 (I) in cells expressing both proteins. Note that in cells expressing both E2F and DP partners the E2F subunit was efficiently accumulated in the nucleus.

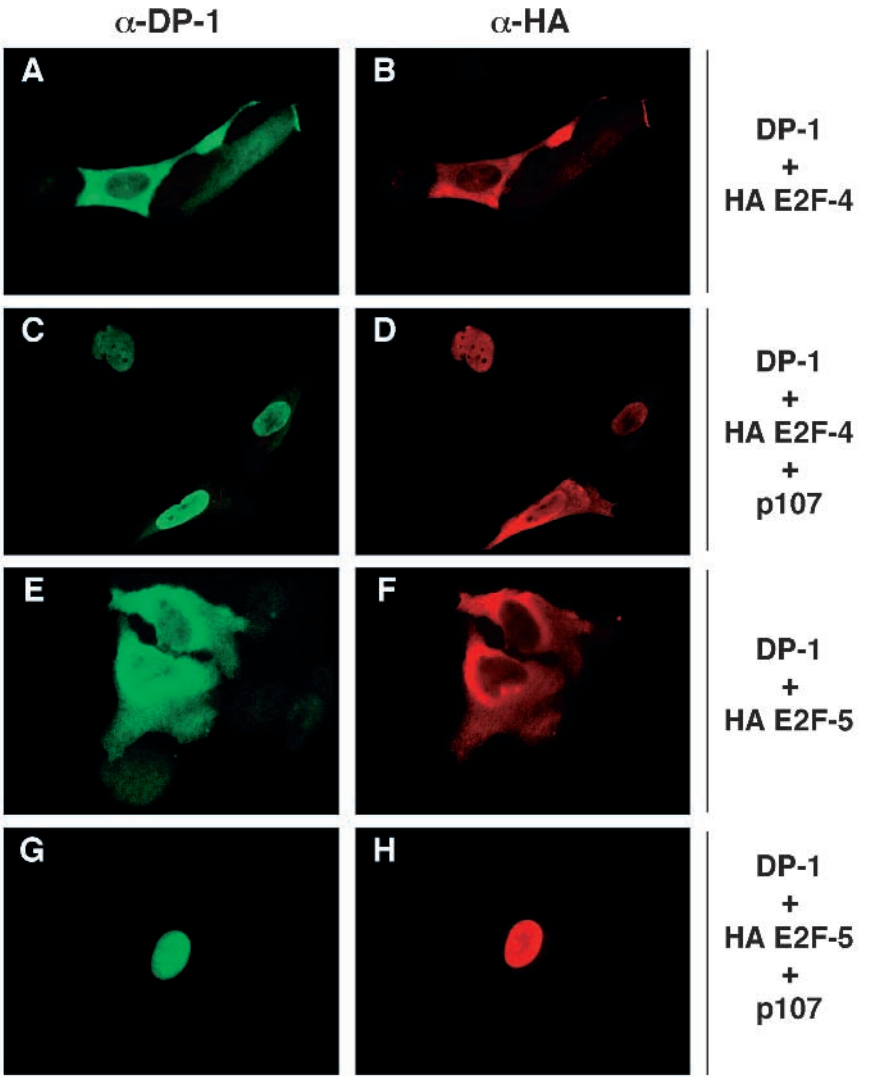

Fig. 3. Pocket proteins mediate the nuclear accumulation of a cytoplasmic E2F heterodimer. U20S cells were transfected with 1 μg of the relevant HA-tagged E2F expression plasmid together with 1 μg pGDP-1 and 1 μg pCMV107 using lipofectamine as described in Materials and Methods. The intracellular distribution of the DP-1 subunit and the HA-tagged E2F heterodimeric partner was detected by immunofluorescence with a rabbit polyclonal anti-DP-1 peptide antibody (A,C,E,G) and an anti-HA monoclonal antibody (B,D,F,H), as described in Materials and Methods. The intracellular distribution of exogenously expressed DP-1 (A) and E2F-4 (B), exogenous DP-1 (C) and E2F-4 (D) co-expressed with p107, exogenous DP-1 (E) and E2F-5 (F) and exogenous DP-1 (G) and E2F-5 (H) co-expressed with p107. Note the nuclear accumulation of E2F and DP-1 subunits in cells co-expressing p107.

Although the data shown relate to p107, similar results were observed for p130 which could recruit a cytoplasmic E2F heterodimer (data not shown). To conclude, these data define a second mechanism in the control of nuclear accumulation of the E2F/DP heterodimer where the NLS is supplied in trans by a pocket protein which is physically associated with the heterodimer. This mechanism could be responsible for the small percentage of cells expressing E2F-4 that exhibited nuclear staining mentioned in the previous section.

Nuclear accumulation by pocket proteins requires the integrity of the E2F activation domain

The specificity of formation of higher order complexes between E2F heterodimers and members of the pocket protein family pRb, p107 and p130 is determined by the E2F subunit (La Thangue, 1994; Lam and La Thangue, 1994). In addition, it has been demonstrated that the pocket protein subunit interacts with the E2F heterodimer via the C-terminal trans activation domain of the E2F protein (Flemington et al., 1993; Helin et al., 1993). To determine whether the nuclear accumulation of the E2F-5 heterodimer by p107 is mediated via the E2F-5 trans activation domain, a mutant E2F-5 protein was constructed with a C-terminal truncation (E2F-5ΔTAD) lacking the marked box and trans activation domains of the protein (Buck et al., 1995). The cellular distribution of this mutant protein was predominantly cytoplasmic, although significant levels of the protein were present in nuclei (Fig. 4B), an observation which may be attributed to passive diffusion of the truncated protein due to its smaller size (about 35 kDa; Gorlich and Mattaj, 1996).

To investigate the ability of E2F-5ΔTAD to allow an E2F heterodimer to accumulate in nuclei, we studied the cellular distribution of p107 and DP-1 when co-expressed with the wild-type or mutant form of E2F-5. As observed earlier, in cells expressing E2F-5 with DP-1 and p107, over 90% of transfected cells exhibited nuclear localization of DP-1 and p107 (Fig. 4C and D, respectively). However, when a parallel experiment was performed with E2F-5ΔTAD, DP-1 failed to accumulate in nuclei in contrast to p107 (Fig. 4E,F). Thus, in the context of the E2F-5/DP-1 heterodimer, the ability of the pocket protein to bind to the E2F component facilitates nuclear accumulation.

To further determine if the E2F-5ΔTAD mutant was compromised in its ability to undergo DP-dependent nuclear accumulation, E2F-5ΔTAD and DP-3δ were co-expressed in U2OS cells; in these conditions over 90% of transfected cells had nuclear

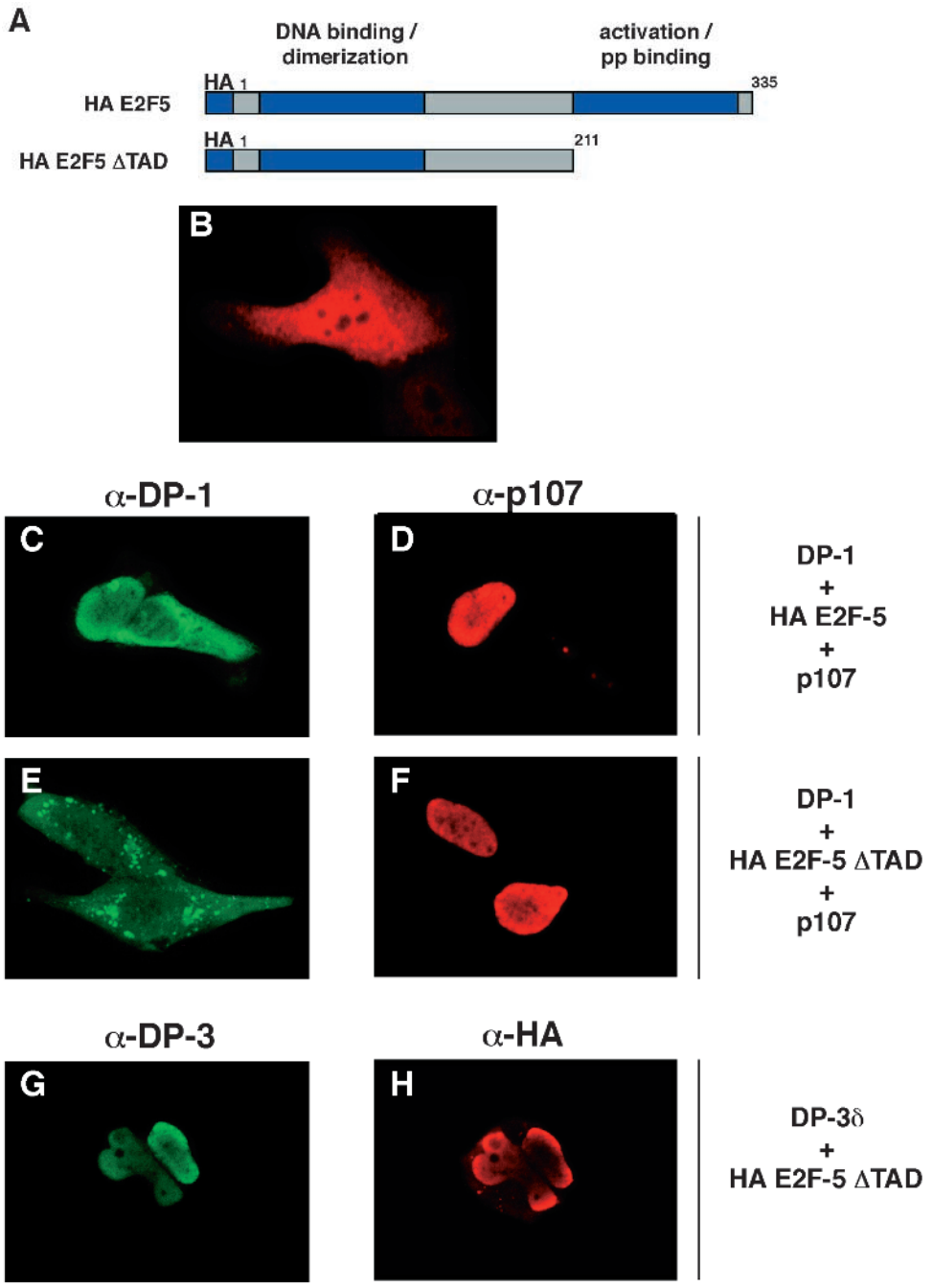

Fig. 4. (A) Diagrammatic representation of the HA-tagged E2F-5 and E2F-5ΔTAD expression vectors, indicating the DNA binding/dimerization and trans activation/pocket protein binding domains. (B) U20S cells were transfected with 1 μg of the HA-tagged E2F-5ΔTAD expression vector using lipofectamine and the intracellular distribution of the exogenously expressed protein was determined by immunofluorescence with an anti-HA monoclonal antibody. (C-F) Cells were transfected with 1 μg of the relevant HA-tagged E2F-5 expression plasmid as indicated, together with 1 μg of pGDP-1 and 1 μg of pCMV107. The cellular distribution of the DP-1 and p107 proteins was determined by immunofluorescence with a rabbit polyclonal anti-DP-1 peptide antibody (C,E) and an anti-p107 monoclonal antibody (D,F). Exogenous DP-1 (C) and p107 (D) expressed together with E2F-5 (note the nuclear accumulation of DP-1 in the cell co-expressing p107), exogenous DP-1 (E) and p107 (F) expressed together with E2F-5ΔTAD (note the lack of nuclear accumulation of DP-1 in cells co-expressing p107). (G and H) U2OS cells were transfected with 1 μg pHA-E2F-5ΔTAD and 1 μg pGDP-3δ using lipofectamine and the intracellular distribution of the exogenously expressed DP and E2F proteins determined by immunostaining using a rabbit polyclonal anti-DP-3 peptide antibody (G) and an anti-HA monoclonal antibody (H) as described. Note the nuclear accumulation of the E2F-5ΔTAD protein in cells co-expressing DP-3δ.

E2F-5ΔTAD (Fig. 4G,H). So far, therefore, the results define two distinct mechanisms of nuclear accumulation, one exerted through an interaction with pocket proteins and the other through the DP partner protein. Moreover, the ability of E2F-5ΔTAD to undergo DP-dependent nuclear accumulation but not pocket protein-dependent accumulation indicates that at the molecular level these processes are mediated through distinct mechanisms.

The intracellular distribution of E2F-5 is under cell cycle control

Since DP-1 is a frequent component of the E2F heterodimer (Bandara et al., 1994; Girling et al., 1993), the results presented would imply that the nuclear accumulation of the E2F-4 and E2F-5 heterodimers should be under cell cycle control, and furthermore predict a nuclear presence mediated through the physical association with pocket proteins in quiescent cells. We tested this idea by studying the intracellular distribution of E2F-5 in NIH3T3 cells which express exogenous E2F-5 as a result of retroviral-mediated gene transfer (see Materials and Methods).

As expected, in asynchronous cultures of exponentially growing cells, E2F-5 was found to be frequently cytoplasmic (Fig. 5A). However, cells arrested as a result of growth in low serum (0.1%) had reduced cytoplasmic levels with E2F-5 showing a predominantly nuclear presence (Fig. 5B). Thus, the intracellular distribution of E2F-5 is under cell cycle control and, based on the earlier studies presented in this study, it is

likely that the nuclear accumulation in quiescent cells is mediated through the physical association of pocket proteins.

In addition, the subcellular distribution properties of endogenous E2F-5 during the cell cycle of human T98G cell line was analysed. Cells were synchronized by the removal of serum for three days, followed by re-stimulation with 10% FCS. The proportion of cells in the G_1, S and G_2/M phases of the cell cycle during serum starvation and re-stimulation was assessed by FACS analysis (Fig. 6A). Samples of asynchronous, serum starved and re-stimulated cells were fractionated into nuclear and low salt cytoplasmic extracts as described previously, using an extraction procedure which has been shown to efficiently separate representative cytoplasmic and nuclear proteins (de la Luna et al., 1996). Extracts were analysed for levels of en-dogenous E2F-5 by immunoblotting with the rabbit polyclonal anti-E2F-5 antibody 2197 (Buck et al., 1995). In asynchronous cells, E2F-5 was found predominantly in the cytoplasmic fraction whereas in serum starved samples E2F-5 levels were higher in the nuclear

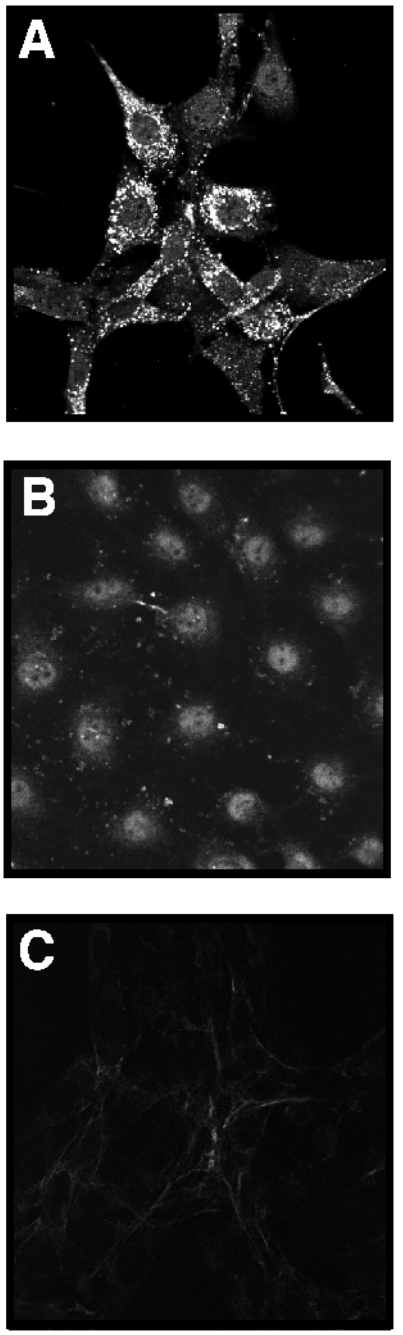

Fig. 5. The intracellular distribution of E2F-5 is under cell cycle control. NIH3T3 cells exogenously expressing HA-E2F-5 were grown in either 10% (A) or 0.1% (B) NBCS as described in Materials and Methods and thereafter the intracellular distribution of E2F-5 assessed by immunofluorescence with the anti-E2F-5 monoclonal antibody MH5. (C) Cells were treated with the secondary anti-mouse reagent alone. Note that in cycling cells (A) that E2F-5 in a major proportion of cells is evident in the cytoplasm and nuclear in the arrested population (B).

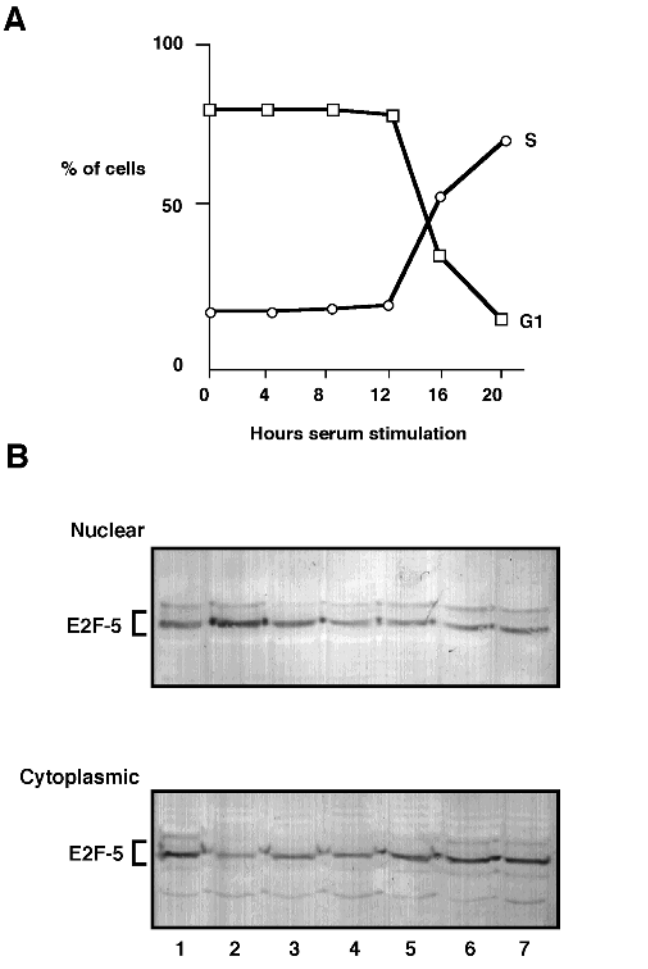

Fig. 6. Analysis of the subcellular distribution of endogenous E2F-5 during the cell cycle of human T98G cells. (A) Human T98G cells were synchronized by serum starvation as described in Materials and Methods. The percentage of cells in the G_1 and S phases of the cell cycle after serum starvation and during subsequent serum stimulation was measured by FACS analysis. (B) Nuclear and cytoplasmic fractions of cells were prepared as described (de la Luna et al., 1996), and approximately 50 μg of cell fractions were immunoblotted using the rabbit anti-E2F-5 antibody 2197 (Buck et al., 1995). The position of endogenous E2F-5 protein is indicated. Track 1, asynchronous T98G culture. Track 2, serum starved T98G cells. Tracks 3-7, T98G cells stimulated with serum for the time indicated in A. Note the change in subcellular distribution of E2F-5 upon serum starvation and during the subsequent cell cycle.

fraction (compare Fig. 6B, tracks 1 and 2). As cells re-entered the cell cycle following serum re-stimulation, nuclear levels of E2F-5 fell slightly and remained constant during the subsequent cell cycle progression, in contrast to the cytoplasmic level of E2F-5 which increased (Fig. 6B, tracks 3-7).

Functional significance of the nuclear accumulation of E2F-5

The results identify two mechanisms which control the nuclear accumulation of cytoplasmic E2F proteins: one exerted through an interaction with an NLS-containing DP partner and the other through formation of a higher order complex with pocket proteins. Consequently, we wanted to determine whether the mechanism responsible for nuclear accumulation of the heterodimer resulted in different effects on the cell cycle. For this, SAOS2 cells were transfected with the relevant expression vectors together with the cell surface marker CD20 (Zhu et al., 1993), grown in serum starvation conditions and then any effects on cell cycle progression monitored by flow cytometry (Fig. 7). When expressed alone neither E2F-5 or DP-3δ caused a significant change in the cell cycle profile whereas p107 had a marginal effect, usually causing less than 3% increase in the G_1 population (data not shown). However, when present together E2F-5 and DP-3δ led to a significant increase in the proportion of cells in the G_2/M phase of the cell cycle (Fig. 7). In contrast, in the same conditions the expression of E2F-5 together with p107 led to a further accumulation of cells in the G_1 phase of the cell cycle. As a control, a fraction of the cells used for flow cytometry were immunostained in parallel for E2F-5 protein which was found to be co-expressed with either DP-3δ or p107 in nuclei (data not shown). Therefore, the two mechanisms responsible for the nuclear accumulation of E2F-5 have distinct biological consequences on cell cycle progression.

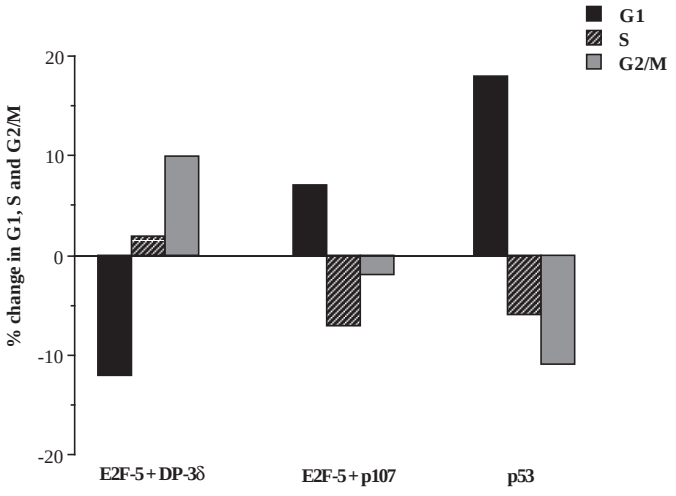

Fig. 7. The two mechanisms of nuclear accumulation of the E2F-5 heterodimer lead to distinct biological consequences on the cell cycle. SAOS2 cells were transfected with 10 μg of HA-tagged E2F-5, DP-1, DP-3δ, p107 or p53 expression plasmids as indicated, together with 10 μg pCMVCD20 as described. Backbone expression vectors were added to normalise the amounts of DNA added in each transfection. After removal of the DNA precipitate, cells were incubated in 0.2% FCS and 40 hours after transfection were harvested. Transfected cells were identified by staining with a FITC-conjugated anti-CD20 antiserum and DNA stained with propidium iodide as described (Zhu et al., 1993). The DNA profiles were calculated using a Becton Dickinson FACScan and Consort 30 software. Cells transfected with CD20 and backbone expression plasmids exhibited a cell cycle profile with approximately 60% cells in the G_1 phase, 15% in the S phase and 20% in the G_2/M phase of the cell cycle. Results are indicated as the percentage change of cells in each phase of the cell cycle relative to CD20 expressing cells transfected with empty expression plasmids. Note that the p53 treatment, which causes cells to accumulate in G_1, serves as a positive control in the assay. The results shown are a representative example of three independent experiments.

Effect of adenovirus E1a on pocket protein-mediated nuclear accumulation of E2F

It is known that the interaction of pocket proteins with the E2F heterodimer is regulated through several distinct mechanisms (La Thangue, 1994; Lam and La Thangue, 1994). One mechanism is mediated through the action of viral oncoproteins, such as adenovirus E1a, which bind to pocket proteins and thereby release E2F, enabling cell cycle progression to ensue (Nevins, 1992). We wished to investigate whether the nuclear accumulation of the E2F heterodimer mediated by the associated pocket protein was affected by adenovirus E1a.

To explore this possibility E1a was expressed in conditions where E2F-5/DP-1 was subject to pocket protein-mediated nuclear uptake. The expression of p107 with the E2F-5/DP-1 heterodimer resulted in approximately 90% of transfected cells exhibiting nuclear p107 and DP-1 (Fig. 8i, A and B). The presence of E1a did not affect this nuclear accumulation since co-expression of E1a in these conditions continued to result in over 80% of transfected cells exhibiting nuclear p107 and DP-1 (Fig. 8i; compare A and B with C and D). Cells transfected with E2F-5, DP-1 and E1a accumulated E1a in the nuclei and DP-1 in the cytoplasm (Fig. 8i, E and F), whereas co-expression of p107 in these conditions resulted in the nuclear accumulation of DP-1 in nuclei which expressed E1a (Fig. 8i, G and H). Immunoprecipitation experiments using cell extracts prepared from cells transfected in parallel confirmed that E1a was expressed at levels sufficient to disrupt the interaction of p107 with the E2F heterodimer (Fig. 8ii, compare tracks 5 and 6 with tracks 7 and 8). Moreover, DNA mobility shift analysis of the same extracts indicated that E1a disrupted a DNA binding complex containing p107 and the E2F-5 heterodimer, releasing the free E2F-5 heterodimer (data not shown). Thus, the release of pocket proteins from the E2F heterodimer by adenovirus E1a does not significantly alter the subcellular distribution of the E2F heterodimer, suggesting that the pocket protein release occurs once the E2F-pocket protein complex has accumulated in the nucleus. This observation also indicates that there is likely to be a means which acts to retain the heterodimer in nuclei once it is no longer associated with pocket proteins.

DISCUSSION

Nuclear accumulation of E2F family members

In a previous study in which we investigated the functional role of an alternatively spliced exon in DP-3, we defined an unexpected level of control involved in regulating the nuclear accumulation of E2F heterodimer (de la Luna et al., 1996). Namely, a sequence of sixteen amino acid residues (the E region)

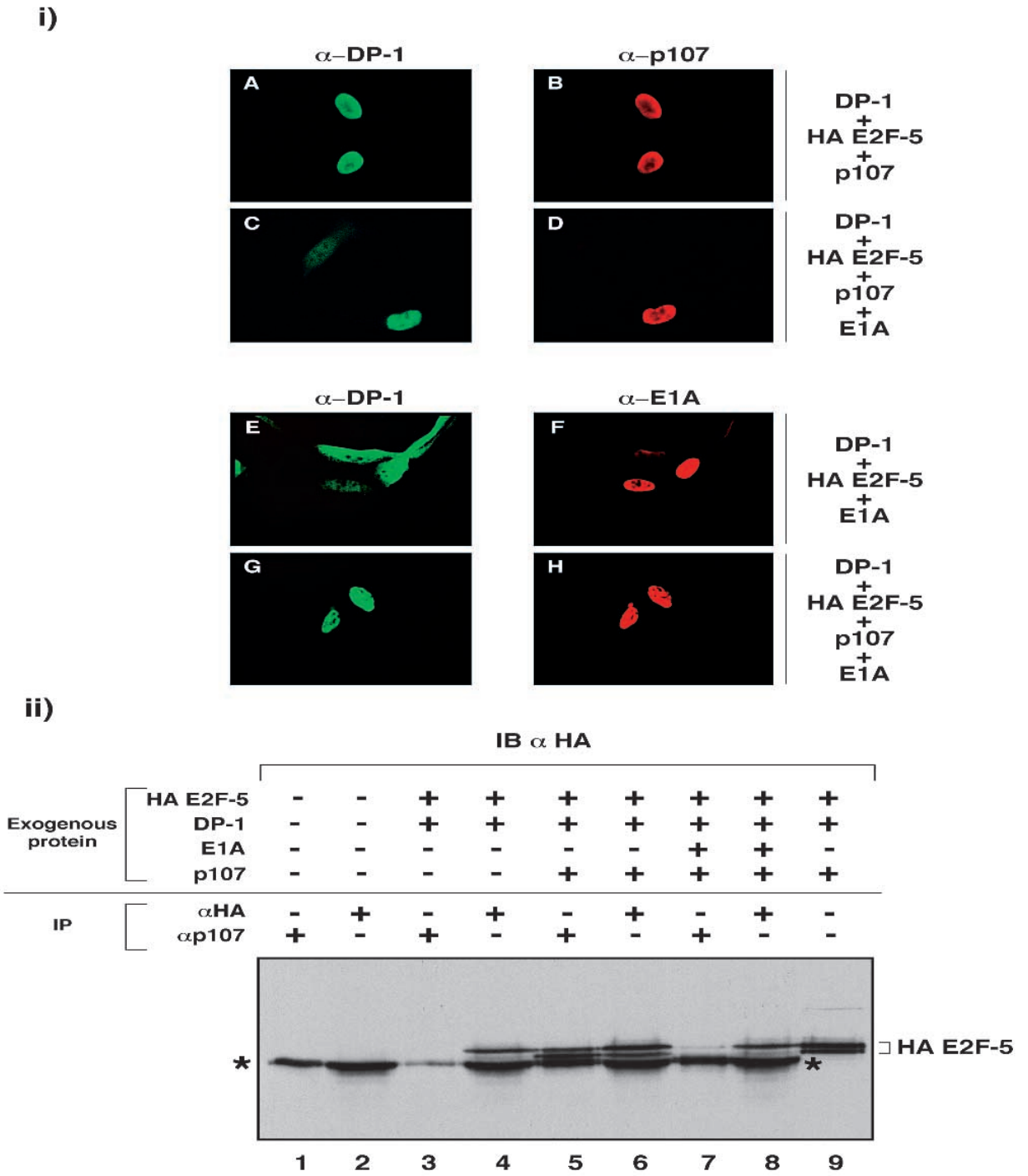

Fig. 8. Pocket protein-dependent nuclear accumulation in the presence of E1a. (i) U2OS cells were transfected with 4 μg of expression plasmids encoding HA-tagged E2F-5, DP-1 and p107 and 8 μg of an expression plasmid encoding E1a using calcium phosphate as described in Materials and Methods. The cellular distribution of the DP-1 and p107 proteins was determined by immunofluorescence with a rabbit polyclonal anti-DP-1 peptide antibody (A,C) and an anti-p107 monoclonal antibody (B,D). The cellular distribution of the DP-1 and E1a proteins was determined by immunofluorescence with a rabbit polyclonal anti-DP-1 peptide antibody (E,G) and an anti-E1a monoclonal antibody (F,H). Cellular distribution of DP-1 (A) and p107 (B) in the presence of E2F-5. Cellular distribution of DP-1 (C) and p107 (D) in the presence of E2F-5 and E1a. Note the nuclear accumulation of DP-1 in cells expressing p107 when co-expressed with E2F-5 and E1a. Cellular distribution of DP-1 (E) and E1a (F) in the presence of E2F-5. Cellular distribution of DP-1 (G) and E1a (H) in the presence of E2F-5 and p107. Note the nuclear accumulation of DP-1 in cells expressing E1a when E2F-5 and p107 are co-expressed. (ii) Immunoprecipitation of HA-tagged E2F-5 and p107 from transfected cell extracts was carried out as described in Materials and Methods. Cells were transfected with the indicated expression plasmids or empty backbone expression vectors at the same ratio as indicated for part i. Extracts were immunoprecipitated with either the anti-p107 antibody SD9 or the anti-HA monoclonal antibody HA11, and proteins detected by immunoblotting with the anti-HA antibody 12CA5. The asterisk indicates a non-specific background band detected in immunoprecipitates of mock-transfected extracts. The position of the HA-E2F-5 protein is indicated.

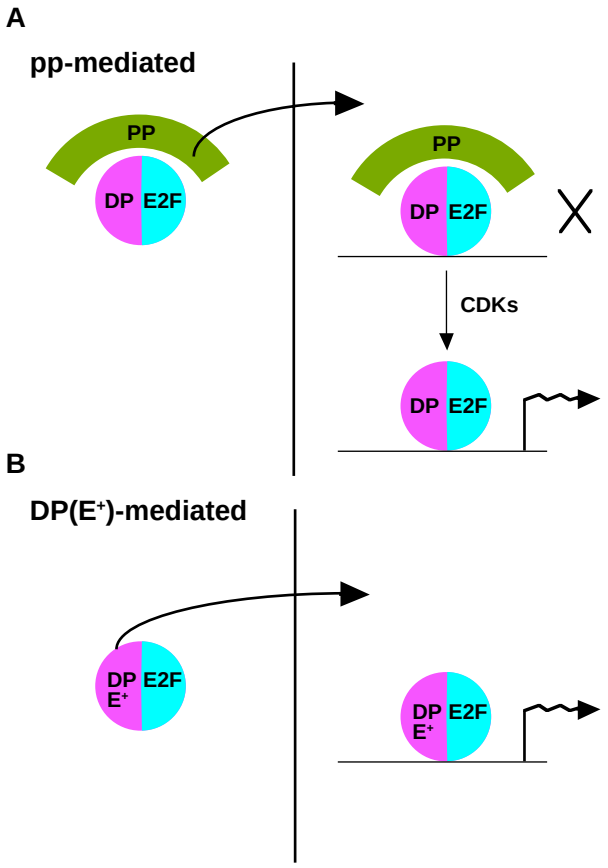

Fig. 9. Summary of mechanisms defined in this study which control the nuclear accumulation of E2F-4 and E2F-5. (A) It is envisaged that the physical association of a pocket protein, such as p107 or p130, with the E2F-4/DP-1 or E2F-5/DP-1 heterodimer, is responsible for mediating nuclear accumulation (indicated by the arrow). In this study, we speculated that pocket protein-mediated uptake allows a dominantly acting repressive form of E2F to enter nuclei and, once target genes have been located, inactivate transcription. During cell cycle progression, the action of cdks enables E2F to be released from pocket protein complexes and thereafter activate target genes. (B) An NLS in the DP-3E+ isoform regulates the nuclear accumulation of the E2F-4/DP or E2F-5/DP heterodimer which can enter nuclei in a transcriptionally active state, in contrast to the mechanism outlined in A. The vertical line indicates the nuclear envelope.

encoded by a 48 bp exon was found to function as an NLS (de la Luna et al., 1996; Ormondroyd et al., 1995). The α and δ isoforms encoded by *DP-3* contain an E region whereas it is absent from the β and γ forms (Ormondroyd et al., 1995). Furthermore DP-1, which lacks a region equivalent to E, fails to accumulate in nuclei when expressed without appropriate E2F partners (de la Luna et al., 1996; Magae et al., 1996).

It is noteworthy that DP-1 in many cell-types is a frequent, if not constitutive, component of the E2F heterodimer (Bandara et al., 1991, 1994; Girling et al., 1993; Wu et al., 1995). Given the absence of a functional NLS in DP-1 (de la Luna et al., 1996; Magae et al., 1996), we wished to gain information on the mechanism of its nuclear accumulation. To this end, we investigated the properties of the different E2F family members. By studying their intracellular distribution, we found that they can be divided into two distinct categories according to their ability to accumulate in nuclei. The first group, exemplified by E2F-1, underwent efficient nuclear accumulation whereas the second, which includes E2F-4 and E2F-5, failed to do so and remained, for the most part, in the cytoplasm. Further analysis has defined the E2F-1 NLS in the N-terminal region, similar to the domain which encompasses a site dedicated to cyclin A binding (Krek et al., 1994; data not shown). Thus, E2F-1 possesses an intrinsic NLS and, consequently, non NLS-containing E2F family members are likely to rely on other proteins to provide NLS activity. These observations reinforce previous studies indicating that the E2F family can be subdivided into two functionally and structurally distinct groups (Krek et al., 1994; Lukas et al., 1996; Mann and Jones, 1996; Zhu et al., 1993). It is possible that the mechanism of nuclear accumulation of the two E2F subfamilies may account for some of their distinct biological properties.

Two mechanisms regulate the nuclear accumulation of E2F-4 and -5

Upon forming a heterodimer with either DP-3α or δ (both E+ isoforms of DP-3), E2F-4 or E2F-5 could undergo nuclear accumulation; the same, however, was not true for DP-1 whereupon the E2F-4 or E2F-5 heterodimer remained cytoplasmic. These data indicate that in the context of E2F, the NLS can be provided by the DP partner. Indeed, comparing this result to the intrinsic ability of E2F-1 to undergo nuclear accumulation, we suggest that the interaction of E2F-4 or E2F-5 with an E+ DP protein could potentially result in a nuclear 'E2F-1-like' activity. Consistent with this idea, the co-expression of E2F-5 with DP-3δ caused cells grown under conditions of serum starvation to undergo cell cycle progression, indicating that this heterodimeric form of E2F provides a growth stimulus. Several studies have shown that E2F-1 can promote progression into S phase (Johnson et al., 1993; Qin et al., 1994) and thus both forms of nuclear E2F, either E2F-1 or E2F-4/E2F-5 with DP-3δ, share the common property of growth stimulation.

Much of the E2F-4 and E2F-5 present in cell extracts has been shown to exist as a heterodimer with DP-1 (Bandara et al., 1993, 1994; Girling et al., 1993; Wu et al., 1995; data not shown). Based on our studies, the E2F-4/DP-1 or E2F-5/DP-1 heterodimer cannot enter nuclei, and thus we were interested to define mechanisms that could account for the nuclear accumulation. One possibility arose when we found that co-expression of the pocket protein could promote the nuclear uptake of E2F-4/DP-1 or E2F-5/DP-1 heterodimers and, consistent with this result, we found E2F-5 to be nuclear during early cell cycle progression with an increased cytoplasmic concentration in cycling cells, a result that agrees with a recent study (Lindeman et al., 1997). Overall, the combined conclusion from these data defines two distinct mechanisms which regulate the levels of nuclear E2F, one in which the NLS is provided by the DP partner and the other where the NLS is supplied by the physical association of a pocket protein.

Importantly, we found that the mechanism of nuclear accumulation had a profound influence on the growth-regulating properties of E2F; when nuclear accumulation was DP E+ mediated, cells received a growth-promoting stimulus, whereas when it was mediated by the pocket-protein complex cell cycle progression could not ensue and cells accumulated in an arrested state. Thus, the mechanism of nuclear accumulation dictates the properties of the E2F heterodimer and the functional consequences on the cell cycle.

The physical interaction of pocket proteins with E2F prevents transcriptional activation (Flemington et al., 1993; Helin et al., 1993). Further studies have documented that the E2F binding site can act in cis to repress the activity of proximal transcription factors (Hsiao et al., 1994; Johnson et al., 1994; Lam and Watson, 1993; Neuman et al., 1994; Zwicker et al., 1995; Zwicker and Muller, 1997) and further that E2F-bound pocket proteins are likely to be involved in exerting these repressive effects (Bremner et al., 1995; Weintraub et al., 1992, 1995). We suggest therefore, based on the studies presented here, that pocket protein-mediated nuclear accumulation of the E2F-4/DP-1 or E2F-5/DP-1 heterodimer provides a mechanism cells use to ensure that a transcriptionally inactive and dominantly repressive form of E2F enters nuclei, thus providing a means to maintain genes regulated by E2F binding sites in a transcriptionally inactive state (Fig. 9A). It is consistent with such an idea that the p130/E2F-4 complex is abundant during early cell cycle progression, in G_0 and early G_1 cells (Cobrinik et al., 1993; Vairo et al., 1995), thus correlating with the transcriptional inactivity of many different E2F target genes (Muller, 1995). We envisage that the action of cdks subsequently releases E2F from pocket protein complexes which allows E2F to then activate the transcription of target genes in a timely fashion. Conversely, DP-mediated nuclear uptake (Fig. 9B) leaves the E2F heterodimer in an active state and thus is likely to be utilised in conditions which favour cell cycle progression.

It is an intriguing aspect of this study that the results may bear on the frequency of mutation of *Rb*, *p107* and *p130* in tumour cells. Indeed *Rb*, rather than *p107* or *p130*, becomes inactivated in tumour cells (Ewen et al., 1991; Horowitz et al., 1990; Lee et al., 1996; Zhu et al., 1993). Perhaps one aspect which influences the susceptibility of *Rb* to mutation reflects the capacity of pRb-regulated E2F members to undergo nuclear accumulation through their intrinsic NLS, even in the absence of pRb. The same would not be true for E2F-4/DP-1 or E2F-5/DP-1 heterodimers if p107 and p130 suffered mutation, since they rely on their pocket proteins for nuclear accumulation and thus would retain a cytoplasmic location. However, we emphasise that we do not wish to imply that we believe this is the sole reason for tumour cells being able to accommodate mutation in *Rb* but, rather, suggest that it may be a contributory factor.

Nuclear accumulation in the presence of adenovirus E1a

Pocket proteins can be displaced from E2F through the action of viral oncoproteins (Nevins, 1992). We assessed whether the adenovirus E1a protein affected the nuclear accumulation of E2F mediated by pocket proteins. To our surprise, co-expression of E1a did not alter the nuclear distribution of the E2F heterodimer even though the pocket protein-E2F complex was dissociated. A possible explanation could arise from considering the location of E1a, which is exclusively nuclear (see Fig. 8). Thus, E1a may displace pocket proteins from E2F-4/DP-1 and E2F-5/DP-1 heterodimers once nuclear accumulation has occurred, thereby converting them to a transcriptionally active state only in the nucleus. That the E2F heterodimer remained nuclear after release by E1a suggests that there is a means which allows nuclear retention of this form of E2F. One possibility to account for nuclear retention would be DNA binding of the heterodimer to target sequences in the promoters of genes.

In conclusion, our study points towards a new level of regulation in the control of E2F activity where subunit composition and interaction with pocket proteins dictates the functional consequence on cell cycle progression through the regulation of nuclear accumulation. It will now be important to identify the genes regulated by these different levels of control and moreover whether these mechanisms are utilised by cells in different environmental conditions.

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