Immortalization of conditionally transformed chicken cells: loss of normal p53 expression is an early step that is independent of cell transformation

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Clones of mortal chicken fibroblasts and erythroblasts transformed by temperature-sensitive *v-src* and *v-erb B* oncoproteins have been developed into immortal cell lines that retain the conditional transformed phenotype. The expressions of two tumor suppressor genes, the retinoblastoma (Rb) gene and the p53 gene, were investigated during senescence, crisis, and cell line establishment. In temperature-sensitive (ts)-*v-erb B* erythroblasts and ts-*v-src* fibroblasts (as well as in *v-myc* macrophages), loss of p53 mRNA or expression of a mutated p53 gene invariably occurred in the early phase of immortalization. In contrast, expression of the Rb gene was unchanged at all stages of immortalization. Inactivation of the original temperature-sensitive oncogene led to loss of the transformed phenotype in fibroblasts and to differentiation in erythroblasts, even in lines that were immortal and lacked p53. The results demonstrate that the process of immortalization is distinct from cell transformation, probably requiring different mutational events.

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The outgrowth of immortal cells from cells of the body that normally have a finite lifespan is generally thought to be a critical step in tumorigenesis. An analogous process occurs when primary cells grown in culture give rise, after senescence and crisis, to permanent cell lines that are immortal. Recently, the so-called tumor suppressor genes or recessive oncogenes, retinoblastoma (Rb) and p53, have been directly implicated in both malignant transformation and transformation/immortalization of cells in culture, as the genes carry deletions, rearrangements, or point mutations in many tumors (Nigro et al. 1989; Takahashi et al. 1989; Mulligan et al. 1990) and cell lines (Hinds et al. 1989). The role of tumor suppressors as negative regulators of cell proliferation is apparent when they are introduced into cells that lack the corresponding wild-type genes. Thus, transfection of a cloned Rb gene into retinoblastomas or osteosarcoma cells in which both copies of endogenous Rb had been inactivated caused loss of the transformed phenotype with respect to cell morphology, growth rate, soft agar colony formation, and tumorigenicity in nude mice (Huang et al. 1988). Likewise, expression of the wild-type p53 gene in human tumor cells and cell lines led to a reversible inhibition of proliferation by abolishing progression from G₀/G₁ into S phase (Diller et al. 1990;

Mercer et al. 1990). The view of these genes as negative growth regulators is strengthened further by the finding that several tumor viruses encode proteins that bind to Rb and p53 and apparently inactivate or promote their degradation (Lane and Crawford 1979; Linzer and Levine 1979; Scheffner et al. 1990).

Although the functions of Rb and p53 proteins are unknown, it has become clear that their behaviour is intimately linked to the cell cycle. Both proteins are post-translationally modified in a cell cycle-dependent fashion. In the case of Rb, interaction with viral oncogene products (e.g., SV40 large T and adenovirus E1A) and susceptibility to negative growth regulation by TGF- β (in epithelial cells) seem to be dependent on these phosphorylation events (Buchkovich et al. 1989; Mihara et al. 1989; Laiho et al. 1990). The p53 protein is phosphorylated by the p34 cdc2 kinase, and the protein accumulates during G₁ (Bischoff et al. 1990). Indirect evidence implicates p53 in controlling the onset of DNA replication during S phase. For example, the p53 protein colocalizes with viral DNA replication complexes in cells infected with herpesvirus (Wilcock and Lane 1991).

Cell cycle regulation of p53 and Rb fits well with their role as negative regulators of cell growth, suggesting that their normal function constitutes a hurdle that the normal cell must cross to progress through the cell cycle. If so, it follows that mutation of tumor suppressor genes, or of oncogenes that interact directly [SV40 large T, ad-

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enovirus E1A and E1B, human papillomavirus (HPV) E6 and E7] or indirectly [*myc*, *fos*, etc.] with tumor suppressor gene products, could remove the hurdle, thereby promoting unrestrained cell growth. In light of these ideas, we wished to ask whether loss or mutation of p53 is an essential step in the immortalization of cells in culture, and if so, whether immortalization was necessarily accompanied by a transformed phenotype. The chicken system offers several advantages in approaching these questions. First, the number of cell generations in culture before the cells enter crisis approaches that of human primary cells (30–50 generations in chickens as compared with 40–60 in humans) and is far greater than in mouse cells (10–15 generations; see Beug and Graf 1977 and references therein). As a result, mortal chicken cells can be cloned and grown in large numbers for analysis. Second, again like human cells, chicken cells are very difficult to immortalize. They remain in crisis for 2–6 months (compared with several weeks in the mouse), and outgrowth of immortal cells does not necessarily occur (Beug et al. 1982b). The advantage of the protracted immortalization period is that it allows us to obtain cells at the early (often unstable) stages of immortalization. Third, and most important, cells that have been transformed by mutant oncogenes encoding temperature-sensitive oncoproteins are available both as cloned mortal cell strains (from various cell lineages) and as immortal cell lines derived from the mortal clone. In these cells, oncoprotein function can be turned on and off at will by a simple shift between the permissive temperature (37°C) and the nonpermissive temperature (42°C). For example, a mortal clone of chick embryo fibroblasts transformed by a temperature-sensitive (ts)-v-*src* mutant exhibits a fully transformed phenotype at 37°C, whereas it is normal in every respect at 42°C (Beug et al. 1978). An immortal cell line derived from this clone retains the temperature-sensitive phenotype (see Results). Similarly, a mortal clone of chicken erythroblasts transformed by v-*erbA*/ts-v-*erbB* oncogenes grows as transformed erythroblasts at the permissive temperature but can be induced to synchronously differentiate into mature erythrocytes if oncoprotein function is inactivated (Beug et al. 1982a,b). As shown below, an immortalized cell line derived from this erythroblast clone can likewise be induced to terminally differentiate upon switching off both oncoproteins (Beug et al. 1982b). The combination of clonal mortal cells and their immortalized derivatives in which oncogenic transformation can be reversed at will provides a unique system for studying the immortalization process in isolation. An equivalent system has not been developed for human cells.

In this study we have used these and other chicken cell lines to study the expression of Rb and p53 mRNA at various stages in the immortalization process. We show that in five independent erythroblast cell lines, as well as fibroblast and macrophage lines, either expression of p53 mRNA was abolished or (more rarely) a mutated mRNA was expressed. In contrast, expression of the Rb gene was not affected. Loss or mutation of p53 expression was first detected in early, still unstable populations of immortal-

ized cells. The loss of p53 function during immortalization did not enhance or alter the transformed phenotype induced in erythroblasts and fibroblasts by their respective conditional oncoproteins. Thus, although the spontaneous immortalization of cells in culture is invariably accompanied by loss or mutation of p53, transformation by oncogenes is independent of the presence or absence of p53.

Results

Reversible transformation of mortal and immortalized fibroblast and erythroblast clones expressing temperature-sensitive oncogenes

Fibroblasts To obtain an immortalized line from clonal temperature-sensitive *src*-transformed fibroblasts, we chose a particularly well-growing clonal cell strain Fa (earlier referred to as TaSp7; Beug et al. 1978) that had been grown from an agar colony of ts-v-*src* (strain Ta)-infected chick embryo fibroblasts. This clone exhibited an in vitro lifespan of >45 generations in culture (Fig. 1A). In the following text, such mortal clonal cell strains will be referred to simply as clones. When tested for its transformed phenotype at permissive (37°C) and nonpermissive (42°C) temperature, the Fa clone exhibited a normal morphology at 42°C [well-developed actin cables (stress fibers) and an extracellular fibronectin network] but showed a typical v-*src*-transformed morphology at 37°C (loss-of-actin filament bundles and reduced expression of an organized fibronectin network) (Fig. 2A). In addition, this clone had been shown to express a number of other fibroblast transformation parameters at 37°C, but not at 42°C (Beug et al. 1978). When these cells were passaged at either 37°C or 42°C, they slowed down in growth rate and displayed large, senescent cells after the same number of passages (~45 population doublings, Fig. 1A), still exhibiting a transformed or normal morphology, respectively (Fig. 2A, insets). After a crisis period of ~3 months, three foci of healthy, slow-growing cells were obtained at 42°C. Only one of these foci could be propagated further, showing a gradually increasing growth rate during the next 1–2 months. After two subclonings of these cells, selecting for expression of a temperature-sensitive phenotype (Fig. 1A; see Materials and methods), the cloned line Fa1 was obtained. Two similar previous trials to grow out a cell line at 42°C and one trial at 37°C were unsuccessful, confirming that immortalization in chicken cells is an extremely rare event.

Further characterization of the Fa1 cell line revealed that like the mortal progenitor, its phenotype was normal at 42°C and transformed at 37°C (Fig. 2B and data not shown). The interchange between normal and transformed phenotypes after temperature switch was also the same for the Fa1 line as for the pre-immortal clone (Fig. 2A, insets; Beug et al. 1978). Thus, immortalization did not significantly affect the v-*src*-induced transformed phenotype in these clonal fibroblasts, with the possible exception of colony formation in agar: The Fa1 cell line was somewhat abnormal in that colony formation was

Figure 1. Time course (in months) of the establishment of the cell lines Fa-1 (A) and Ea1 and Ea2 (B), and schematic representation of the mortal and immortalized fibroblast and erythroblast populations obtained during isolation of these lines. The status of p53 expression is indicated as follows: (+) Normal-sized p53 message; (-) no p53 message; (+*) truncated p53 message; (-*) p53 with inactivating point mutation; (?) not analyzed. The derivation of both fibroblast and erythroblast clones from semisolid medium and the approximate time points of cloning are indicated.

reduced rather than enhanced at the permissive temperature (data not shown).

Erythroblasts The Ea1 erythroblast cell line (elsewhere referred to as HD3) was isolated from an erythroblast clone (Ea) that had been nonproductively infected with a temperature-sensitive mutant (ts34) of the *ts-v-erbB/v-erbA*-containing avian erythroblastosis virus (AEV) (Fig. 1B; see Beug et al. 1982b). Inactivation of the oncoproteins leads to reversal of transformation and complete differentiation into mature erythroblasts (Beug et al. 1982a). To compare the mortal clone Ea and the immortalized line Ea1 in this respect, the two cell types were cultivated in pH 8 differentiation medium at 42°C (to inactivate *ts-v-erbB* oncoprotein function) and in the presence of the protein kinase inhibitor H7 (to suppress *v-erbA* protein function by preventing its phosphorylation; Glineur et al. 1990). Both the mortal clone Ea and the immortalized line Ea1 completely differentiated into mature erythrocytes within 4–5 days at 42°C (Fig. 3). This again demonstrates that immortalization per se had no detectable effect on the expression of the transformed erythroblast phenotype induced by *v-erbB* and *v-erbA* oncogenes.

Expression of p53 and Rb mRNA in mortal vs. immortalized fibroblast and erythroblast clones

Cells from the Fa fibroblast clone (25 population doublings) and from the Fa1 line (passage 53 after second subcloning; see Materials and methods) were grown for 2 days at 37°C and 42°C, respectively and analyzed for p53, Rb and, as a control, β -actin expression, using Northern blots of total and poly(A)⁺ RNA. In addition, Fa cells were passaged at 42°C until they essentially stopped growing and displayed senescent cells (Fig. 2A, insets) before preparing RNA (Fa aged). Figure 4A shows that the cell strain Fa expresses both p53 and Rb message, whereas no p53 mRNA was detectable in the Fa1 line. The Fa1 line did, however, express normal levels of Rb and β -actin mRNA. This pattern was seen in normal and transformed cells grown at both growth temperatures. The absence of p53 mRNA in Fa1 cells was verified by analyzing poly(A)⁺ RNA (data not shown). On the other hand, two different, independently derived populations of senescent, essentially stationary Fa cells still expressed p53 mRNA at normal levels (Fig. 4B), suggesting that the events leading to loss of p53 expression are not a consequence of senescence.

Figure 2. Reversible transformation of a mortal fibroblast clone Fa (A) and a derivative immortal cell line Fa1 (B) as assayed by cell morphology, expression of actin cables, and expression of fibronectin at the permissive and nonpermissive temperature. (A) Phase micrographs of clone Fa grown at 42°C (mostly flat morphology) and 37°C (spindle-like transformed morphology) are shown in the upper panels. (Insets) Stationary, aged cells passaged at the respective temperature until senescence. The aged cells still display a (somewhat aberrant) normal (42°C) and transformed (37°C) morphology. The middle and lower panels show photographs of the same fields of cells stained with TRITC-phalloidin (actin) and anti-fibronectin antibody followed by FITC-labeled second antibody (fibronectin) and photographed using filter combinations to view FITC and TRITC fluorescence, respectively (Beug et al. 1978). Note abundant actin cables and fibronectin network at 42°C but not at 37°C. (B) Cells from the Fa1 cell line were photographed at 42°C and 37°C as in A. (Insets) Cells shifted from 42°C to 37°C (left) or from 37°C to 42°C (right), indicating that the cell line had retained the ability of the Fa clone to transform rapidly or normalize upon temperature shift. Note similar loss of actin cables and fibronectin network upon shift to 37°C as seen for the Fa clone.

Very similar results were obtained in erythroblasts (Fig. 1B). Cells of the mortal strain Ea expressed p53 and Rb RNA, both as transformed erythroblasts at 37°C and as partially mature reticulocytes after 2 days at 42°C. In contrast, equivalent cell preparations from line Ea1 contained no detectable levels of p53 mRNA but contained normal levels of Rb message (Fig. 5A). Again, senescent Ea cells (characterized by very slow growth, large irregular cells, and accumulating dead cells) still expressed p53 mRNA (Fig. 5A, Ea aged).

Loss or mutation of p53 is correlated with immortalization of erythroblasts

In contrast to the fibroblast system, where only one cell line was obtained, two further immortal erythroblast cell lines were available for which the parent clonal cell strain could be analyzed (Ea2 and Eb1, earlier referred to as HD2 and HD4, respectively; Beug et al. 1982b). In addition, one further erythroblast line, for which no parent clone was available, was tested (Ec1; earlier, HD1). Finally, three separate mortal erythroblast clones that were generated at the same time as Ea and Eb, but had not been developed into cell lines, were included in the analysis. All tested clones expressed p53 mRNA of ap-

parently normal size at normal levels (Fig. 5; Table 1). In contrast, the immortal line Eb1 exhibited undetectable levels of p53 mRNA (verified by the use of poly(A)⁺ RNA; Table 1 and data not shown). The two immortal lines Ea2 and Ec1, however, did express p53 mRNA. The Ec1 line expressed abnormally high levels of a heavily truncated p53 RNA, as suggested by its reduced size (1 kb instead of 1.5 kb; Fig. 5B). The Ea2 line, on the other hand, expressed an apparently normal-sized p53 mRNA at levels similar to those in the Ea clone (Fig. 5B). Interestingly, the Ea2 line was derived from the same erythroblast clone (Ea) as Ea1 but was independently established by cultivation at 42°C (Fig. 1B) and, thus, by selection for cells that would grow rather than differentiate at the nonpermissive temperature.

Expression of a mutated p53 gene in line Ea2

To check whether expression of a normal-sized p53 mRNA by line Ea2 represents an exception to the apparent correlation between immortalization and loss or mutation of p53, we isolated parts of the p53 gene from both Ea and Ea2 by polymerase chain reaction (PCR) amplification of cDNA prepared from the respective RNAs and obtained DNA sequence (Fig. 6). A 586-bp fragment (en-

Figure 3. Comparison of transformed phenotype at 37°C and differentiation at 42°C of erythroblast clone Ea and its derivative cell line Ea1. Cells were shifted to 42°C for 4 days (Ea) and 5 days (Ea1) or kept at 37°C as described in Materials and methods. Photographs of cytospin preparations stained with neutral benzidine plus histological dyes and viewed under blue light (left parts of upper and lower panels) to reveal hemoglobin staining [see Materials and methods] or under green light (right parts of upper panels) to reveal histological details of the immature cells at 37°C are shown. Note similar appearance of both immature and mature cells from clone Ea and line Ea1.

compassing codons 90–286 of a total of 367) showed a single-base alteration from TGC at codon 224 in strain Ea to TCC at the same position in Ea2. This result was obtained in two independent experiments, thus ruling out a PCR artifact. The affected triplet encodes cysteine in the Ea cDNA and serine in the Ea2 cDNA. Comparison with the deduced amino acid sequences of mouse, human, *Xenopus*, and chicken p53 proteins showed that amino acid 224 is in a highly conserved region [region IV; Soussi et al. 1990] and is invariably cysteine. Sequencing of the three other highly conserved regions, II, III, and V, failed to show any other mutation [region I of Soussi et al. (1990) is not highly conserved in the chicken sequence]. We conclude that the immortal Ea2 line expresses p53 mRNA carrying a mutation in a highly conserved region of the protein, suggesting that this protein is either nonfunctional or altered in its function (see Discussion). The parental, mortal clone Ea does not carry this mutation. Attempts to detect trace amounts of the unmutated p53 RNA by sequencing pooled PCR products from Ea2 cells were unsuccessful, suggesting that the normal allele, if present, is inactive.

Loss or mutation of p53 is an early step in the immortalization process

During establishment of the Ea1 cell line, aliquots of cells had been frozen at different stages: senescence; outgrowth of labile cell populations during crisis; and early phases of cell line establishment. As shown in Figure 1B, clone Ea cultivated at 37°C underwent senescence after ~40 divisions, resulting in a drastic decrease in growth rate, the appearance of irregularly sized, large cells, and cell disintegration. At this stage (Fig. 1B), cells still expressed a normal-sized p53 mRNA (Fig. 5A; Ea aged). After 2 months of crisis, a very small proportion of the cells started outgrowing as clumps of healthy, small erythroblasts. Because these cell populations did not tol-

erate cloning and were lost easily during operations to purify them from dead cells (which constituted the majority of these cell populations), they were cultivated in several parallel dishes and aliquots were frozen down when sufficient cell numbers were reached. RNA samples prepared from two such line candidates (Lcs) that were frozen down within a month were probed for p53 expression (Fig. 1B, EaLc1 and EaLc2). As shown in Figure 5C, a truncated p53 mRNA was present in EaLc1 cells, whereas an apparently normal-sized p53 RNA was still present in EaLc2.

One month later, we had the first indications that a stable immortalized cell line was growing out. Part of that cell population (referred to as Ea1a) had been successfully infected with helper virus to prove its nonproducer nature (Graf 1973 and data not shown). These Ea1a cells represent an early stage of immortalization, as they were frozen 1 month before the Ea1 cell line was successfully cloned (see Fig. 1B). Northern blot analysis showed that Ea1a cells no longer expressed p53 mRNA (Fig. 5B). Early passages of the cloned immortal Ea1 cell line that were frozen in 1979, as well as samples that have been passaged for >100 generations in our laboratory invariably lacked p53 RNA but expressed Rb RNA at apparently normal levels (Fig. 5A and data not shown). Thus, the loss of p53 expression must have occurred somewhere during the crisis/early immortalization period.

Very similar results, although documented by fewer cell samples, were obtained during immortalization of the wild-type AEV-transformed erythroblast clone Eb. Whereas cells from the Eb clone still grew for >10 population doublings after thawing and expressed normal-sized p53 message, cells frozen during early stages of immortalization (EbLc1) already lacked p53 mRNA (Table 1).

Finally, our notion that loss and/or mutation of the p53 gene is an event early in cell line establishment was

Figure 4. Loss of p53 mRNA during immortalization of fibroblast clone Fa. (A) RNA was prepared from mortal (M) Fa cells and from immortal (I) Fa1 cells growing at 37°C (transformed phenotype) or 42°C (nontransformed phenotype; see Fig. 2). A single Northern blot of total RNA was probed sequentially with part of the chicken Rb gene cDNA, the chicken β -actin cDNA, and the p53 cDNA, as described in Materials and methods. The sizes of the appropriate bands for each probe are indicated by labeled arrowheads. The large Rb band is smeared downward in this preparation owing to degradation that affects this 4.6-kb RNA more than the smaller actin (2-kb) and p53 (1.5-kb) mRNAs. The presence of normal levels of full-length Rb RNA in the Fa1 line is corroborated in B. (B) RNA from two samples of senescent Fa cells (Fa aged; see Fig. 1A) compared with Fa1 RNA after probing with Rb or p53 probes.

corroborated by analyzing cell samples frozen during the immortalization of line Ec1. Growing cells of the mortal clone Ec were not available, but cell populations frozen early during immortalization (EcLc1 from November 1977 and EcLc2 from January 1978) already expressed a heavily truncated p53 message, as did the immortal Ec1 cell line analyzed from cells frozen 10 years later (Table 1).

Unfortunately, we were unable to obtain a continuously growing cell population from any of the above line candidates (EaLc1, EaLc2, EbLc1, EcLc1, EcLc2). A trivial explanation for this is that these slow-growing, highly unstable cell populations did not tolerate freezing and thawing, particularly because they had been stored in liquid nitrogen for >14 years. Because we had no problems growing the established lines Ea1, Ea2, Eb, and Ec, which had been frozen for a similarly long time, it is possible that loss of p53 represents an early step in immortalization of chicken erythroblasts that is necessary, but perhaps not sufficient, for full immortalization. Evidence for the notion that immortalization is a multistep process has recently been obtained in other systems (Wright et al. 1989; Strauss et al. 1990).

Loss of p53 expression during immortalization of v-myc-transformed macrophages

Immortalization is thought to be induced or accelerated by nuclear oncogenes (e.g., *myc*, *fos*, adenovirus E1A, SV40 large T) but not by cytoplasmic ones (e.g., the *src* and *erbB* oncogenes used here). We therefore tested whether and how easily immortal cell lines could be isolated from avian fibroblasts and macrophage clones

Figure 5. Changes in p53 mRNA during immortalization of erythroblast cells. (A) RNA was prepared from mortal (M) Ea cells and immortal (I) Ea1 cells grown at 37°C or 42°C, and also from senescent Ea cells (Ea aged) at 37°C. Northern blots were probed as described in Materials and methods. (B) Immortal erythroblast lines Ea2 and Ec1 (see text) show, respectively, a normal-sized p53 mRNA and a truncated p53 mRNA in the Northern blot assay. (C) EaLc1 and EaLc2, representing early outgrowths of senescent Ea cells, show, respectively, a truncated p53 mRNA and an apparently normal p53 mRNA.

Table 1. Expression of p53 and Rb mRNA in chicken cells during immortalization

Erythroblast strain or line	p53	Rb
Clone Eb	+	+
Clone Ed	+	+
Clone Ee	+	+
Clone Ef	+	+
EbLc1	-	+
Eb1	-	+
EcLc1	+ ^a	+
EcLc2	+ ^a	+

Expression of mRNAs excluding data presented in Fig. 5 and summarized in Fig. 1B.

^aA truncated p53 mRNA of ~1 kb was detected.

transformed by the v-*myc*-expressing MC29 virus [Graf 1973]. V-*myc*-transformed cells from a normal chicken fibroblast clone that exhibited an in vitro lifespan of >45 generations in culture (clone 1/77; Beug and Graf 1977) could not be developed into a cell line, although the senescent cells were kept in culture for >3 months. In contrast, using a pool of v-*myc* oncogene-transformed macrophage clones, a continuous macrophage cell line Mal (earlier referred to as HD11; Beug et al. 1982b; Leutz et al. 1984) could be grown after a senescence period of 1 month and a prolonged crisis period (2 months), suggesting that v-*myc*-transformed cells in the chicken are not easier to immortalize than cells transformed by cytoplasmic oncogenes. Although the mortal macrophages (Mal) expressed normal levels of p53, the immortal cell line Mal failed to do so (Fig. 7). This result could be verified by analysis of poly(A)⁺ RNA (data not shown). As expected, Mal cells frozen down shortly after immortalization also failed to express p53 mRNA (not shown). These results confirm our notion that loss (or mutation) of p53 is a consistent feature of the immortalization process in chicken cells, regardless of the transforming oncogene or the cell lineage of origin.

Discussion

Loss of wild-type p53 is a necessary early event in immortalization of chicken cells

Our results strongly suggest that in cultured chicken cells the expression of wild-type p53 is incompatible with cell immortality. Previous comparisons between cells at the end of the immortalization process and primary cells have noted frequent alterations of p53 gene expression in the immortal lines [e.g., Friend erythroleukemia cell lines; Munroe et al. 1990]. By following the progression to immortality of individual primary cell clones, we implicate the loss of normal p53 as a pivotal event in the immortalization process. Cloned transformed primary cells of fibroblast, erythroblast, and macrophage origin all expressed apparently normal p53 mRNA through the stages of normal proliferation and

Figure 6. A point mutation in a conserved region of the p53 protein has occurred in the immortal erythroblast line Ea2. A segment of the p53 mRNA was amplified directly from RNA of the parental clone Ea and its derivative immortal line Ea2, using the primers described in Materials and methods. Portions of the autoradiographs that were used to determine the sequence of the amplified segments in Ea and Ea2 are shown, together with the sequence surrounding codon 224 [boxed].

senescence. On the other hand, spontaneously immortalized cells that eventually arose from the primary cells in crisis all exhibited loss or alteration of p53 mRNA. The transition from crisis to immortality was investigated by analyzing four early (uncloned) outgrowing cell populations from three independent erythroblast cultures. One of these line candidates did not express p53 mRNA, and the other two showed a heavily truncated message. The results indicate that loss or mutation of normal p53 expression is a very early event in the evolution of immortal cell lines and may therefore be a critical step in the transition to immortality. Our results do not prove that the observed changes in p53 are sufficient in themselves to cause immortalization. To address this point, we are currently introducing the mutated p53 genes of Ea2 and Ec1 into transformed erythroblast clones by retroviral vectors.

The p53 gene product may have a dual function in oncogenesis [Levine et al. 1991]. On one hand, wild-type p53 functions as a tumor suppressor gene, acting as a negative regulator of cell proliferation. Thus, uncontrolled growth might ensue if p53 expression is abolished by deletion of the gene or inactivation of the protein. On the other hand, certain mutations in p53 seem to convert it into a dominant oncogene, leading to cell transformation in cooperation with cytoplasmic oncogenes (Eliyahu

Figure 7. Loss of p53 expression during progression from the macrophage cell clone Ma to the immortal cell line Ma1. Northern blots of RNA from each cell type were probed with Rb and p53 probes as described in Materials and methods.

et al. 1984; Hinds et al. 1989) and promoting immortalization of rodent fibroblasts (Jenkins et al. 1984; Rovinski and Benchimol 1988). Whereas a mutated, dominantly acting p53 gene is the most common alteration in mammalian cell lines and tumors (Levine et al. 1991), complete loss of p53 expression was observed most often in chicken cells. The reason for this difference may be that the chicken cells that we used already expressed an externally introduced transforming oncogene (*erbB* in erythroblasts, or *v-myc* in macrophages). In the presence of the transforming oncogene, simple loss of p53 expression might be sufficient for immortalization. Compatible with this hypothesis is the finding that one of the few erythroblast cell lines expressing a mutated p53 (line Ea2) was selected under conditions where the transforming oncogene was inactive (i.e., at the nonpermissive temperature). Thus, the Ea2 line probably emerged under a strong selective pressure not only to lose normal p53 but also to acquire a transforming oncogene (to replace the inactivated *ts-v-erbB*). A testable prediction is that the mutation at position 224 of the p53 gene contributes to both of these requirements. The same reasoning may explain the puzzling fact that line Ea1 (which was selected for its ability to differentiate at 42°C, i.e., for the absence of an oncogene other than *ts-v-erbB*) had lost p53, whereas a cell preparation frozen early during estab-

lishment of this line contained a truncated p53 (Figs. 1B and 5C). It is likely that immortalized cells originating from such cells were discarded upon cloning of the Ea1 line, because they failed to differentiate.

It should be noted that no such selective pressure should be operative in fibroblasts, which proliferate similarly in the presence or absence of a transforming *v-src* oncoprotein. This may explain why the p53 gene was deleted rather than mutated in the Fa1 cell line, which was also selected at 42°C, because the pre-immortal Fa cells exhibited a high growth rate and a long in vitro lifespan regardless of the presence or absence of a functional *v-src* oncoprotein (H. Beug and T. Graf, unpubl.; see Beug and Graf 1977). It is possible that the particularly long lifespan of the TaSp7 clone was the result of activation of an oncogene in the mortal ancestors of Fa1 prior to selection.

What is the mechanism of p53 mRNA loss during immortalization of our chicken cell lines? The simplest possibility is that a genetic alteration has rendered the gene nonfunctional, for example, by complete or partial deletion. Preliminary studies of the truncated mRNA in line Ea1 (Fig. 5) indicate that sequences encoding the amino-terminal half of the p53 protein are missing in this transcript. Whether this is the result of deletion in the gene or a mutation affecting splicing is not yet known. Most intriguing, however, are the majority of cell lines that lack p53 mRNA altogether. Preliminary examination of the p53 gene in the nonexpressing erythroid line Ea1 by Southern blots showed no evidence of gross rearrangement of the gene, although small deletions or point mutations would not have been detected (E. Ulrich, unpubl.). Another possibility is that the mechanism of gene inactivation is epigenetic. It is known that a variety of genes in cultured cells are inactivated by DNA methylation at CpG islands, which are normally constitutively methylation free (Antequera et al. 1990; Jones et al. 1990). Recently, it has been found that the CpG island at the Rb gene is sometimes methylated in retinoblastomas (Greger et al. 1989; Sakai et al. 1991). Southern blot analysis (E. Ulrich, unpubl.) shows that p53 sequences are nonmethylated in both mortal Ea cells and immortal Ea1 cells, making it less likely, though not impossible, that DNA methylation is involved.

Is loss of p53 a necessary step in immortalization? Although no exceptions have been found in our chicken cell lines from fibroblastic, erythroid, and myeloid lineages, the situation seems to be more complicated in avian lymphoid cells transformed by the *v-rel* oncogene, a mutated transcription factor related to NF-κB (Ghosh et al. 1990). Initially these cells were thought to represent instantaneously immortalized, very immature, pre-B/pre-T-like cells (Lewis et al. 1981). Recently, however, these cells were shown to frequently undergo senescence after a greatly prolonged in vitro lifespan of 60–80 population doublings when passaged as rather small cell populations (Morrison et al. 1991). When passaged as larger cell populations, some clones could be passaged to >150 doublings. In preliminary experiments, all of these

rel-transformed clones expressed an apparent unchanged p53 (E. Ulrich and G. Boehmelt, unpubl.). It is therefore tempting to speculate that *v-rel* replaces or cooperates with a recessive oncogene, leading to cellular changes such as the loss of p53.

Is immortalization a necessary step in oncogenesis?

The results presented here favor the view that immortalization and malignant transformation are separate, but interdependent, sets of mutational events. First, the fibroblast and erythroblast clones described were reversibly transformed at the outset (owing to the temperature sensitivity of the original oncogenes) but were nevertheless mortal. This was also true *in vivo*, because wild-type AEV was found to induce mortal erythroleukemia cells in chicks (Graf et al. 1977). Second, transformation could still be reversed after immortalization had occurred. At the nonpermissive temperature, erythroblasts differentiated to give erythrocytes, whereas fibroblasts reverted to a typical fibroblast morphology. By this and several other criteria, the behavior of these cell lines before (Ea and Fa) and after (Ea1 and Fa1) immortalization was similar or even identical (see Figs. 2 and 3). It is therefore tempting to see the escape from crisis, due at least in part to the loss of normal p53, as an event principally independent of transformation.

Are immortalization and transformation independent events in tumorigenesis? Attempts to answer this question must take into account that the frequency of immortalization of a given cell type varies widely in different animal species and is inversely correlated to the *in vitro* lifespan of primary cells. Thus, immortalization seems to be relatively frequent in mice and rats as well as certain birds (Japanese quail), whose primary cells exhibit short *in vitro* lifespans (10–20 generations). On the other hand, primate (including human) cells and chicken cells exhibit much longer lifespans and are difficult or even impossible to immortalize (Hayflick 1965; Macieira-Coelho 1976, 1980). For these findings to be relevant *in vivo*, it is necessary that immortalization be a common event during tumorigenesis in mice but a rare event in chickens and humans. This is exactly what is found. Most tumors isolated from mice, including leukemias, consist of cells that are already immortalized. Oncogene-transformed mouse or rat fibroblasts that are mortal have usually been found to become immortalized when they are recovered from a tumor (H. Land, pers. comm.). In contrast, we have been unable to detect immortalized cells in tumors and leukemias induced in chickens by various oncogene-containing retroviruses. Rather, these cells underwent senescence in culture after *in vitro* lifespans that were typical for chicken cells. Furthermore, the isolation of cell lines from the cultured tumor cells was as difficult as from *in vitro*-transformed cells and involved a clearly defined crisis period (Metz and Graf 1991; H. Beug and T. Graf, unpubl.). In humans, the situation is complicated by the fact that primary cells derived from most human tumors (e.g., carcinomas) are very reluctant to grow in culture, rendering it diffi-

cult to decide whether the cells that eventually grow out had to undergo a crisis before doing so. In spite of this limitation, a variety of human myeloid and lymphoid leukemias have been isolated from patients and shown to consist of nonimmortalized cells that undergo a typical crisis period before progressing (in some cases) to immortalized lines (H. Messner, pers. comm.). Therefore, we propose that in contrast to the prevailing dogma, immortalization is not a prerequisite for tumorigenesis but, rather, a separate set of mutational events (Wright et al. 1989; Strauss et al. 1990) that may or may not contribute to transformation. Our hypothesis does not question the established fact that the frequency of mutational events involved in immortalization is enhanced by a multitude of transforming oncogenes. Nor does it conflict with the assumption that the mutational events necessary (but not sufficient) for full immortalization represent important steps in tumorigenesis. One such step certainly could be the loss or mutation of p53, occurring in human tumors of many different kinds. Our result that *v-rel* transformation of chicken fibroblasts and lymphoid cells leads to a two- to threefold increase of *in vitro* lifespan without causing instantaneous immortalization may help to identify other events that contribute to immortalization.

Materials and methods

Cells and cell culture

The origins of the fibroblast clone TaSp7 (transformed with the Ta temperature-sensitive mutant of the Bryan high-titer strain of Rous sarcoma virus) has been described earlier (Beug et al. 1978). The erythroblast clones Ea (ts34A6), Eb (9D4), Ed (ts34NPB4), Ee (ts151AEVcl3), and Ef (ts186AEVcl5) were isolated according to standard procedures (Graf et al. 1978) between 1978 and 1980. The isolation of the cell lines Ea1 (HD3), Ea2 (HD2), Eb1 (HD4), and Ec1 (HD1) has been described earlier (Beug et al. 1982b). Further details of the origin of various erythroblast populations derived during the establishment of these lines are described in the text. The origin of the cell line Ma1 (HD11) from a pool of MC29 (RAV-2)-transformed macrophage clones (Graf 1973) has been described (Beug et al. 1979; Leutz et al. 1984).

Fibroblasts, macrophages, and erythroblast cell lines were cultivated in standard growth medium, a modification of Dulbecco's modified Eagle medium (Graf 1973) supplemented with 8% fetal bovine serum, 2% chicken serum, and 25 mM HEPES (pH 7.3). Erythroblast clones were grown in CFU-E medium (Radke et al. 1982) lacking anemic serum.

Isolation of the cell line Fa1

TaSp7 fibroblasts were passaged at 42°C (Beug and Graf 1977) until they were senescent. They were then seeded in three tissue culture flasks (250 ml) in 15 ml of standard growth medium and subjected to partial medium changes every second to third day. After ~8 weeks, small islands of small, healthy looking cells appeared in two of the three flasks (two islands in one flask and one in the second). During the next 4 weeks, these islands grew slowly, but only one could be propagated further; the other two underwent senescence after passage. This cell population

was subjected to cloning after passage for an additional 4 weeks, and the clones were screened for a temperature-sensitive phenotype. However, the clone initially selected (L3) became rapidly heterogenous and was therefore subjected to a second subcloning and screen for a bona fide temperature-sensitive phenotype. The cloned line that was finally selected (TaSp7 L3/17) displayed a fairly stable phenotype provided it was cultivated at 42°C for limited time periods (<2 weeks).

Transformation parameters

Analysis of fibroblast transformation parameters was done as described in Beug et al. (1978).

Erythroblast differentiation assay

Cells of the clone Ea and the cell line Ea1 were seeded in pH 8 medium [Zenke et al. 1988] supplemented with 3% high-titer anemic serum, insulin (1 µg/ml), and 15 µM (Ea) or 25 µM of the protein kinase inhibitor H7 [Glineur et al. 1990] and incubated at 42°C for 3–4 days (Ea) or 4–5 days (Ea1). Controls were kept at 37°C in the same medium lacking anemic serum. Cells were then cytocentrifuged onto slides, stained with histological dyes plus neutral benzidine, and photographed under blue light to reveal histochemical staining for hemoglobin, as described in Beug et al. (1982a).

Northern blot analysis

Cells (1×10^7 to 2×10^7 in the case of fibroblasts and macrophages; 2×10^7 to 2×10^8 in the case of erythroblasts) were lysed in guanidinium-rhodanide (4 M GITC, 25 mM sodium-citrate at pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) according to Chomczynski and Sacchi (1987). Total RNA (10 µg) was then separated on formaldehyde-agarose gels, blotted to GeneScreen membranes, and hybridized to the following probes: pBA13, which contains a *Hind*III fragment of the chicken β -actin cDNA clone [Kost et al. 1983]; pGdp53, which corresponds to the predicted coding sequence of chicken p53 cloned in pKCR3 [Soussi et al. 1988]; and pGdRb2, which contains the central 1.6-kb *Eco*RI fragment of the cDNA for chicken Rb. The Rb clone was isolated from a library of chicken embryo cDNA cloned in λ gt11 [Sap et al. 1986]. The 1.6-kb fragment is flanked at the 5' side by a 0.9-kb *Eco*RI fragment and at the 3' side by a 2.0-kb fragment. These three fragments encompass the Rb open reading frame [E. Ulrich, unpubl.]. Hybridization was at 65°C according to Church and Gilbert (1984), and filters were washed twice in $2 \times$ SSC and once in $0.1 \times$ SSC at 65°C. Blots were exposed on Kodak XAR5 film for 1–7 days.

Isolation of wild-type and mutant p53 by PCR

Primers were initially designed to amplify conserved regions II–V on a single fragment of 614 bp on the basis of the sequence of Soussi et al. (1988). The sequences of the primers that were complementary to p53 were GACTTCGACTTCCGGGTGGGGTTCGTGGAGG and TAGCCACGCCCCGGCCCCGC-CCCTCTTGCG. Flanking sequences provided *Eco*RI adaptors for cloning. Total RNA (10 µg) from clone Ea and line Ea2 was amplified using 1-min denaturation at 94°C and then 2 min at 55°C, followed by 3 min at 72°C. The resulting fragment was isolated from an agarose gel and cloned in the *Eco*RI site of pUC19. Sequencing of the amplified fragment by the chain termination method [Tabor and Richardson 1987] revealed a point

mutation in RNA from line Ea2. To corroborate this, primers flanking the putative mutation were constructed and additional PCR reactions were performed. The sequences of the primers that were complementary to p53 were ACCACGAGCGCT-GCGGGGGG and CCCTACTTGGCGCGGGGTA. The resulting fragment of 242 bp was either cloned and sequenced as described above or pooled PCR products were sequenced directly.

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