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Mammalian $TAF_{II}30$ is required for cell cycle progression and specific cellular differentiation programmes

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The two alleles of the 30 kDa TATA-binding protein associated factor ($TAF_{II}30$) gene, have been targeted by homologous recombination in murine F9 embryonal carcinoma cells and subsequently disrupted using a Cre recombinase–loxP strategy. The $TAF_{II}30$-null cells are not viable, but are rescued by the expression of human $TAF_{II}30$. Cells lacking $TAF_{II}30$ are blocked in G_1/G_0 phase of the cell cycle and undergo apoptosis. In agreement with the G_1 arrest phenotype, the expression of cyclin E is impaired and the retinoblastoma protein is hypophosphorylated in the $TAF_{II}30$-null cells. Interestingly, retinoic acid (RA) treatment prevented $TAF_{II}30$-null cell death and induced primitive endodermal differentiation. In contrast, the RA- and cAMP-induced parietal endodermal differentiation was impaired in the $TAF_{II}30$-null cells. Thus, $TAF_{II}30$ is not indispensable for class II gene transcription in general, but seems to be required for the expression of a subset of genes.

Keywords: apoptosis/conditional gene knock-out/Cre recombinase/proliferation/TFIID

Introduction

Correct initiation of transcription of protein-encoding genes by RNA polymerase II (Pol II) requires the transcription factor TFIID, which is comprised of TATA-binding protein (TBP) and a number of TBP-associated factors (TAF_{II}s) (Tansey and Herr, 1997). The exact role of TAF_{II}s in transcription initiation is unknown. Initial *in vitro* transcription studies suggested that TAF_{II}s may work as general co-activators to mediate the transcriptional activation of the different activators (Goodrich *et al*., 1996). It was also shown that recombinant TBP can substitute *in vitro* for TFIID in reconstituted transcription systems, but does not support transcription from TATA-less promoters (Pugh and Tjian, 1991; Zhou *et al*., 1992).

Evidence that TAF_{II}s function as co-activators in mammalian cells has been provided by studies showing that $hTAF_{II}28$ and $hTAF_{II}135$ enhance transcriptional activation by nuclear receptors (May *et al*., 1996; Mengus *et al*., 1997). Further evidence that TAF_{II}s are required for transcriptional activation came from genetic studies of *D.melanogaster* which showed that mutations in $dTAF_{II}$s lead to specific defects in transcriptional activation in the embryo (Zhou *et al*., 1998).

In yeast (y) strains lacking functional $yTAF_{II}$s, a variety of inducible genes are normally transcribed *in vivo* (Apone *et al*., 1996; Moqtaderi *et al*., 1996a; Walker *et al*., 1996). Nevertheless, TAF_{II}s do play an essential role in yeast, as mutant strains are not viable (Moqtaderi *et al*., 1996b). Thus, TAF_{II}s may not generally be required for activator-dependent transcription, but seem to be essential for the transcription of certain genes.

Functionally distinct TFIID populations, sharing common TAF_{II}s but differing in their specific TAF_{II} composition, have been isolated from mammalian cells (Bell and Tora, 1999). TFIID complexes that either contain (TFIIDβ) or lack (TFIIDα) $hTAF_{II}30$ have been separated from mammalian cells extracts (Jacq *et al*., 1994; Mengus *et al*., 1995). Human $TAF_{II}30$ is present in only ~50% of TFIID complexes and is therefore considered a 'specific' TAF_{II} (Jacq *et al*., 1994). Recently, a novel multiprotein complex containing neither TBP nor TBP-like factor (TLF), but comprising $hTAF_{II}30$ and several other TAF_{II}s, has been characterized (Wieczorek *et al*., 1998). This complex, called TBP-free TAF_{II}-containing complex (TFTC), can replace TFIID in *in vitro* transcription assays (Wieczorek *et al*., 1998) and contains histone acetyl transferase (HAT) activity (Brand *et al*., 1999). Moreover, $TAF_{II}30$ and its yeast homologue $yTAF_{II}25$ are present in several other TAF_{II}–HAT-containing complexes, the yeast SAGA and the human PCAF/GCN5 complexes (Grant *et al*., 1998; Ogryzko *et al*., 1998).

To understand the functions of mammalian TAF_{II}s in the regulation of Pol II transcription *in vivo*, and more specifically that of $TAF_{II}30$, we have inactivated both alleles of the *$TAF_{II}30$* gene in murine embryonal carcinoma F9 cells. The F9 cell line provides an interesting model system to analyse the role of a given transcription factor during either cell proliferation and/or cell differentiation. Treatment of F9 cells with all-*trans* retinoic acid (RA) induces primitive endodermal differentiation (Strickland and Mahdavi, 1978), whereas treatment with RA and dibutyryl c-AMP (bt_2cAMP) induces a parietal endodermal phenotype (Strickland *et al*., 1980). Moreover, the RA-induced differentiation of F9 cells is accompanied by a dramatic decrease in the proliferation rate, an increase in the fraction of cells arrested in G_1/G_0 phase of the cell cycle and an increase in the number of apoptotic cells (Clifford *et al*., 1996, and references therein). The RA-induced differentiation is mediated by the RA receptors (RARs) and the retinoic X receptors (RXRs) (Chiba *et al*., 1997a, and references therein). The two *$TAF_{II}30$* alleles have been targeted here by homologous recombination (HR) and subsequently disrupted by using a Cre recombinase–loxP strategy (Sauer and Henderson, 1990). F9 cells

with both *TAF$_{II}$30* alleles inactivated are not viable, but can be rescued by expression of human TAF$_{II}$30. Furthermore, we present evidence that TAF$_{II}$30 is required for cell cycle progression through G_1 phase. TAF$_{II}$30-null cells undergo massive apoptosis which is prevented by RA-induced differentiation. Interestingly, parietal, but not primitive endodermal differentiation is strongly impaired in the TAF$_{II}$30-null cells. Taken together, our results indicate that TAF$_{II}$30 plays an important and selective role in establishing gene expression patterns.

Results

Conditional targeting of the TAF$_{II}$30 gene in F9 cells

To inactivate the *TAF$_{II}$30* gene by HR, we first cloned and characterized the mouse gene (Figure 1). The structure of the mouse (*m*) *TAF$_{II}$30* gene is very similar to the human TAF$_{II}$30 gene (TAF2H; Scheer *et al*., 1995), i.e. it consists of five exons (Figure 1). The *mTAF$_{II}$30* coding sequence deduced from the genomic sequence is 759 bases long (89% identical to the human cDNA) and encodes a 218 amino acid protein that is 90.3% identical to the human TAF$_{II}$30 protein.

To perform a conditional disruption of the *mTAF$_{II}$30* gene in F9 cells, we used a Cre–loxP strategy, similar to that previously described (Sumi-Ichinose *et al*., 1997). We constructed two targeting vectors (Figure 1B and C), in which a loxP site (hereafter termed L) was inserted into the intron located upstream of exon 2, while a neomycin-resistance gene expression cassette (tk-neo, hereafter termed N) flanked by two loxP sites ('floxed') was inserted into the intron located downstream of exon 2. Thus, following targeting of the wild-type (WT) alleles by HR, the Cre recombinase will allow the excision of exon 2 as well as the floxed selection marker cassette, resulting in the deletion of the sequences encoding amino acid residues 78 (alanine) to 130 (isoleucine) and the creation of a frame shift in the beginning of exon 3 with a stop codon (Figure 1 and data not shown). Thus, the putative truncated protein produced from the disrupted gene will lack the evolutionary highly conserved C-terminal domain of TAF$_{II}$30.

To target the first allele of the *TAF$_{II}$30* gene, F9 cells were electroporated with the pTAF$_{II}$30$^{(L:LNL)a}$ vector (Figure 1B). Southern blotting and genomic PCR analyses revealed clones that had one targeted *TAF$_{II}$30* allele [TAF$_{II}$30$^{(L:LNL)a/+}$; see Figures 1B, 2A and C]. As the presence of the marker gene in the targeted *mTAF$_{II}$30* allele might affect its expression, we established a cell line in which the floxed neomycin-resistance gene was excised before targeting the second allele. To this end, TAF$_{II}$30$^{(L:LNL)a/+}$ cells were electroporated with the Cre recombinase expression vector. Clones were obtained in which either exon 2 and the selection marker cassette (TAF$_{II}$30$^{-La/+}$ cells) or only the selection marker cassette [TAF$_{II}$30$^{(L:L)a/+}$ cells] were deleted (see Figure 2A and C). Cells from one of the TAF$_{II}$30$^{(L:L)a/+}$ clones were then electroporated with the second targeting vector (Figure 1C) and cells containing the second targeted *TAF$_{II}$30* allele were identified (Figures 1, 2B and C). Note that in the second targeting vector, the three loxP sites were in the opposite orientation when compared with the previously used vector.

To excise the sequences located between the loxP sites, the TAF$_{II}$30$^{(L:L)a/(L:LNL)b}$ cells (Figure 2B) were electroporated with the Cre recombinase expression vector. No clones with both alleles inactivated [TAF$_{II}$30$^{-La/-Lb}$ or TAFII30$^{-La/-(\ LNL)b}$] were obtained out of 560 analysed clones, but 30 clones had one inactivated *TAF$_{II}$30* allele (Figure 2B). Amongst these clones, all the possible different genotypes for the two *TAF$_{II}$30* alleles were present (Figure 2B and C and data not shown). Thus, it appears that either one of the two alleles of the mouse *TAF$_{II}$30* gene can be independently inactivated, but that inactivation of both TAF$_{II}$30 alleles may be lethal.

Inactivation of both TAF$_{II}$30 alleles impairs F9 cell viability

To investigate whether the inactivation of both *TAF$_{II}$30* alleles impairs the proliferation of F9 cells, TAF$_{II}$30$^{-La/(\ L:L)b}$ cells were electroporated with the Cre recombinase expression vector and the cell population was genotyped by PCR using DNA extracted over an 11 day period (Figure 3). Southern blotting was used for the detection of the (L:L)b and –Lb alleles (Figure 3B). The intensity of the 706 bp signal corresponding to the (L:L)b allele was similar in all the samples analysed. In contrast, although the 233 bp signal corresponding to the –Lb allele could be detected 1 day after Cre recombinase electroporation, it decreased at day four, and finally disappeared totally between days five and seven (Figure 3B and data not shown). Thus, F9 cells with both TAF$_{II}$30 alleles inactivated were obtained, but were apparently severely affected in their proliferation and could not be cloned.

Conditional rescue of TAF$_{II}$30$^{-La/-Lb}$ cells with human TAF$_{II}$30

To demonstrate unequivocally that the failure to obtain an F9 cell line in which both *mTAF$_{II}$30* alleles are inactivated, was due to an absolute requirement of the TAF$_{II}$30 protein for cell proliferation, and to allow further studies of TAF$_{II}$30 function, we generated a Dox-inducible TAF$_{II}$30 expression system. We stably integrated an expression cassette encoding the reverse tetracycline-controlled transactivator (rtTA; Gossen *et al*., 1995) in the genome of the TAF$_{II}$30$^{-La/(\ L:L)b}$ cells, as well as another cassette in which the human *TAF$_{II}$30* cDNA is under the control of tetracycline-operators, resulting in the TAF$_{II}$30$^{-La/(\ L:L)b}$:R cell line. The inducibility of hTAF$_{II}$30 protein synthesis by the tetracycline analogue Dox was verified by Western blotting analysis using the anti-hTAF$_{II}$30 monoclonal antibody 2F4 (Jacq *et al*., 1994), which is specific for the human protein. Whereas no hTAF$_{II}$30 protein could be detected in TAF$_{II}$30$^{-La/(\ L:L)b}$:R F9 cells grown in the absence of Dox, the amount of hTAF$_{II}$30 protein after Dox treatment of these cells was comparable to that of mTAF$_{II}$30 in WT F9 cells (Figure 3C). To inactivate both *mTAF$_{II}$30* alleles, TAF$_{II}$30$^{-La/(\ L:L)b}$:R cells were electroporated with the Cre recombinase expression vector and cultured in the presence of Dox. Under these conditions we obtained several clones in which exon 2 was deleted on both alleles, as verified by Southern blotting and genomic PCR analyses (TAF$_{II}$30$^{-La/Lb}$:R; Figure 3D). Inter-

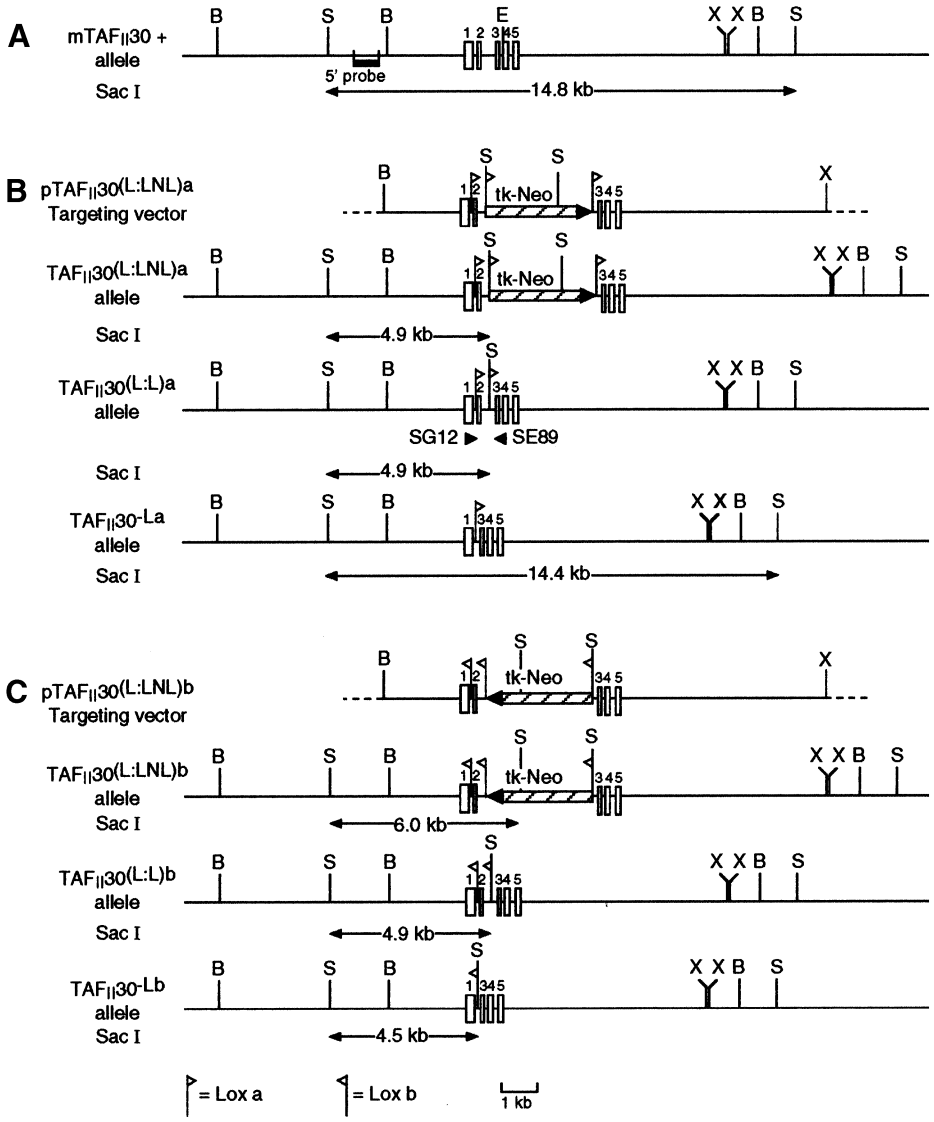

Fig. 1. Strategy for targeting the mouse *TAF$_{II}$30* gene. (**A**) Structure of the mouse *TAF$_{II}$30* gene (mTAF$_{II}$30^{+} allele). Exons 1 to 5, and the 5′ probe corresponding to a 0.8 kb *Eco*RV–*Kpn*I fragment are indicated. (**B**) First allele targeting. The targeting vector pTAF$_{II}$30$^{(L:LNL)a}$ is represented. The loxP sites (open triangles) and the tk-Neo cassette (tk-Neo) are indicated. The expected genomic maps after HR [TAF$_{II}$30$^{(L:LNL)a}$ allele] and Cre-mediated excision of loxP-flanked DNA segments [TAF$_{II}$30$^{(L:L)a}$ and TAF$_{II}$30^{-La} alleles] are shown. (**C**) Second allele targeting. The targeting vector pTAF$_{II}$30$^{(L:LNL)b}$ is represented. The loxP sites (open triangles) and the tk-Neo cassette, in opposite orientations relative to those in the pTAF$_{II}$30$^{(L:LNL)a}$ vector, are indicated. The expected genomic maps after HR [TAF$_{II}$30$^{(L:LNL)b}$ allele] and Cre-mediated excision of loxP-flanked DNA segments [TAF$_{II}$30$^{(L:L)b}$ and TAF$_{II}$30^{-Lb} alleles] are shown. The horizontal arrowheads indicate the location of the primers used for PCRs (see Figures 2C and 3D). DNA fragments obtained after *Sac*I-digestion and detection with the 5′ probe (see Figures 2C and 3D) are also indicated: B, *Bam*HI; S, *Sac*I; X, *Xho*I; and E, *Eco*RI (E is only indicated in the mTAF$_{II}$30^{+} allele). The solid line in the targeting vectors corresponds to the DNA fragment used for gene targeting. Dotted lines represent plasmid sequences.

estingly, in the TAF$_{II}$30$^{-La/-Lb}$:R cells neither a truncated *mTAF$_{II}$30* mRNA nor a truncated protein could be detected by Northern and Western blotting analyses, respectively (Figure 3D). Therefore, the –La and –Lb alleles are true null alleles.

To investigate the role of TAF$_{II}$30 in cell proliferation, we cultured the TAF$_{II}$30$^{-La/-Lb}$:R cells in the absence and presence of Dox. One day after Dox withdrawal, the hTAF$_{II}$30 protein level decreased by ~50% and became undetectable from the second day after Dox withdrawal (Figure 4A), whereas in the presence of Dox the hTAF$_{II}$30 levels were constant over several weeks (Figure 4A and data not shown). The growth rates of TAF$_{II}$30$^{-La/-Lb}$:R cells grown either in the presence or absence of Dox were similar for the first 2 days, but in the absence of Dox the cells became arrested after 3 days, and almost all the cells were dead after 6 days. Whereas cells grew to confluency in the presence of Dox [Figures 4B and 7A (b) and (c)]. Taken together, these results demonstrate that the *TAF$_{II}$30* gene product is indispensable for the viability of undifferentiated F9 cells.

Interestingly, even 5 days after Dox withdrawal, the protein levels of other TFIID and TFTC components (such as mTBP and mTAF$_{II}$135, mTAF$_{II}$100, mTAF$_{II}$80 and mTAF$_{II}$55) were not affected in TAF$_{II}$30-null cells (Figure 4A and data not shown).

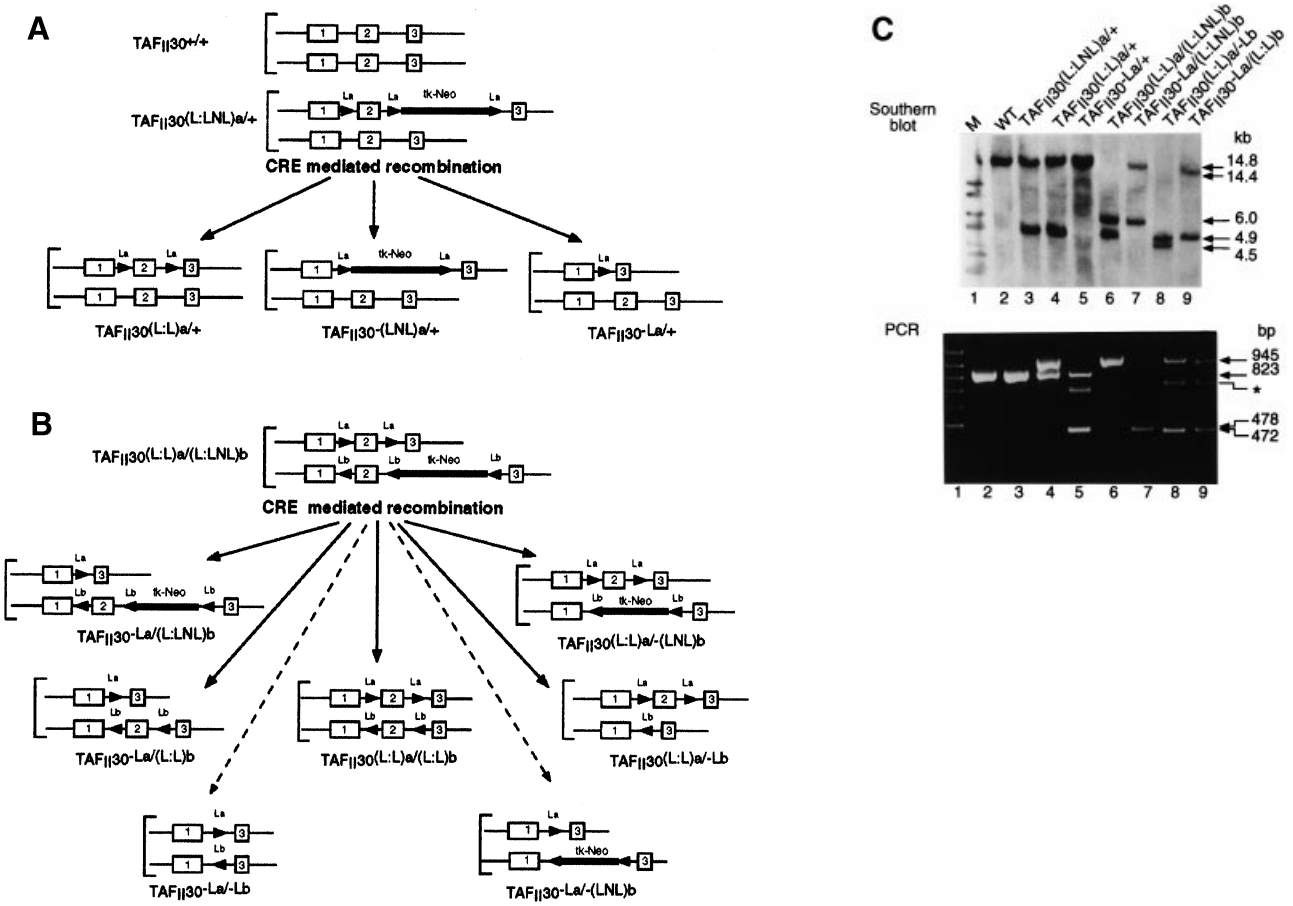

Fig. 2. Nomenclature and characterization of the genotypes of the different F9 cell lines targeted in the *TAF$_{II}$30* locus. Schematic representation of the different TAF$_{II}$30 mutant cell lines obtained after targeting the first (**A**) and the second (**B**) alleles and after Cre-mediated recombination. The exons are represented as boxes (1–3) and the loxP sites in orientation a (La) or b (Lb) as triangles. The genotypes of cells indicated by dotted arrows were not obtained. (**C**) Southern blot of *Sac*I-digested genomic DNA from the different cell lines (as indicated) hybridized with the 5′ probe (see Figure 1). Lane 2, +/+ (14.8 kb); lane 3, (L:LNL)a/+ (4.9 and 14.8 kb); lane 4, (L:L)a/+ (4.9 and 14.8 kb); lane 5, –La/+ (14.4 and 14.8 kb); lane 6, (L:L)a/(L:LNL)b (4.9 and 6 kb); lane 7, –La/(L:LNL)b (14.4 and 6 kb); lane 8, (L:L)a/–Lb (4.9 and 4.5 kb); and lane 9, –La/(L:L)b (14.4 and 4.5 kb). Genomic DNA extracted from the indicated cell lines was analysed by PCR using the primers indicated in Figure 1B. The SG12–SE89 primer pair amplifies an 823 bp fragment from the WT and the (L:LNL)a/+ alleles (lanes 2 and 3), 945 and 823 bp fragments from the (L:L)a/+ alleles (lane 4), 823 and 478 bp fragments from the –La/+ alleles (lane 5), 945 bp fragment from the (L:L)a/(L:LNL)b alleles (lane 6), 478 bp fragment from –La/(L:LNL)b alleles (lane 7), 472 and 945 bp fragments from the (L:L)a/–Lb alleles (lane 8), and 478 and 945 bp fragments from the –La/(L:L)b alleles (lane 9), respectively. The * indicates a non specific band. The 1 kb ladder DNA (M; Gibco-BRL) is shown in lane 1.

TAF$_{II}$30 is required for G$_1$–S phase cell cycle progression

The cell cycle profile of the TAF$_{II}$30$^{-La/-Lb}$:R cells grown in the absence or presence of Dox was investigated by flow cytometry. In agreement with the proliferation rate studies (Figure 4B), no significant differences in the cell cycle profiles were seen between TAF$_{II}$30$^{-La/-Lb}$:R cells grown for 2 days in the absence or presence of Dox (Figure 4C). In contrast, 5 days after Dox withdrawal the TAF$_{II}$30-null cells accumulated in G$_1$/G$_0$ phase. The proportion of cells in G$_1$/G$_0$ phase increased from ~39 to 61% (Figure 4C).

We then investigated the mRNA level of different cell cycle-related cyclin genes in TAF$_{II}$30-null cells. Reverse transcription–polymerase chain reaction (RT–PCR) analysis was used to obtain a semi-quantitative estimation of the expression of different genes (see Materials and methods). Cyclin E expression was progressively reduced from the third to the fifth day after Dox withdrawal (~6.6-fold reduction at the fifth day), when compared with either the expression in cells grown in the presence of Dox or WT cells (Figure 5A and B). In contrast, the expression of the other tested cyclins was either not, or was only weakly altered after the suppression of hTAF$_{II}$30 synthesis (Figure 5A and B). Cyclin E, which is expressed at maximal levels in the G$_1$–S transition and interacts with its catalytic partner *cdk2*, is a key regulator of G$_1$ progression during the cell cycle (Sherr, 1994; Reed, 1997). The strong reduction of cyclin E expression in TAF$_{II}$30-null cells is in good agreement with the results obtained by flow cytometry indicating that TAF$_{II}$30-null cells accumulate in G$_1$ phase.

Phosphorylation of retinoblastoma protein (pRb) in G$_1$ inactivates its growth inhibitory function, allowing cell cycle progression (Bartek *et al.*, 1997). As cyclin E–*cdk2* complex collaborates with the D-type cyclin–*cdk4/6* complexes for pRb phosphorylation (Hatakeyama *et al.*, 1994; Lundberg and Weinberg, 1998), we investigated the phosphorylation state of pRb in the TAF$_{II}$30-null cells. In WT and TAF$_{II}$30$^{-La/-Lb}$:R cells expressing hTAF$_{II}$30 protein, ~70% of pRb was phosphorylated (Figure 5C, lanes 1 and 2). In contrast, the level of pRb phosphorylation

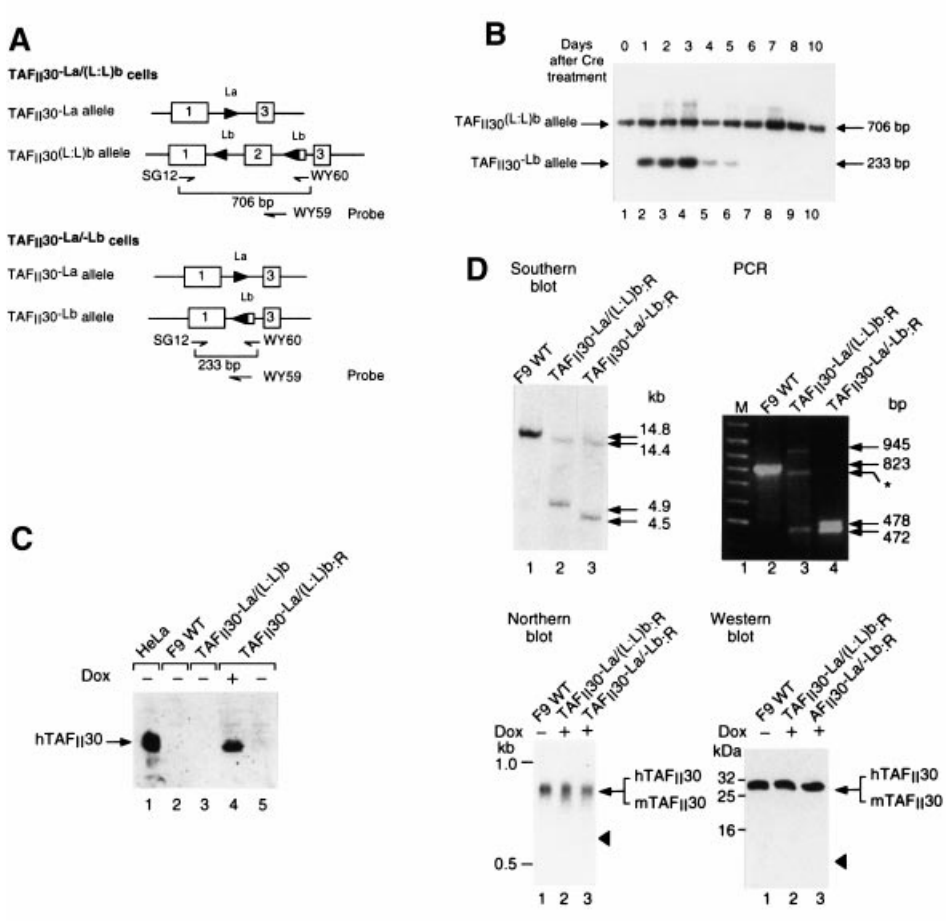

Fig. 3. $TAF_{II}30^{-La/-Lb}$ cells are not viable, but can be rescued by human $TAF_{II}30$. (**A**) The PCR strategy to identify the different alleles is outlined. Exons are indicated as numbered boxes. LoxP sites in orientation a (La) are indicated as black triangles. The oligonucleotides used either as PCR primers (SG12 and WY60; see Materials and methods) or as a probe WY59 (5′-GGAGGGCATCGTCGACATAA-3′) are represented by arrows. The loxP site in orientation b (Lb) with its surrounding sequences, and recognized by the PCR primer WY60, is shown as black triangles fused to an open box. The length of the amplified fragments from the different alleles is given. (**B**) Detection of $TAF_{II}30^{-La/-Lb}$ cells by PCR. $TAF_{II}30^{-La/(L:L)b}$ cells were electroporated with the pSG–Cre expression vector, plated at a density of 2.5×10^3 cells/well in 6-well plates and collected from days zero to 10 and stored at –80°C. Detection of the $TAF_{II}30^{(L/L)b}$ and $TAF_{II}30^{-Lb}$ alleles was performed according to the Materials and methods, as depicted in (A). (**C**) Dox-induced expression of $hTAF_{II}30$ in $TAF_{II}30^{-La/(L:L)b}$:R cells. Cell extracts were prepared from cells grown in the presence or absence of Dox, as well as from HeLa and F9 cells, as indicated. Twenty-five micrograms of each protein extract was loaded per lane and analysed by Western blotting using the anti-$hTAF_{II}30$ monoclonal antibody 2F4 (Jacq *et al*., 1994), which is specific for the human protein. (**D**) Characterization by Southern, Northern and Western blotting and PCR amplification of $TAF_{II}30^{-La/-Lb}$:R cells. Southern blotting was performed on *Sac*I-digested genomic DNA isolated from the different cell lines (as indicated), and hybridized with the 5′ probe (see Figure 1). Lane 1, WT (14.8 kb); lane 2, –La/(L:L)b (14.4 and 4.5 kb); and lane 3, –La/–Lb (14.4 and 4.5 kb). Genomic DNA extracted from the indicated cell lines was analysed by PCR using the primers indicated in Figure 1B. The SG12–SE89 primer pair amplifies an 823 bp fragment from the WT alleles (lane 2), 945 and 472 bp fragments from the –La/(L:L)b alleles (lane 3), and 472 and 478 bp fragments from the –La/–Lb alleles (lane 4), respectively. The asterisk indicates a non specific band. The 1 kb ladder DNA (M; Gibco-BRL) is shown in lane 1. The Northern blotting was carried out by standard methods using cytoplasmic RNA isolated from the indicated cell lines and hybridized with a $mTAF_{II}30$ cDNA probe. The Western blotting was carried out with protein extracts isolated from the indicated cell lines using the 1H8 anti-$TAF_{II}30$ mAb, directed against the N-terminal end of $TAF_{II}30$ (Wieczorek *et al*., 1998). The arrowheads on the Northern and Western blots indicate the sizes of the putative truncated $mTAF_{II}30$ RNA and protein, respectively.

decreased after stopping $hTAF_{II}30$ expression, and after 5 days of culture in the absence of Dox, pRb was essentially unphosphorylated (Figure 5C, lane 6). Note that the expression of pRb mRNA was not affected in the $TAF_{II}30$-null cells (data not shown). Taken together, the above results show that $TAF_{II}30$ is required for G_1–S phase cell cycle progression and cyclin E expression.

$TAF_{II}30$-null F9 cells undergo apoptosis

When the $TAF_{II}30^{-La/-Lb}$:R cells were grown for 5 days in the absence of Dox, ~82% of the cells had a sub-2N size DNA content, whereas only ~6% of such particles were detected in the presence of Dox, suggesting that most of the $TAF_{II}30$-lacking cells die by apoptosis (Figure 4C). During apoptosis endonucleases that preferentially cut DNA are activated. The characteristic DNA 'ladder' of fragmented DNA appeared 4 days after growing the $TAF_{II}30^{-La/-Lb}$:R cells in the absence of Dox and its amount increased with time (Figure 6C, lanes 17, 21 and 25). The translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is another feature of apoptotic cells. Annexin V binds specifically to PS of cells and therefore allows detection of cells early in apoptosis (Philippe *et al*., 1997). To distinguish early apoptotic from late apoptotic cells we used annexin V in combination with

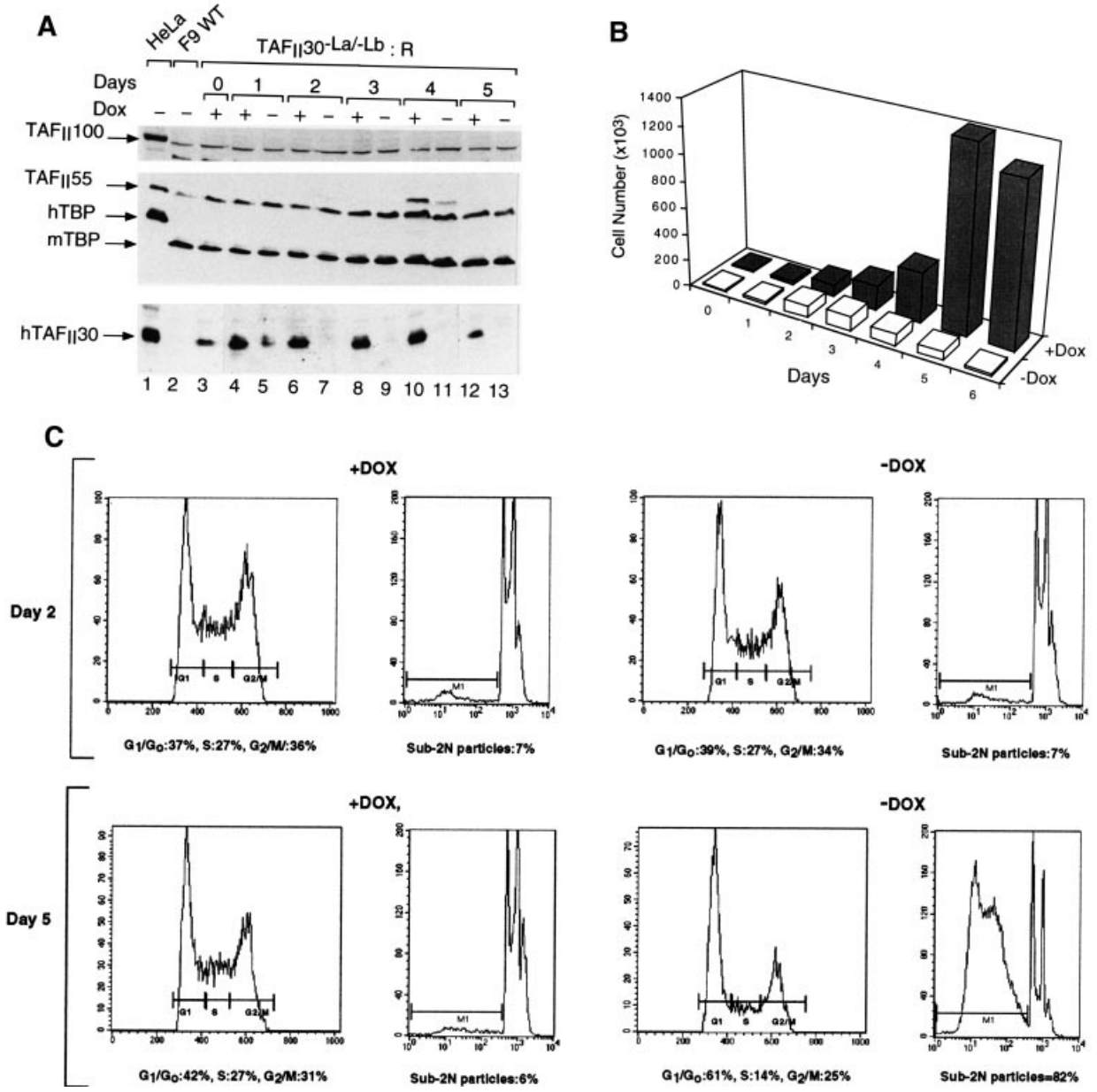

Fig. 4. $TAF_{II}30$-null cells are impaired in their proliferation and accumulate in G_1 phase of the cell cycle. (**A**) Analysis of $TAF_{II}30$, TBP, $TAF_{II}55$ and $TAF_{II}100$ protein levels in HeLa, F9 WT and $TAF_{II}30^{-La/-Lb}$:R cells grown in the presence (+) or absence (–) of Dox. Whole-cell extracts were prepared from the various cell lines as indicated, and 25 μg of each protein extract was loaded on to a 10% gel and analysed by Western blotting using mAbs raised against TBP, $hTAF_{II}55$ and $TAF_{II}100$ (see Wieczorek *et al*., 1998). (**B**) 10^4 cells were plated on day zero in the presence (+Dox) or absence (–Dox) of Dox and counted for 6 days. The number of cells grown in the presence (black columns) and absence (white columns) of Dox are indicated. (**C**) $TAF_{II}30^{-La/-Lb}$:R cells were grown in the presence (+Dox) or absence (–Dox) of Dox for 2 and 5 days as indicated, as subconfluent cultures. Cells were fixed, stained with PI and analysed by FACS. The *x*-axis indicates the DNA content and the *y*-axis the cell number. For each histogram ~20 000 particles have been gated. For each +Dox and –Dox sample, the living cells (*x*-axis is a linear scale) and all the particles (*x*-axis is a log scale) were gated in the right and left panels, respectively. The percentage of cells in G_1/G_0, S and G_2/M are indicated (accurate to within ± 5%). M1 represents the sub-2N size DNA-containing particles.

propidium iodide (PI). The proportion of pre-apoptotic particles was similar when $TAF_{II}30^{-La/-Lb}$:R cells were grown for 2 days in the absence or presence of Dox (Figure 6A, 4 and 10% annexin V+, PI– cells, respectively). However, 5 days after Dox withdrawal >60% of the $TAF_{II}30^{-La/-Lb}$:R cells were pre-apoptotic, whereas when the cells were grown in the presence of Dox for 5 days, only 16% were pre-apoptotic (Figure 6A). Taken together, these results demonstrate that F9 cells undergo apoptosis in the absence of $TAF_{II}30$ expression.

Parietal, but not primitive endodermal differentiation is impaired in $TAF_{II}30$-null cells

To analyse the function of $TAF_{II}30$ during RA-induced differentiation, $TAF_{II}30^{-La/-Lb}$:R cells were grown for 60 h as monolayers in the presence or absence of Dox, and then grown further in the presence or absence of 1 μM RA. As for WT cells, RA induced a severe decrease of the proliferation rate of $TAF_{II}30^{-La/-Lb}$:R cells expressing $hTAF_{II}30$ (+Dox) (Figure 6D and data not shown) and induced a primitive endodermal-like morphological

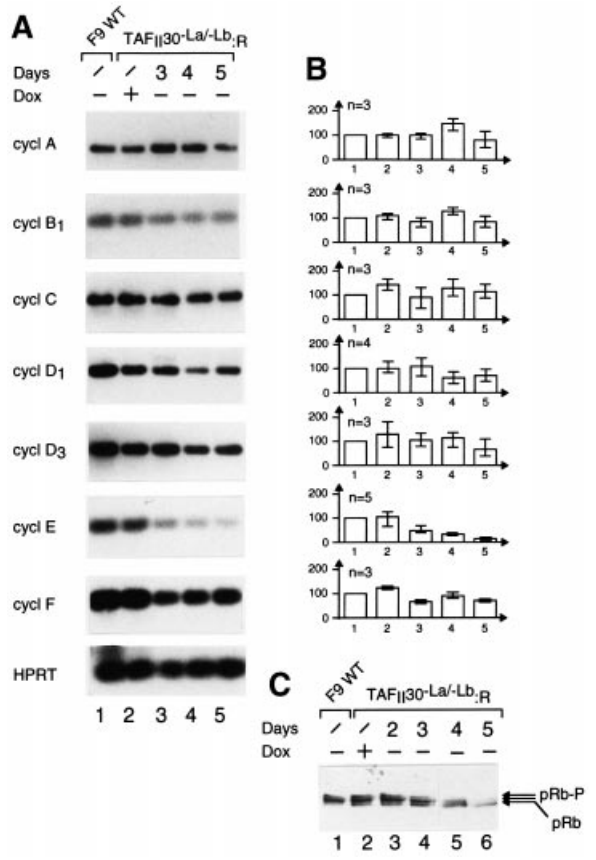

Fig. 5. Cyclin E expression and phosphorylation of pRb is impaired in $TAF_{II}30$-null cells. (**A**) Total cytoplasmic RNA from WT or $TAF_{II}30^{-La/-Lb}$:R cells, grown either in the presence of Dox (+) or for the indicated number of days without Dox (–), was subjected to RT–PCR analysis for the following cyclin genes (number of PCR cycles in parentheses): cyclin A (25), cyclin B1 (15), cyclin C (20), cyclin D1 (18), cyclin D3 (15), cyclin E (15), and cyclin F (18). The hypoxanthine phosphoribonucleotide transferase (HPRT) gene (10 PCR cycles) was used as an internal control. (**B**) RT–PCR analysis of the cyclin genes was performed as in (A) for at least three independent experiments [the number of experiments (*n*) is indicated]. The mRNA levels of the different cyclin genes are expressed relative to the amount present in the WT cells, which was taken as 100%. The error bars represent the minimal and maximal values obtained. The number under each bar corresponds to the same lane in (A). (**C**) Whole-cell extracts from WT or $TAF_{II}30^{-La/-Lb}$:R cells, grown either in the presence of (+) or for the indicated days without (–) Dox, were subjected to Western blot analysis using the anti-pRb G3-245 mAb (PharMingen). The hypophosphorylated (pRb) and the hyperphosphorylated (pRb-P) forms of pRb are indicated.

differentiation (Figure 7). Whereas undifferentiated $TAF_{II}30$-null cells died within 5–7 days of Dox withdrawal [Figures 4B, 6D and 7A (c); data not shown], surprisingly the RA treatment of $TAF_{II}30$-null cells prevented rapid cell death and induced a primitive endodermal morphological differentiation, similar to that observed with $TAF_{II}30$-containing cells [Figure 7A (e) and (f)]. Thus, the genes which are required for this differentiation pathway are expressed in the $TAF_{II}30$-null cells. Furthermore, several RA-inducible genes, i.e. *RAR*β*2*, *Hox-1*, *Stra4* and *Stra8* (Chiba *et al*., 1997b) were similarly induced in $TAF_{II}30$-null and WT cells after 2 days of RA treatment (data not shown). Moreover, the RA-treated $TAF_{II}30$-null cells survived for >10 days (Figure 6D). Interestingly, in the presence of RA the $TAF_{II}30^{-La/-Lb}$:R cells arrested with a similar time course when grown in the absence or presence of Dox (Figure 6D). The RA treatment of $TAF_{II}30^{-La/-Lb}$:R cells grown in the presence of Dox resulted in a moderate increase (from 16 to 22%) in pre-apoptotic cells (compare +Dox 5 days in Figure 6A with B), in agreement with previous studies performed on WT F9 cells (Clifford *et al*., 1996). Note that this low level of apoptosis could not be visualized in the DNA ladder assay used in this study (Figure 6C and data not shown). In contrast, the RA treatment of the $TAF_{II}30^{-La/-Lb}$:R cells in the absence of Dox resulted in a strong reduction in pre-apoptotic cells (from 61 to 20%) as detected by fluorescence-activated cell sorting (FACS) analysis (compare –Dox 5 days in Figure 6A with B) or by the absence of fragmented apoptotic DNA 'ladder' (Figure 6C, compare lanes 17 with 18, 21 with 22, and 25 with 26).

To induce parietal endodermal differentiation, WT and $TAF_{II}30^{-La/-Lb}$:R cells were grown in monolayer culture in the absence or presence of Dox and treated with 1 μM RA and 250 μM bt_2cAMP. The morphological changes which normally accompany RA + cAMP-induced differentiation of WT cells were observed in $TAF_{II}30^{-La/-Lb}$:R cells expressing $hTAF_{II}30$ [Figure 7A (h)]. In contrast, $TAF_{II}30$-null cells (–Dox), although they differentiated into primitive endoderm-like cells, were strongly impaired in parietal endodermal differentiation. The parietal differentiation of WT and mutant cells was also investigated by determining the expression of the thrombomodulin (TM) gene, a marker of parietal endodermal differentiation (Niforas *et al*., 1996; C.Rochette-Egly, personal communication). As expected, after 10 days of RA+cAMP treatment, TM was strongly stimulated in WT and mutant cells expressing $hTAF_{II}30$ (+Dox) (>90-fold, Figure 7B). In contrast, in the $TAF_{II}30$-null cells (–Dox) the induction of TM was very low (<6-fold) and the levels of TM were at least five times lower than in the WT or $hTAF_{II}30$ expressing (+Dox) cells (Figure 7B). Taken together, these results indicate that $TAF_{II}30$-null cells are strongly impaired in parietal, but not primitive endodermal differentiation.

Discussion

$TAF_{II}30$-null F9 cells are not viable

In this study, we performed conditional disruption of the *$TAF_{II}30$* gene in F9 murine embryonal carcinoma cells, using a Cre/LoxP strategy. No F9 cell clone disrupted for the two alleles could be isolated, indicating that undifferentiated $TAF_{II}30$-null F9 cells are impaired in their proliferation. This was confirmed by a study of the genotype of a $TAF_{II}30^{+/-}$ [$TAF_{II}30^{-La/(L/L)b}$] F9 cell population after Cre-mediated disruption of the second allele. Although $TAF_{II}30^{-/-}$($TAF_{II}30^{-La/-Lb}$) cells could be detected during the 5 days which followed Cre recombinase transfection, their number did not increase with time and they eventually disappeared after a week, whereas $TAF_{II}30^{+/-}$ cells proliferated normally. The Cre-mediated disruption of the targeted alleles encodes a truncated protein lacking the C-terminal evolutionary conserved domain. Importantly, neither the truncated mRNA nor the protein could be detected in heterozygous cells ($TAF_{II}30^{+/-}$) or in rescue cells in which both alleles are

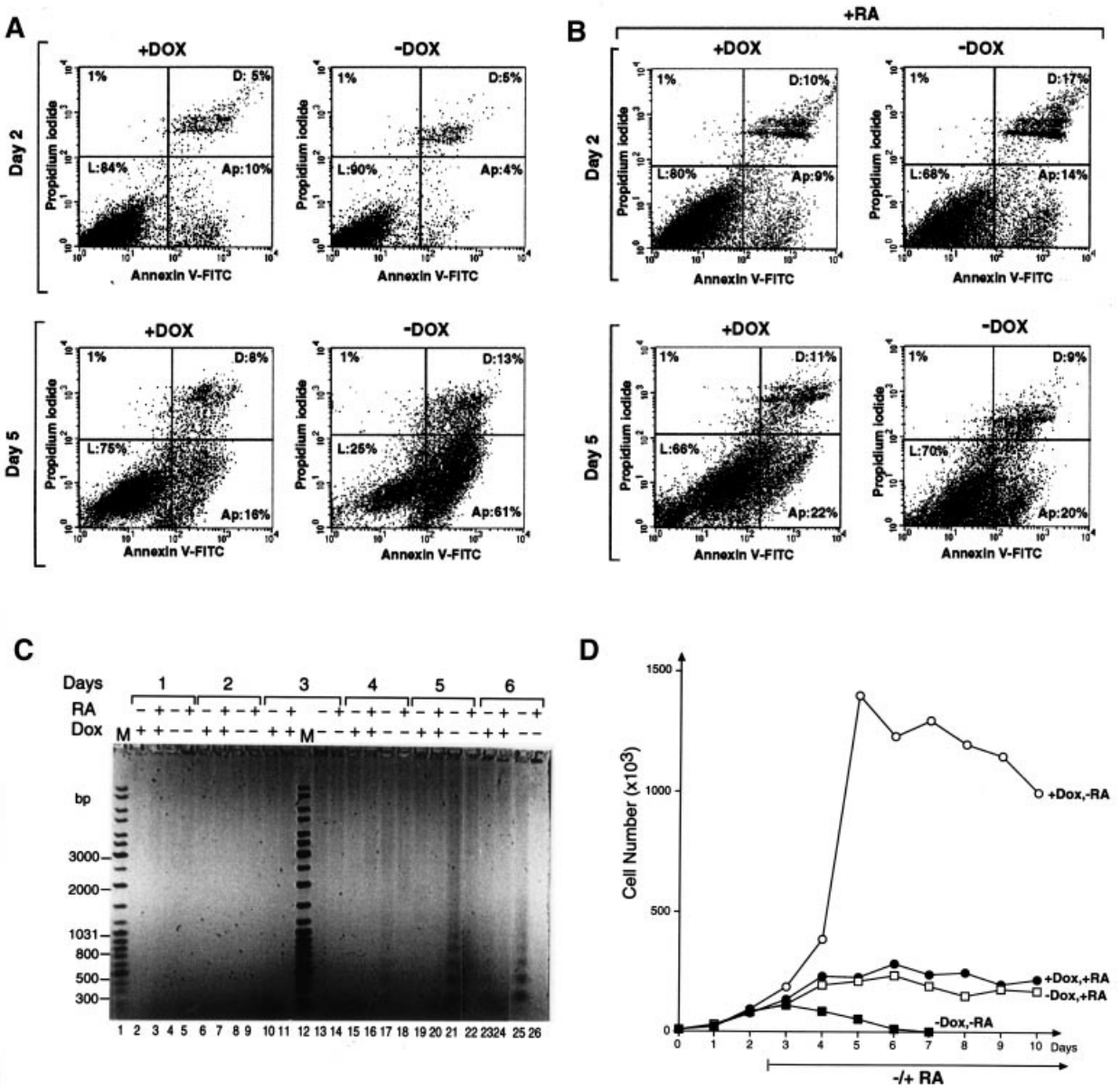

Fig. 6. $TAF_{II}30$-null cells die by apoptosis but RA strongly reduces the rate of apoptosis of the $TAF_{II}30$-null cells. $TAF_{II}30^{-La/-Lb}$:R cells, grown in the presence (+Dox) or absence (–Dox) of Dox, and either in the absence (**A**) or presence (**B**) of 1 μM RA for the indicated number of days, were labelled with annexin V–fluorescein isothiocyanate (annexin V–FITC) and PI, and subjected to FACS. The results in (A) and (B) are represented as dual parameter scatter plots. The values indicated for the percentage of living (L; annexin V–, PI–), early apoptotic (Ap; annexin V+, PI–) and dead (D; annexin V+, PI+) cells are accurate to within ± 5%. (**C**) $TAF_{II}30^{-La/-Lb}$:R cells were grown in the presence (+Dox) or absence (–Dox) of Dox, and in the presence (+RA) or absence (–RA) of retinoic acid, as indicated. Low mol. wt DNA (see Materials and methods) was electrophoresed in a 1.2% agarose gel, followed by ethidium bromide staining. The DNA marker (M) size is expressed in bp. (**D**) 10^4 $TAF_{II}30^{-La/-Lb}$:R cells were plated on day zero. Cells were grown in the presence (+Dox) or absence (–Dox) of Dox and 1 μM RA (added 60 h after plating) as indicated. Cells were counted each day for 10 days.

inactivated. Therefore, inactivation of both *$TAF_{II}30$* alleles results in $TAF_{II}30$-null F9 cells.

Human $TAF_{II}30$ can functionally replace its mouse counterpart

To analyse further the function of $TAF_{II}30$ in F9 cells, we have generated an F9 cell line expressing the human $TAF_{II}30$ protein in a conditional manner, in which both *$TAF_{II}30$* alleles have been inactivated ($TAF_{II}30^{-La/-Lb}$:R cells). $TAF_{II}30^{-La/-Lb}$:R cells expressing $hTAF_{II}30$ are viable, in contrast to $TAF_{II}30$-null cells, showing that $hTAF_{II}30$ can functionally replace $mTAF_{II}30$ in F9 cells and suggesting that $hTAF_{II}30$ can enter into the different murine $TAF_{II}30$-containing complexes such as TFIID, TFTC and PCAF/GCN5 complexes. This is in good agreement with the high homology between the mouse and human proteins. However, some differences between $TAF_{II}30^{-La/-Lb}$:R cells expressing $hTAF_{II}30$ and WT cells were observed. For example, their RA-induced morphological primitive endodermal differentiation is similar, although not identical, and parietal endodermal differentiation of the rescue cells is delayed by 48–56 h (data not shown). This partial rescue might reflect the amino acid differences between the N-terminal region of the mouse

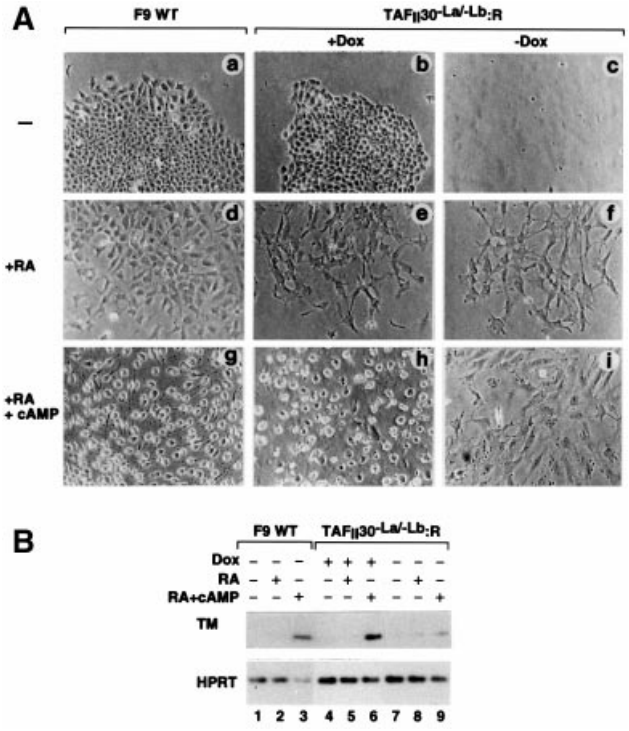

Fig. 7. Parietal but not primitive endodermal differentiation is impaired in $TAF_{II}30$-null cells. (**A**) WT [(a), (d) and (g)], and $TAF_{II}30^{-La/-Lb}$:R cultured in the presence [+Dox; (b), (e) and (h)] or absence [–Dox; (c), (f) and (i)] of Dox were treated 60 h after plating with either control vehicle [(a), (b) and (c)], 1 μM RA [(d), (e) and (f)] or 1 μM RA + 250 μM bt_2cAMP [(g), (h) and (i)]. Cells were photographed [8, 10 and 12 days following the plating for (a–f), (g) and (h–i), respectively] under a phase-contrast microscope at 125× magnification. (**B**) Cytoplasmic RNA from WT or $TAF_{II}30^{-La/-Lb}$:R cells (grown either in the presence or absence of Dox) and treated with RA alone or RA + cAMP (as indicated) for 10 days, was subjected to RT–PCR analysis to test the expression of the parietal marker gene TM. The HPRT gene was used as an internal control.

and the human protein and/or the absence of regulation of the transgene expression.

$TAF_{II}30$ is required for G_1/S progression of the cell cycle and cyclin E expression

The conditional expression of $hTAF_{II}30$ in $TAF_{II}30^{-/-}$ cells allowed us to study its role in the cell cycle regulation. FACS analysis showed that $TAF_{II}30$-null cells accumulate in G_1/G_0 phase. Molecular analyses revealed that cyclin E expression is down-regulated after suppression of $TAF_{II}30$ expression, while expression of cyclin-dependent kinase inhibitor p21 is enhanced (data not shown). Consistent with the decreased cyclin E and increased p21 expression, we found that phosphorylation of pRb is decreased in $TAF_{II}30$ lacking cells. These changes are all possible causes of the G_1 cell cycle arrest phenotype of the $TAF_{II}30$ lacking cells. Taken together, these results suggest that $TAF_{II}30$ may regulate cyclin E expression and that only certain genes are regulated (directly or indirectly) by $TAF_{II}30$.

Interestingly, growth arrest of *$yTAF_{II}90$* and *$yTAF_{II}150$* (Tsm1) mutant yeast strains occurs at the G_2/M boundary of the cell cycle, whereas *$yTAF_{II}145$* mutant strains arrest in G_1 phase (Apone *et al*., 1996; Walker *et al*., 1996). $yTAF_{II}145$ has been shown to be required for transcription of G_1 cyclins and certain B-type cyclin genes (Walker *et al*., 1997). Furthermore, a hamster cell line containing a temperature-sensitive mutation in $TAF_{II}250$ undergoes G_1 arrest upon shifting to the restrictive temperature (Sekiguchi *et al*., 1991), resulting in reduced transcription of G_1 cyclin genes such as cyclin A and D1 (Wang and Tjian, 1994; Suzuki-Yagawa *et al*., 1997; Wang *et al*., 1997). Thus, different TAF_{II}s may specifically regulate distinct cyclin genes to control cell proliferation.

RA prevents cell death of $TAF_{II}30$-null cells and induces primitive, but not parietal endodermal differentiation

Our results indicate that cells lacking $TAF_{II}30$ are not simply arrested in G_1/G_0 phase of the cell cycle, but undergo apoptosis (Figures 4C, 6A and C). The analysis of the expression of 80 genes (on a mouse cDNA expression array) involved in the regulation of apoptosis, revealed that the mRNA levels of 14 genes were up-regulated (i.e. encoding caspase 3, 6 and 11, BAD and BID) and five genes were down-regulated (encoding i.e. the p55 cell division control protein and the glutathione *S*-tranferase Mu 1 protein) in $TAF_{II}30$-null cells when compared with cells expressing $hTAF_{II}30$ (data not shown). Whether $TAF_{II}30$ is directly or indirectly involved in the regulation of these genes is unknown. The lack of $TAF_{II}30$ may induce apoptosis indirectly by deregulating distinct cell cycle check-points, i.e. pRb and/or E2F activities. A recent study has suggested that another 'specific' TAF_{II}, $hTAF_{II}105$, is involved in activation of anti-apoptotic genes by serving as a co-activator for NF-κB, a factor known to protect cells from apoptotic stimuli (Yamit-Hezi and Dikstein, 1998). It is therefore conceivable that TAF_{II}s may regulate anti-apoptotic events through different pathways.

Interestingly, after RA-treatment, $TAF_{II}30$-null cells did not die, but differentiated into primitive endodermal-like cells, and arrested with a similar time course to the RA-treated WT F9 cells (Figure 6D). Therefore, in contrast to WT cells (where RA not only induces differentiation, but also antiproliferation and apoptosis), RA-induced differentiation prevents $TAF_{II}30$-null cell death by strongly reducing apoptosis. These data suggest that when the cells are programmed to proliferate, a G_1 check-point, where the $TAF_{II}30$ function is indispensable, does not function properly and apoptosis is triggered. On the other hand, when cells are programmed to differentiate by RA, the G_1 check-point is not used since the cells enter G_0, and thus $TAF_{II}30$-null cells differentiate into primitive endodermal cells and survive. Strikingly however, the genes that are needed to trigger the morphological changes leading to primitive endodermal differentiation, are correctly expressed in $TAF_{II}30$-null cells. In contrast, parietal endodermal differentiation is strongly impaired in $TAF_{II}30$-null cells. Parietal endodermal differentiation is apparently achieved in two steps, an initial RA-induced differentiation into primitive endoderm, followed by a cAMP-induced differentiation switch from primitive to parietal endoderm (Strickland *et al*., 1980). Thus, $TAF_{II}30$ may be involved in the regulation of certain cAMP-induced genes. In any event, our results indicate that $TAF_{II}30$ is not needed for the transcription of genes required for the differentiation pathways leading to the primitive endodermal differentiation of F9 cells and for

their survival in the G_0 state. Interestingly, several RA-responsive genes can be activated in $TAF_{II}30$-null cells to similar levels to those in WT cells (data not shown), indicating that $TAF_{II}30$-containing complexes are not absolutely required for transcriptional activation by RAR/RXR heterodimers. In conclusion, we have identified a cellular context in which mammalian cells can survive in the absence of $TAF_{II}30$, indicating that all genes necessary for primitive differentiation and survival of F9 cells can be activated without $TAF_{II}30$. In contrast to the yeast TAF_{II} knock-out experiments (Moqtaderi *et al*., 1996a; Walker *et al*., 1996), the expression of different $mTAF_{II}$s and mTBP was not affected in $TAF_{II}30$-null cells (Figure 4B) further suggesting that the TFIID, TFTC and PCAF/GCN5 complexes may function without $TAF_{II}30$.

Our results demonstrate that mammalian TAF_{II}s are not generally required for activator-dependent transcription, but are essential for the transcription of specific genes. Further *in vitro* experiments will be needed to understand the exact molecular mechanisms by which $TAF_{II}30$ in the different $TAF_{II}30$-containing complexes, such as TFIID, TFTC and PCAF/GCN5, modulates the transcription of a subset of genes.

Materials and methods

Cloning of the mouse $TAF_{II}30$ gene and determination of exon–intron boundaries

A mouse embryonic stem (ES) cell genomic library constructed in λGEM12 phage was screened with a 300 bp human $TAF_{II}30$ cDNA probe (from position 355 to 654; Jacq *et al*., 1994). A 16 kb DNA fragment containing the entire mouse *$TAF_{II}30$* gene and its flanking sequences was subcloned in pBluescript II SK+ (pBSK, Stratagene) and mapped with restriction enzymes (Figure 1). The exon–intron boundaries of the mouse (*m*) *$TAF_{II}30$* gene were determined by DNA sequencing.

Targeting of the $TAF_{II}30$ gene in F9 cells

The targeting vectors $pTAF_{II}30^{(L:LNL)a}$ and $pTAF_{II}30^{(L:LNL)b}$ were constructed as follows. The 3.2 kb *Bam*HI–*Eco*RI restriction fragment, containing exons 1, 2 and 3 of the *$mTAF_{II}30$* gene (Figure 1A), was subcloned into the corresponding sites of pBEX, resulting in pBEX–TAF1-3. pBEX is a pBSK derived vector containing a new polylinker (*Bam*HI, *Eco*RI and *Xho*I), obtained by cloning the oligonucleotides 5′-GGTACCGTGGAATTCAGCTCGAGAGTAC-3′ and 5′-TCTCGAGCTGAATTCCACGGTACCAGCT-3′ into the *Sac*I and *Kpn*I sites of pBSK. A *Sal*I site was introduced between exons 1 and 2, and a *Not*I site between exons 2 and 3 by site directed mutagenesis of pBEX–TAF1-3 with the oligonucleotides 5′-CCCCGGCACCTAGTCGACGATGCCCTCCTCCTCCCT-3′ and 5′-GGCAGAGGCAGGCGGCCGCTGAGTTCAAAGCCAG-3′, respectively, resulting in pBEX–TAFSN. The oligonucleotides 5′-TCGACATAACTTCGTATAATGTATGCTATACGAAGTTATGGTACC-3′ and 5′-TCGAGGTACCATAACTTCGTATAGCATACATTATACGAAGTTATG-3′, containing a loxP site (labelled as ^{La} or ^{Lb} in the following constructions, depending on their orientation), were cloned into the *Sal*I site of pBEX–TAFSN, resulting in $pBEX–TAF^{La}N$ and $pBEX–TAF^{Lb}N$. To clone the floxed tk-Neo cassette (Metzger *et al*., 1995) into the *Not*I site of $pBEX–TAF^{La}N$ and $pBEX–TAF^{Lb}N$ vectors, a polylinker containing the following restriction sites (*Not*I, *Cla*I, *Bgl*II, *Xba*I, *Sac*I and *Not*I; 5′-GCGGCCGCTAAATCGATTCGAGATCTCAAGTCTAGAGACGAGCTCTAAGCGGCCGCGTAC-3′) was cloned in the *Sac*I and *Kpn*I sites of pBSK, resulting in pBSK–NBXN. The 3.2 kb *Bam*HI–*Xba*I fragment containing the floxed tk-Neo cassette, isolated from pHR56 (Metzger *et al*., 1995), was cloned into the corresponding sites of pBSK–NBXN, resulting in $pN^{LNL}–XN$. The 3 kb *Not*I fragment containing the floxed tk-Neo cassette excised from this vector and introduced into the *Not*I sites of $pBEX–TAF^{La}N$ and $pBEX–TAF^{Lb}N$ vectors, resulted in $pBEX–TAF_{II}30^{(L:LNL)a}$ and $pBEX–TAF_{II}30^{(L:LNL)b}$, respectively. In the $pBEX–TAF_{II}30^{(L:LNL)a}$ vector the three loxP sites are in the (a) orientation and in the $pBEX–TAF_{II}30^{(L:LNL)b}$ vector the three loxP sites are in the opposite, (b) orientation. To obtain the final targeting vectors, the 6.7 kb *Eco*RI–*Xho*I DNA fragment containing *$mTAF_{II}30$* exons 4 and 5 and its 3′ flanking sequences, was inserted into the $pBEX–TAF_{II}30^{(L:LN)La}$ and $pBEX–TAF_{II}30^{(L:LNL)b}$, resulting in the targeting vectors $pTAF_{II}30^{(L:LNL)a}$ and $pTAF_{II}30^{(L:LNL)b}$ (Figure 1). The *Cre* expression vector pSG–Cre was obtained by cloning a PCR-amplified *Cre* gene (Metzger *et al*., 1995) into the *Eco*RI–*Bgl*II sites of pSG5.

F9 cells were electroporated with 5 μg of the 13.8 kb *Bam*HI–*Xho*I fragment, isolated either from $pTAF_{II}30^{(L:LNL)a}$ or $pTAF_{II}30^{(L:LNL)b}$ vectors (Figure 1), or with 5 μg of the pSG–Cre expression vector (Clifford *et al*., 1996). The selection of neomycin-resistant clones, Cre recombinase mediated excision and Southern blot analysis were performed as described (Metzger *et al*., 1995). The 5′ probe corresponds to a 0.8 kb *Eco*RV–*Kpn*I genomic fragment, located outside the genomic sequences present in the targeting vector (Figure 1A). The primers used for the identification of the genotypes (Figures 2C and 3C) and their locations were as follows: SG12 (5′-CTGCCCACTAGCACGGCC-3′) located in the first exon, SE89 (5′-CAGTCTAACCTGCTCCGAG-3′) located between exons 3 and 4, and WY60 (5′-CGCTTAGAGCTCGTCTCTAG-3′) specific to the junction between the Loxb site and intron 2 (Figures 1 and 3).

A Dox-inducible $hTAF_{II}30$ expression system in F9 $TAF_{II}30^{-La/(L:L)b}$ cells

The expression vector pDG1–rtTA, in which the expression of the rtTA (Gossen *et al*., 1995) is under the control of the PGK promoter, was constructed by inserting the 1044-bp *Eco*RI–*Bam*HI fragment isolated from pUHD172–1Neo (Gossen *et al*., 1995) into the corresponding sites of the pDG1 expression vector. An *Eco*RI–*Bgl*II human *$TAF_{II}30$* cDNA fragment was excised from pXJ–TAF30 (Jacq *et al*., 1994) and cloned into the *Eco*RI–*Bam*HI sites of the pUHD10-3 vector (Gossen and Bujard, 1992), resulting in the expression vector pUHD10–TAF30 in which the expression of $hTAF_{II}30$ is under the control of the tet-operator. The $TAF_{II}30^{-La/(L:L)b}$ F9 cells were co-electroporated with 5 μg of pDG1–rtTA and linearized by *Aat*II and with 0.5 μg of *Xho*I-digested pPGK-hygro vector (te Riele *et al*., 1990). After hygromycin selection, rtTA positive clones were selected by genomic PCR using the primers QY74 (5′-CACGCTTCAAAAGCGCACGT-3′) and WK31 (5′-CAATACAGTGTAGGCTGCTGCTC-3′), and by Western blotting analysis using three anti-VP16 monoclonal antibodies (2GV4, 5GV2 and 5GV7; White *et al*., 1992). A rtTA positive $TAF_{II}30^{-La/(L:L)b}$ cell line was co-electroporated with 5 μg of the 2 kb *Aat*II–*Hin*dIII DNA fragment isolated from pUHD10–TAFII30 and with 0.5 μg of the neomycin-resistance cassette (a 1.6 kb *Bgl*II DNA fragment isolated from the vector pKJ-1B; pKJ-1B was obtained by cloning the oligonucleotide 5′-AATTAGATCT-3′ into the *Eco*RI site of pKJ1; McBurney *et al*., 1991). After neomycin selection, cells were treated with 1 μg/ml Dox for 3 days, and $hTAF_{II}30$ positive clones were selected by Western blot analysis using the human $TAF_{II}30$-specific antibody 2F4 (Jacq *et al*., 1994).

Western blot analysis

Cells resuspended in 20 mM Tris–HCl pH 7.5, 2 mM DTT, 20% glycerol, 0.4 M KCl, 1 mM PMSF and protease inhibitor cocktail (2.5 μg/ml of leupeptin, pepstatin, chymostatin, antipain and aprotinin), were lysed by three cycles of freeze–thaw and cell debris was eliminated by centrifugation. Proteins were loaded on a 10% SDS–polyacrylamide gel, separated by electrophoresis and electroblotted. The blots were then treated with monoclonal antibodies followed by incubation with peroxidase-conjugated goat anti-mouse IgG antibody (Jackson Immuno Research). Chemiluminescence detection was performed according to the manufacturer's instructions (Amersham).

PCR amplification of genomic DNA and RT–PCR

Genomic DNA was prepared as follows: cells grown in 6-well plates were collected in 500 μl of lysis buffer (50 mM Tris–HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 1% SDS and 0.2 mg/ml proteinase K) and incubated overnight at 55°C. The lysate was extracted with phenol/chloroform and chloroform and precipitated with ethanol at room temperature. PCR amplifications were performed in 50 μl reactions containing 67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 1.5 mM $MgCl_2$, 10% DMSO, 0.2 mM dNTPs, primers (12.5 pmol each), 1 μl of proteinase K-treated cell extract or 20 ng of purified genomic DNA and 2.5 U *Taq* polymerase. The amplification conditions were: 94°C for 5 min followed by 35 cycles at 94°C for 30 s and 55°C for 30 s, and finally one cycle at 94°C for 30 s, 50°C for 30 s and 72°C for 5 min.

Cytoplasmic RNA extraction following cell lysis with 0.5% NP-40

and semi-quantitative RT–PCRs were performed as described (Chiba *et al*., 1997b). The PCR primers used for amplification of the different transcripts are available upon request. To make sure that amplifications were in the linear range, aliquots of PCR products were taken from each reaction at three different cycles between cycles 15 and 25, depending on the gene analysed (Freeman *et al*., 1994). Control experiments were performed to determine the range of PCR cycles over which amplification efficiency remained linear and to verify that the amount of PCR product was directly proportional to the amount of input RNA (data not shown). End-labelled oligonucleotides were generated for probing the PCR products on Southern blots. Transcript levels were quantified with a BAS 2000 bio-imaging analyser (Fuji Ltd) and were normalized to the corresponding HPRT levels.

Analysis of F9 cell growth and differentiation

Cells were seeded in 10-cm culture dishes at a density of 10^4 cells/well at day zero, in the presence or absence of 1 μg/ml Dox. The medium was changed every second day, and 1 μM RA or 1 μM RA + 250 μM bt_2cAMP was added as indicated. Cells were counted with a particle counter (Coulter Z2) and their differentiation status was examined under light microscopy as described (Clifford *et al*., 1996).

Flow cytometric quantitation of cell cycle and apoptosis, and extraction of low mol. wt DNA

Flow cytometric quantitation of cell cycle was carried out as described in Clifford *et al*. (1996), except that instead of ethidium bromide, propidum iodide (PI) was used at a final concentration of 25 μg/ml. Flow cytometric quantitation of apoptosis using annexin V together with PI was as described (Lecoeur and Gougeon, 1996; Philippe *et al*., 1997). PI stains non-fixed cells in late-apoptotic and necrotic stages as these cells have lost their membrane inegrity. Briefly, 0.2×10^6 cells were resuspended in 50 μl of AV buffer (10 mM HEPES–NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and incubated with 1 μl FITC-labelled AV (CALTAG Laboratories) for 30 min at room temperature in the dark. Five hundred microlitres of AV buffer containing 5 μg/ml PI was added for 5 min (in the dark) and the samples were analysed on a FACScan (Beckton Dickinson) equipped with a single argon ion laser. A minimum of 10 000 cells per sample were analysed with the CELLQuest software (Beckton Dickinson).

Extraction of low mol. wt DNA from apoptotic cells was done in 0.2 M phosphate–citrate buffer pH 7.8, as described by Gong *et al*. (1994).

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