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Expression of p24, a novel p21$^{Waf1/Cip1/Sdi1}$-related protein, correlates with measurement of the finite proliferative potential of rodent embryo fibroblasts

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ABSTRACT **Normal mammalian fibroblasts undergo a limited number of divisions when cultured *in vitro* before entering a state of replicative senescence. The molecular basis for the determination of the finite mitotic potential is not known. Nevertheless, simian virus 40 T antigen, among other oncogenes, is able to prevent senescence in rodent embryo fibroblasts. T antigen immortalized cells are dependent upon this protein for maintaining growth once their normal mitotic life span has elapsed. Even though the mechanism that measures the finite mitotic potential of rodent fibroblasts is not known, it has been shown that it continues to function normally in the presence of this immortalizing gene. Accumulation of cyclin-dependent kinase inhibitors such as p21$^{Waf1/Cip1/Sdi1}$ could potentially be a component of the mechanism that determines the finite life span. Here we show that accumulation of p21$^{Waf1/Cip1/Sdi1}$ does not correlate with this biological counting mechanism, but we have identified p24, a p21$^{Waf1/Cip1/Sdi1}$-related protein, whose accumulation does correlate with the measurement of the finite proliferative potential of rodent embryo fibroblasts and suggest that sequestration might be a mechanism by which its activity is regulated.**

Normal mammalian fibroblasts cultured *in vitro* undergo a limited number of divisions before entering a senescent phase in which they can be maintained for long periods but cannot be induced to divide (1–3). While the mechanism that regulates the finite proliferative potential is not known, it has been suggested to be limited either by random accumulation of cell damage or by a genetic program (4–6). The cell damage hypothesis suggests that as cells divide they randomly accumulate mutations, karyotypic changes, and other forms of genetic damage which lead to changes in the expression of positive and negative regulators of cell growth or to a predisposition to karyotypic instability, resulting in loss of proliferative potential (4, 5). The processive loss of telomeric DNA and other essential sequences from the ends of chromosomes has recently been proposed to contribute to senescence (7, 8). Even though human diploid fibroblasts in culture loose about 50 bp of their telomeric DNA per population doubling, it remains to be directly demonstrated that the finite life span is measured by this progressive shortening of telomeres (8). The genetic program hypothesis suggests that an internal biological clock measures the finite life span so that upon its completion cells cease dividing and enter into the postmitotic state of replicative senescence (5, 6, 9).

Even though senescence has been extensively studied, the underlying molecular basis for the entry into this state is not known. In rodent cells it can be overcome by the expression of viral and cellular immortalizing genes (10, 11). Simian virus 40 T antigen represents one such example; it is able to induce both rat and mouse embryo fibroblasts to divide indefinitely (12–14), but such cells are absolutely dependent upon it for maintaining growth (15). Inactivation of T antigen results in the cells undergoing a rapid and irreversible growth arrest and entering a state that mimics senescence (15, 16). We have also shown that mouse embryo fibroblasts, only become dependent upon T antigen for maintenance of proliferation when their normal mitotic life span has elapsed and that the biological clock that measures the mitotic potential continues to function normally in the presence of this immortalizing gene (17). These results strongly suggested that random accumulation of cell damage was unlikely to be the factor that limits fibroblast division but supported the hypothesis that senescence was regulated via a genetic program.

The genetic program could potentially involve components of the mitotic cell cycle. This is considered largely to be regulated by cyclin-dependent kinases (Cdks), originally identified in yeast as genes whose inactivation causes cell cycle arrest (18). Activation of Cdks is complex and involves phosphorylation/dephosphorylation of Cdks themselves, binding to cyclins and inhibition of kinase activity by association with a family of molecules known as the Cdk inhibitors (19). One such inhibitor, p27^{Kip1}, inhibits cyclin E/cdk2 and cyclin A/cdk2 kinase activities and is induced in response to transforming growth factor β and by contact inhibition (20, 21). This protein shares homology to another Cdk inhibitor, p21$^{Waf1/Cip1/Sdi1}$, in the region involved in binding to cyclin/Cdk complexes (22). P21$^{Waf1/Cip1/Sdi1}$ was identified as a gene transcriptionally up-regulated by wild-type p53 (23) and by virtue of its interaction with cdk2 in a yeast two-hybrid screen (24). Because transfection of p21$^{Waf1/Cip1/Sdi1}$ into cells inhibits DNA synthesis, it has been proposed that the growth-inhibitory function of p53 may be mediated via this protein (23). It also binds proliferating cell nuclear antigen (PCNA); because PCNA is a component of DNA polymerase δ, it has been suggested that this interaction may promote DNA repair (25, 26). P21$^{Waf1/Cip1/Sdi1}$ was also isolated as a gene that was up-regulated when human diploid fibroblasts underwent replicative senescence and thus proposed to be a component of the mechanism that limits their finite proliferative potential (27).

Thus our goal was to investigate whether p21$^{Waf1/Cip1/Sdi1}$ was involved in regulating the mitotic life span of rodent embryo fibroblasts. We began by determining whether it was up-regulated when the conditionally immortal tsA58 T antigen

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Abbreviations: Cdk, cyclin-dependent kinase; REF, rat embryo fibroblast; TMEFs, transgenic mouse fibroblast cultures prepared from embryos derived from homozygous H-2K^{b}tsA58 males × normal females; NMEFs, normal mouse fibroblast cultures prepared from embryos derived from males × normal females; IFN, interferon; GST, glutathione *S*-transferase; MEFs, mouse embryo fibroblasts.

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derived rat embryo fibroblast (REF) cell lines undergo senescence. Next we analyzed cultures of REFs as they underwent senescence upon serial passaging. H-2K^{b}tsA58 embryonic fibroblasts were also studied because we have previously used these cells to show that the measurement of the life span continues normally in the presence of T antigen. We have found that accumulation of p21$^{Waf1/Cip1/Sdi1}$ does not correlate with the biological counting mechanism but have identified a protein p24, related to p21$^{Waf1/Cip1/Sdi1}$, whose accumulation correlates with the measurement of the finite proliferative potential.

MATERIALS AND METHODS

Cells and Cell Culture. tsa14, tsa129, and SV4 cells were maintained in DMEM supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. REFs were prepared from 13-day-old Sprague–Dawley rat embryos and grown in the same medium. Mouse embryo fibroblast cultures were prepared from 12- to 13-day-old embryos from transgenic homozygous H-2K^{b}tsA58 males $\times$ normal females (TMEFs) and normal males $\times$ normal females (NMEFs) and grown in the DMEM with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 units/ml of recombinant murine γ-interferon (IFN) when appropriate. TMEFs and NMEFs were serially passaged as described (17). All media and components were obtained from GIBCO/BRL. Mice were killed in accordance with Home Office Regulations.

Isolation of the Rat p21 cDNA. The rat p21 gene was isolated by screening a λ ZAP cDNA library prepared from mRNA extracted from tsa14 cells 72 h after a shift up to 39.5°C using a probe specific for the rat p21 gene. The probe was a 250-bp PCR fragment amplified from the same library using oligonucleotide primers designed from human and mouse p21 nucleotide sequence; the oligonucleotides were 5′-CTAGGGCTGCCCAAG-3′ and 5′-TTTGGAGTGGTAGAAATCTGT-3′. The 250-bp fragment was labeled by random priming and used to screen the library. Ten positive clones were obtained, plaque purified, and sequenced.

Preparation of Glutathione *S*-Transferase (GST)–p21 Fusion Protein. The full-length rat p21 coding sequence was inserted in frame into the bacterial expression vector pGEX2T (Pharmacia) and used to overproduce and purify the GST–p21 fusion protein by standard procedures.

Western Blot Analysis. Preparation of protein extracts and their analysis by immunoblotting was performed by standard procedures (28).

Immunoprecipitation Analysis. Cells were lysed in RIPA buffer (0.15 M NaCl/0.01 M Tris·HCl, pH 7.2/1% Triton X-100/0.1% SDS/1% sodium deoxycholate/5 mM EDTA) containing 50 mg/ml leupeptin, 100 mg/ml aprotinin, 100 mg/ml phenylmethylsulfonyl fluoride, and 50 mM NaF. Equal amounts of protein were immunoprecipitated using appropriate antibodies, and the immune complexes were collected using protein A-Sepharose. The immune complexes were washed several times before boiling in 6$\times$ sample buffer (0.35 M Tris·HCl/10.28% SDS/36% glycerol/0.6 M 2-mercaptoethanol) and fractionated on a 10% SDS/polyacrylamide gel. The fractionated proteins were transferred to Hybond C and blotted by standard procedures.

RESULTS

Identification of p24. Analysis of p21 mRNA has shown that it is up-regulated about 2- to 3-fold when REFs undergo senescence (16). It is also up-regulated when conditionally immortal cell lines derived by immortalizing REFs with the thermolabile tsA58 T antigen undergo senescence (16). To determine whether the increase in the level of mRNA affected the level of protein, crude extracts from two conditionally immortal cell lines (tsa14 and tsa129) were analyzed by Western blotting. To control for changes in expression due to the different growth temperatures, protein extracted from SV4 cells that proliferate at both 33°C and 39.5°C (15, 16) was analyzed. Extracts were prepared and immunoblotted using p21-specific antibodies; CP74, 471, and 472. Even though the increase in protein was less than the increase in mRNA (16), a 2- to 5-fold increase was consistently observed in the tsa cells using CP74 and 472, upon inactivation of T antigen (Fig. 1). However, 471, which immunoblots GST–p21 fusion protein and immunoprecipitates p21, unexpectedly detected a protein (designated p24) that migrated slower than p21 and its level did not change upon inactivation of T antigen.

Accumulation of p21, p24, and p27 upon Serial Passaging of REFs. To distinguish between the possibilities that p24 was a modified form of p21, or that p24 was p27^{Kip1}, protein extracts prepared from serially passaged REFs were analyzed for p21, p24, and p27. p24 was reproducibly up-regulated (Fig. 2); it was undetectable in extracts prepared from cells before plating (t0) and reached a maximal level corresponding to the onset of senescence. Expression of authentic p21 was also increased upon passaging, but the increase occurred earlier and reached a plateau well before the cells stopped dividing (compare CP74, 472, and 397 with 471 in Fig. 2). In contrast, p27 declined from a high level in

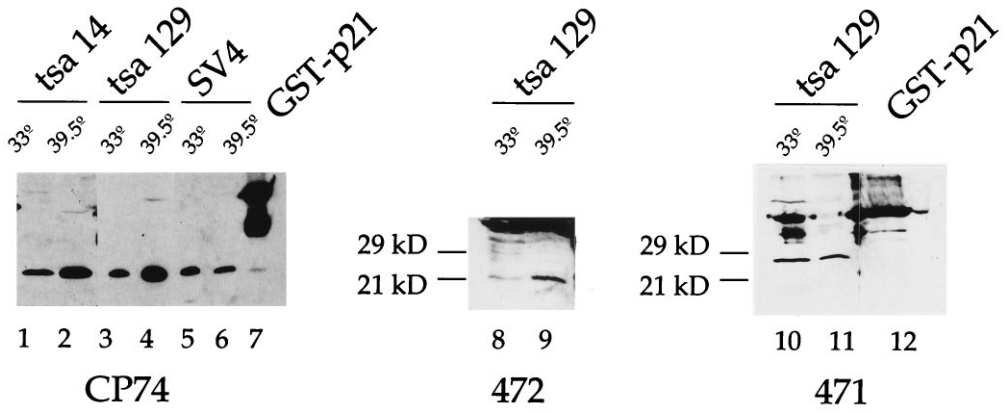

FIG. 1. Expression of p21 by Western blot analysis. Total protein (30 μg) extracted from tsa14, 129, and SV4 cell lines cultured at 33°C and at 39.5°C were analyzed by immunoblotting using CP74, a mAb specific for p21 (lanes 1–7; provided by E. Harlow, Massachusetts General Hospital). Two affinity-purified rabbit polyclonal peptide antibodies, 471 and 472 (purchased from Santa Cruz Biotechnology), were also used to analyze extracts prepared from tsa129 cells (lanes 8–12). Enhanced chemiluminescence system was used for detection. The GST–rat p21 fusion protein was used as positive control. Surprisingly this fusion protein is recognized by the 471 antibody whereas endogenous p21 is not detected.

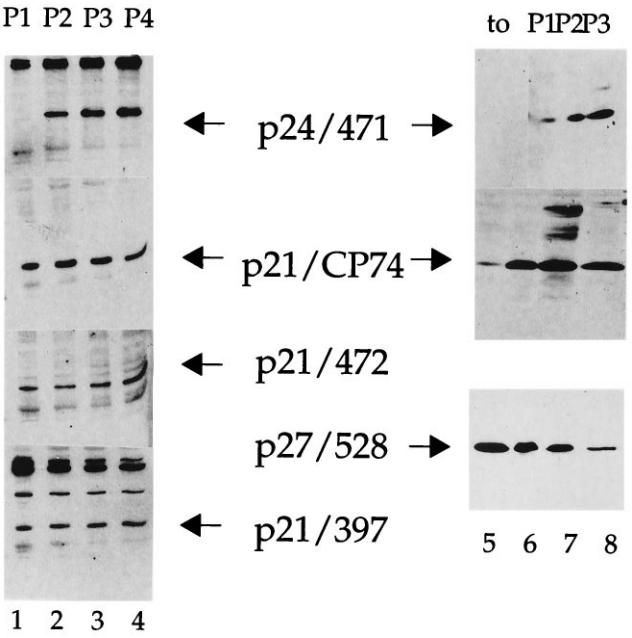

FIG. 2. Protein analysis in REFs upon serial passaging. REFs prepared from 13-day-old Sprague–Dawley rat embryos were passaged at 33°C every 4 days until they ceased dividing. Total protein extracts were prepared, and 30 μg of protein was analyzed by Western blotting using a variety of antisera against p21 (CP74, 472, and 397), p24 (471), and p27 (528). Results obtained from two independent experiments are presented; the cultures ceased dividing at passage 4 (P4) (*Left*) (lanes 1–4) and at passage 3 (P3) (*Right*) (lanes 5–8). Cultures were considered senescent when the cell number did not alter upon replating. t0 extracts were prepared from cells before plating. Antibodies 397 and 528 are affinity purified rabbit polyclonal peptide antibodies purchased from Santa Cruz Biotechnology.

t0 cells to a lower level in senescent fibroblasts (Fig. 2), and thus p24 was unlikely to be p27.

Accumulation of p21 and p24 upon Serial Passaging of Mouse Embryo Fibroblasts (MEFs). Because p24 accumulated upon passaging of REFs and its level was unaltered upon inactivation of T antigen in tsa129 cells, it raised the possibility that p24 might be a component of the biological clock that measures the mitotic potential of rodent embryo fibroblasts. To explore this possibility we used H-2K^{b}tsA58 MEFs, a system in which expression of tsA58 T antigen can be regulated at the level of transcription as well as temperature (17, 29).

TMEFs and NMEFs were prepared from 12- to 13-day-old embryos and serially cultivated in the presence of mouse γ-IFN at 33°C until NMEFs ceased dividing. Under these growth conditions TMEFs yield immortal cultures that can be passaged indefinitely whereas NMEFs stop dividing (17, 29). A gradual accumulation of p24 with a peak around P6 when the NMEFs ceased dividing was observed in both NMEFs and TMEFs (Fig. 3*a*). As for the REF cultures, the increase in p21 expression occurred more rapidly and reached a steady state earlier; the maximum level of p21 in TMEFs was lower that in NMEFs (Fig. 3*a*), an unsurprising result since p53 is complexed by T antigen.

Because TMEFs and NMEFs grown at 39.5°C exhibit the same finite proliferative potential, the passaging experiment was repeated and protein extracts were prepared and analyzed. p24 accumulated with the same kinetics in both TMEFs and NMEFs at 39.5°C (Fig. 3*b*). It also accumulated in TMEF cultures serially cultivated at 33°C plus γ-IFN and reached a maximal level when NMEFs ceased dividing. This was followed by a reduction in the level of p24 to a steady-state level that did not change upon further passaging and also upon inactivation of T antigen (Fig. 3*c*). This profile of p24 expression correlated remarkably well with the growth kinetics of these cultures. When TMEFs are cultured at 33°C in the presence of γ-IFN, there is always an initial rapid growth period that is followed by a small but reproducible decline in the growth rate to a relatively constant level (17). Interestingly the decline in growth rate occurs at about the same time as NMEFs cease dividing and express the maximal level of p24. In other experiments where NMEFs ceased dividing later (P6), the peak of p24 occurred later at P6, and the steady-state level of p24 was not achieved until passage 11 (data not shown). In this experiment Western blot analysis for T antigen showed that a steady-state level of T antigen was attained by passage 4 (data not shown). After 12 passages, inactivation of T antigen did not alter the level of p24 but p21 was up-regulated (Fig. 3*c*).

P24 Is Not a p21 Gene Product. To unequivocally determine whether p24 was derived from the p21$^{Waf1/Cip1/Sdi1}$ gene, protein extracts prepared from p21$-/-$ MEFs (30) were analyzed. Results presented in Fig. 3*d* show that although p21 was undetectable in these cells, p24 was present at levels comparable to TMEFs and senescent NMEFs. Thus p24 is not a product of the p21$^{Waf1/Cip1/Sdi1}$ gene.

T Antigen Is Coimmunoprecipitated by 471 But Not by Other p21 Antibodies. We have found that antibody 471 coprecipitates T antigen. Unlabeled extracts prepared from growing tsa129 cells were immunoprecipitated using antibody 471 and analyzed by immunoblotting with a mixture of T antigen-specific mAbs (419 and 416; ref. 31) or a p21 specific monoclonal antibody (SX118). Because T antigen is known to be complexed to p53 (32, 33), extracts immunoprecipitated with 421, a p53-specific mAb (31), were analyzed as a positive control. Two antibodies that immunoprecipitate authentic p21 (SX118 and 397) and a p27 antibody (528) were included as controls. The results in Fig. 4*a* show that 471 coprecipitates T antigen whereas antibodies that precipitate either p21 or p27 do not coprecipitate T antigen. This can be competed by an excess of the immunogenic peptide (Fig. 4*b*). Antibody 471 also immunoprecipitates authentic p21 which can be detected by SX118 (Fig. 4*b*). Because there is no homology between the 471 peptide and T antigen, it suggests that T antigen was precipitated as a consequence of interacting with a protein recognized by 471.

DISCUSSION

Here we have undertaken an analysis to determine whether the Cdk inhibitor p21$^{Waf1/Cip1/Sdi1}$is a component of the biological clock that measures the finite proliferative potential of rodent embryo fibroblasts. We have found that accumulation of p21 does not correlate with the biological clock but have identified p24, a protein that accumulates when rodent embryonic fibroblasts are serially passaged *in vitro* both in the presence and absence of T antigen. The timing and pattern of accumulation of this protein also correlate very closely with the counting mechanism that measures the finite proliferative potential of these cells. p24 shares some homology to p21$^{Waf1/Cip1/Sdi1}$ since it was identified using an affinity-purified p21 antibody but is not a product of the p21$^{Waf1/Cip1/Sdi1}$ gene. It may also be present in a complex with T antigen. These results suggest that p24 may be a component of the biological clock that measures the finite life span of rodent embryo fibroblasts and that its activity may be modulated by sequesteration.

Identifying the mechanism that regulates the proliferative potential of normal cells has important implications for studying a variety of processes such as aging and cancer. Even though the molecular mechanism is not understood, recent work showing that cell lines conditionally immortalized with T antigen undergo senescence upon its inactivation (15, 16) and that measurement of the finite life span continues in the presence of T antigen (17) has suggested that the loss of proliferative potential may involve two processes: a biological

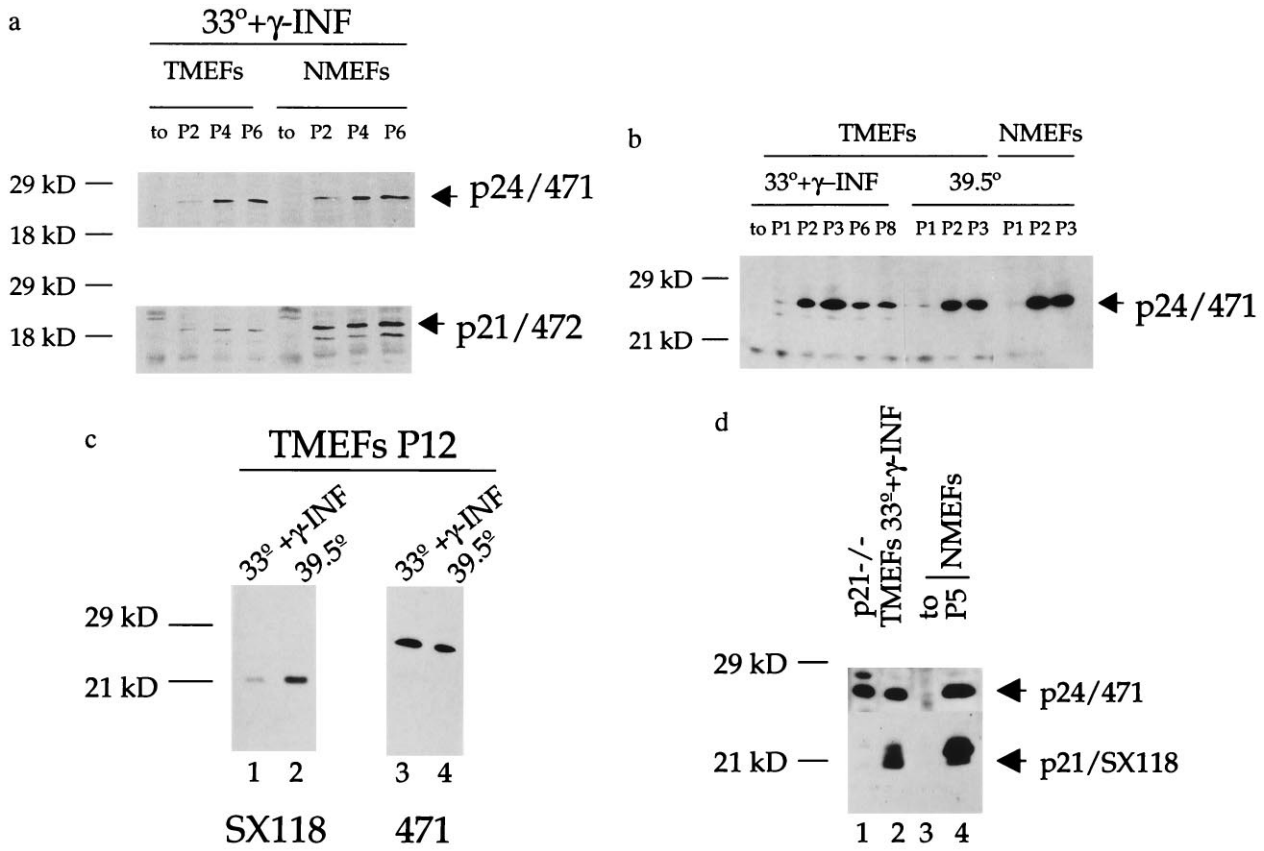

FIG. 3. Expression of p21 and p24 in MEFs. (*a*) Fibroblast cultures were prepared from 12- to 13-day-old embryos from homozygous H-2k^{b}tsA58 males × normal females (TMEFs) and normal males × normal females (NMEFs). The H-2K^{b}tsA58 mice harbor the tsA58 (41) early region coding sequences under the control of the mouse major histocompatibility complex H-2K^b class 1 promoter (29). Expression from this promoter can be enhanced by exposure of the cells to γ-IFN. Fibroblasts derived from these mice are dependent upon the presence of γ-IFN as well as the permissive temperature for growth (17, 29). Embryo fibroblasts were prepared and grown in medium containing 10 units/ml γ-IFN where indicated (17). Cells were plated at a density of 1×10^6 cells per 10-cm dish or 2.7×10^6 cells per 15-cm dish. Cultures were serially cultivated every 4 days using this passaging regime until the cell number obtained after growth for 4 days was the same as the number of cells plated. At this stage the cells had ceased dividing and were considered to have become senescent. Total cell extracts were prepared 2 days after plating and analyzed for p24 and p21 by immunoblotting using 30 μg protein. (*b*) TMEFs were serially cultivated at 33°C plus γ-IFN or 39.5°C in the absence of γ-IFN. NMEFs were passaged at 39.5°C without γ-IFN. Total cell extracts were prepared and analyzed for p24 by immunoblotting. (*c*) Extracts were prepared from passage 12 TMEFs cultured at either 33°C plus γ-IFN or after shift up to 39.5°C for 3 days. Total protein (30 μg) was analyzed for p21 and p24 by immunoblotting with antibodies SX118 and 471. SX118 is another mouse mAb specific for p21 (provided by X. Liu, Ludwig Institute for Cancer Research, London). (*d*) Expression of p21 and p24 was analyzed in extracts prepared from p21−/− MEFs (30) as well as TMEFs and NMEFs. Total protein (50 μg) was analyzed initially with antibody 471 to detect p24 and then reprobed with antibody SX118 to detect p21.

clock that counts the number of divisions that a cell undergoes and entry into the postmitotic state of senescence (16). Accumulation of inhibitors of cell division has long been postulated as a mechanism for regulating the finite life span (35). The Cdk inhibitor p21$^{Waf1/Cip1/Sdi1}$ was recently proposed to be involved in regulating the proliferative life span of human diploid fibroblasts because it was found to be up-regulated in senescent human diploid fibroblasts and inhibited DNA synthesis upon microinjection into young cells (27). It is also transcriptionally up-regulated by p53 (23) and thus proposed to be a central component of the pathway that results in growth arrest in response to DNA damage (36) and depletion of nucleotide pools (37). Our results show that when rodent embryo fibroblasts are passaged *in vitro*, p21 does accumulate but the maximal accumulation occurs before the cultures cease dividing. Of course it is possible that even though the p21 protein is expressed maximally before the cells have ceased dividing the protein is not fully functional but requires other posttranscriptional modifications to become inhibitory. However, when expression of p21 was analyzed in H-2K^{b}tsA58 TMEFs, we found that the level of p21 was much lower than NMEFs, and this level is up-regulated upon inactivation of T antigen. These results are not surprising because p21 is up-regulated by p53 (23) and T antigen sequesters p53 (32, 33). However, they are consistent with those of Tahara *et al.* (38) who found that the level of p21 mRNA increases during senescence of human diploid fibroblasts immortalized with T antigen but at a much lower level than compared with cells that did not contain T antigen. Because we have shown that the biological clock that measures the finite mitotic life span continues in the presence and absence of T antigen at the same rate (17), these results for p21 suggest that it is not a good marker for the counting mechanism. However, because there is a rapid elevation in the level of p21 upon inactivation of T antigen and this coincides with cells undergoing senescence, it suggests that p21 may be involved in regulating the entry into the postmitotic state of senescence.

During the course of our investigations we identified a p21-related protein, p24, by cross reaction with a p21 peptide antibody. This protein is not a product of the p21$^{Waf1/Cip1/Sdi1}$ gene. Analysis of the accumulation profile of this protein showed that it correlated much better with the counting mechanism; the accumulation occurred at the same rate in the presence and absence of T antigen and correlated with the growth kinetics of the fibroblasts. We have found that when TMEFs are grown in the presence of γ-IFN at 33°C (conditions

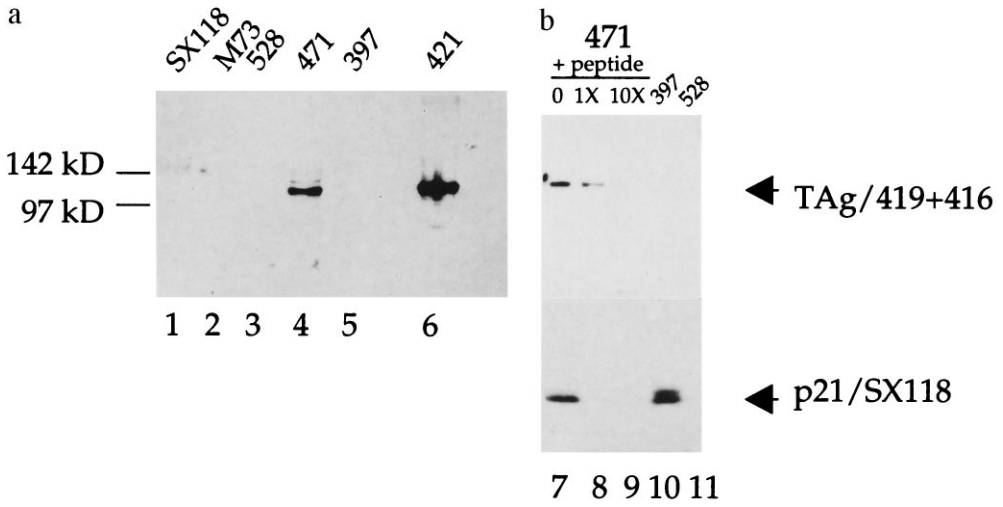

FIG. 4. Coprecipitation of T antigen. (*a*) An equal amount RIPA cell lysate prepared from tsa129 cells cultured at 33°C was immunoprecipitated using antibodies specific for p21 (SX118, 397), p27 (528), or p53 (421). mAb M73 specific for the adenovirus E1A protein was used as a negative control (34). The immunoprecipitated proteins were fractionated and analyzed for the presence of T antigen by immunoblotting using a mixture of T antigen-specific mAbs 419 and 416. (*b*) RIPA cell lysate prepared from tsa129 cells at 33°C was immunoprecipitated with 471 in the presence of increasing amounts of cold peptide used to raise this antiserum. 1× corresponds to an equal amount of peptide, whereas 10× is 10-fold higher amount of peptide. The blocking experiments were performed by preincubating antibody 471 with the competing peptide. The immunoprecipitates were first analyzed for the presence of T antigen and then reprobed for p21 using SX118.

that result in levels of T antigen sufficient for immortalization), there is always a rapid initial phase of growth followed by a reproducible decline in the growth rate, after which it remains relatively constant. Interestingly, the decline in growth rate coincides with the NMEFs ceasing proliferation. It is interesting that the highest level of p24 is observed at the time when the growth rate declines and NMEFs cease dividing. After this the level of p24 decreases to a lower level which does not alter thereafter even upon inactivation of T antigen. This suggests that, if by analogy to p21, high levels of p24 are growth inhibitory, then the finite life span is regulated by accumulation of p24 and that immortalization would require modulation of p24 activity. This could be achieved by sequestration. Antibody 471 coimmunoprecipitates T antigen, but T antigen is not coprecipitated by other p21 antibodies and thus is not complexed with p21. One candidate for the protein that coprecipitates T antigen is p24. However, this remains to be verified because it is possible that T antigen was coprecipitated as a consequence of its association with another protein because antibody 471 does recognize other proteins in Western blots; unequivocal proof will only be obtained when the gene has been isolated and more specific antibodies prepared.

Several important issues are raised by our data. What causes the accumulation of p24? p21 has been shown to be up-regulated by p53 in response to DNA damage (36) and nucleotide depletion (37) whereas accumulation of p24 is p53-independent. Is p24 associated with T antigen and does this interaction involve a domain of T antigen that is required for immortalization? Another important question that needs to be addressed is whether this protein may be involved in regulating the life span of other cell types and other species. We have found that p24 is present in human fibroblasts as well as epithelial cells (data not shown). Faragher *et al*. (39) have suggested that the gene responsible for Werner syndrome, a rare autosomal disorder, may be a counting gene that controls the number of divisions that cells undergo before terminal differentiation. This gene has now been isolated and predicted to encode a helicase of 1432 amino acids (40). Because this protein is larger than p24, it would be interesting to study the regulation of p24 in Werner fibroblasts and determine if there is a link between p24 and this putative helicase.

In conclusion, we have identified a protein, p24, which accumulates when rodent embryo fibroblasts are serially passaged *in vitro*. The timing and pattern of accumulation of this protein correlate very closely with the counting mechanism that measures the finite proliferative potential of these cells. This suggests that p24 may be a component of the biological clock that measures the finite life span of rodent embryo fibroblasts and that its activity may be modulated by sequesteration.

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1. Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell Res.* **25,** 585–621.
2. Todaro, G. J. & Green, H. J. (1963) *Cell Biol.* **17,** 299–313.
3. Lan, S. (1971) Ph.D. thesis (Univ. of London, London).
4. Cristofalo, V. J, Phillips, P. D. & Brooks, K. M. (1985) *Basic Life Sci.* **35,** 241–253.
5. Goldstein, S. (1990) *Science* **249,** 1129–1133.
6. Pereira-Smith, O. M., Spiering, A. L. & Smith, J. R. (1989) in *Growth Control During Cell Aging,* eds. Wang, E. & Warner H. R. (CRC, Boca Raton, FL), pp. 123–136.
7. Harley, C. B., Futcher, A. B. & Greider, C. W. (1990) *Nature (London)* **345,** 458–460.
8. de Lange, T. (1994) *Proc. Natl Acad. Sci. USA* **91,** 1882–2885.
9. Bayreuther K., Rodemann, H. P., Homenel, R., Dittmann, K., Albeiz, M. & Francz, P. I. (1988) *Proc. Acad. Sci. USA* **85,** 5112–5116.
10. Hunter, T. (1991) *Cell* **64,** 249–270.
11. Gonos, E. S., Powell, A. J. & Jat, P. S. (1992) *Int. J. Oncol.* **1,** 209–213.
12. Colby, W. & Shenk, T. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5189–5193.
13. Petit, C. A., Gardes, M. & Feunteun, J. (1983) *Virology* **127,** 74–82.
14. Jat, P. S. & Sharp, P. A. (1986) *J. Virol.* **59,** 746–750.
15. Jat, P. S. & Sharp, P. A. (1989) *Mol. Cell. Biol.* **9,** 1672–1681.
16. Gonos, E. S., Burns, J. S., Mazars, G. R., Kobrna, A., Riley, T. E. W., Barnett, S. C., Zafarana, G., Ludwig R. L., Ikram, Z., Powell, A. J. & Jat P. S. (1996) *Mol. Cell. Biol.* **16,** 5127–5138.
17. Ikram, Z., Norton, T. & Jat, P. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6448–6452.
18. Morgan, D. O. (1995) *Nature (London)* **374,** 131–134.

19. Sherr, C. J. & Roberts J. M. (1995) *Genes Dev.* **9,** 1149–1163.
20. Koff, A., Ohtsuki, M., Polyak, K., Roberts, J. M. & Massague, J. (1993) *Science* **260,** 536–539.
21. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Tempst, P. & Massague, J. (1994) *Cell* **78,** 59–66.
22. Toyoshima, H. & Hunter, T. (1994) *Cell* **78,** 67–74.
23. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. & Vogelstein B. (1993) *Cell* **75,** 817–825.
24. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993) *Cell* **75,** 805–816.
25. Shivji, M. K., Grey, S. J., Strausfeld, U. P., Wood, R. D. & Blow, J. J. (1994) *Curr. Biol.* **4,** 1062–1068.
26. Li, R., Waga, S., Hannon, G. J., Beach, D. & Stillman, B. (1994) *Nature (London)* **371,** 534–537.
27. Noda, A., Ning, Y., Venable, S. F., Pereira, S. O. & Smith, J. R. (1994) *Exp. Cell Res.* **211,** 90–98.
28. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
29. Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L. & Kioussis, D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5096–5100.
30. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. (1995) *Cell* **82,** 675–684.
31. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. C. (1981) *J. Virol.* **39,** 861–869.
32. Lane, D. P. & Crawford, L. V. (1979) *Nature (London)* **278,** 261–263.
33. Linzer, D. I. H. & Levine, A. J. (1979) *Cell* **17,** 43–52.
34. Harlow, E., Franza, R. & Schley, C. (1985) *J. Virol.* **55,** 533–546.
35. Lumpkin, C. K., Jr., McClung, J. R., Pereira-Smith, O. M. & Smith, J. R. (1986) *Science* **232,** 393–395.
36. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrel, M., Hill, D. E., Wang, K. G., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W. & Vogelstein B. (1994) *Cancer Res.* **54,** 1169–1174.
37. Linke, S. P., Clarkin, K. C., Di Leonardo, A., Tsou, A. & Wahl G. M. (1996) *Genes Dev.* **10,** 934–947.
38. Tahara, H., Sato, E., Noda, A. & Ide T. (1995) *Oncogene* **10,** 835–840.
39. Faragher, R. G. A., Kill, I. R., Hunter, J. A. A., Pope, F. M., Tannock, C. & Shall, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 12030–12034.
40. Yu, C.-E., Oshima, J., Fu, Y.-H., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G. M., Mulligan, J. & Schellenberg G. D. (1996) *Science* **272,** 258–262.
41. Tegtmeyer, P. (1975) *J. Virol.* **15,** 613–618.