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Direct derivation of conditionally immortal cell lines from an *H-2K*^{b}-tsA58 transgenic mouse

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ABSTRACT **Studies on cell lines have greatly improved our understanding of many important biological questions. Generation of cell lines is facilitated by the introduction of immortalizing oncogenes into cell types of interest. One gene known to immortalize many different cell types *in vitro* encodes the simian virus 40 (SV40) large tumor (T) antigen (TAg). To circumvent the need for gene insertion *in vitro* to generate cell lines, we created transgenic mice harboring the SV40 TAg gene. Since previous studies have shown that TAg expression in transgenic mice is associated with tumorigenesis and aberrant development, we utilized a thermolabile TAg [from a SV40 strain, tsA58, temperature sensitive (ts) for transformation] to reduce the levels of functional TAg present *in vivo*. To direct expression to a broad range of tissues, we used the mouse major histocompatibility complex *H-2K*^{b} promoter, which is both widely active and can be further induced by interferons. tsA58 TAg mRNA was expressed in tissues of all animals harboring the hybrid construct. Development of all tissues was macroscopically normal except for thymus, which consistently showed hyperplasia. Fibroblast and cytokeratin$^+$ thymic epithelial cultures from these mice were readily established without undergoing crisis and were conditionally immortal in their growth; the degree of conditionality was correlated with the levels of tsA58 TAg detected. One strain of *H-2K*^{b}-tsA58 mice has been bred through several generations to homozygosity and transmits a functional copy of the transgene.**

Although the use of cell lines has been of central importance in the development of cellular and molecular biology, the limited number of available cell lines and the difficulty in obtaining new ones have impeded many areas of study. The increasing realization of the value of cell lines has been associated with a continual evolution in relevant technologies. Initially, cell lines were obtained only as tumor cells or as spontaneously immortalized variants of cells that grew readily in tissue culture (1). More recently, transfection and retroviral-mediated gene insertion of immortalizing genes have been used to facilitate the production of cell lines from various tissues (2–11). However, transfection requires a large number of target cells to ensure that some cells of interest stably integrate the chosen DNA in a position suitable for expression. Viral-mediated gene transfer can be carried out with fewer cells by cocultivation of target cells with virus-producing feeder layers; however, this method still requires that target cells are dividing to achieve integration of the selected DNA into the genome (2). Moreover, both of these technologies require the growth of cells for extended periods of time in culture, under selective pressure, to obtain sufficient numbers of cells expressing the immortalizing gene to allow experimentation. In addition, lines from putatively identical cells have different sites of gene integration and often express markedly different behaviors and levels of expression of the immortalizing gene.

An additional problem associated with the introduction of immortalizing genes into cells is that these genes can alter normal cellular physiology (1, 12), a problem that is also relevant to the isolation of cell lines from transgenic animals (e.g., refs. 28–30, 34, 35). This problem theoretically can be overcome through the use of conditional immortalizing genes, which allow the generation of continuously proliferating cell lines capable of differentiation after inactivation of the immortalizing gene. For example, the simian virus 40 (SV40) mutant temperature-sensitive (ts) strain tsA58, which encodes a thermolabile large tumor (T) antigen (TAg) capable of immortalization only at the permissive temperatures, has been used in the generation of a variety of conditionally immortal cell lines (13–17). However, introduction of conditional immortalizing genes *in vitro* still suffers from the problems discussed above for transfection and infection of wild-type genes.

To overcome some of the difficulties in the generation of cell lines, an approach was developed that facilitates and ensures the presence of a conditional oncogene in all of the cells of interest at a common integration site. Thus, transgenic mice were generated that harbor SV40 strain tsA58 early region coding sequences under the control of the mouse major histocompatibility complex *H-2K*^{b} class I promoter (18–21). This promoter is active at various levels in different tissues of the body but can be induced to higher levels of expression in almost all cells by exposure of the cells to interferons (IFNs) (21–23). Skin fibroblast cultures derived from these mice were conditional in their growth, as has been demonstrated for rat embryo fibroblasts immortalized by infection with a recombinant retrovirus that transduces the tsA58 TAg (15). Work with transfection and viral-mediated gene insertion has consistently indicated that techniques developed through the use of fibroblast populations can be transferred readily to other cell systems. This is also the case with the cells obtained from these transgenic mice, and cytokeratin$^+$ thymic epithelial cell lines that were also established readily from these animals.

MATERIALS AND METHODS

Construction of the Transgene. The 5′ flanking promoter sequences and the transcriptional initiation site of the mouse *H-2K*^{b} class1 gene were fused to the SV40 tsA58 early region coding sequences. The 4.2-kilobase (kb) *Eco*RI–*Nru* I fragment encompassing the *H-2K*^{b} promoter sequences was ligated to the 2.7-kb *Bgl* I–*Bam*HI fragment derived from the tsA58 early region gene and pUC19 double-digested with

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Abbreviations: TAg, large tumor (T) antigen; ts, temperature sensitive; IFN, interferon; mAb, monoclonal antibody; SV40, simian virus 40.

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*Eco*RI and *Bam*HI. The Bgl I site was blunted by using the Klenow fragment of *Escherichia coli* DNA polymerase I to allow fusion to the *Nru* I site. For microinjection, the *H-2K*^{b}-tsA58 DNA fragment was isolated free of vector sequences by digestion with *Eco*RI and *Sal* I (24). All DNA manipulations were carried out by standard procedures (25).

RNA Blot-Hybridization (Northern) Analysis. RNA was prepared and analyzed by hybridization to a ^{32}P-labeled SV40 early region fragment using standard procedures (25, 26).

Cloning and Proliferation Assays. Skin fibroblasts were prepared as described (27) and grown in Dulbecco's modified Eagle's medium supplemented with 100 units of penicillin, streptomycin, and recombinant murine γ interferon (IFN-γ, Genzyme) per ml. For colony assays, 10^3 cells derived from cultures grown at 33°C in the presence of IFN-γ were replated in 6-cm tissue culture dishes in the absence of IFN-γ at 33°C to allow adherence under identical conditions. Growth conditions were changed after 24 hr to indicated conditions (Fig. 2). Cultures were refed twice weekly for 14 days and stained with 2% methylene blue; colonies then were counted blind. For proliferation assays, 10^4 cells were similarly plated, and dishes were analyzed after 7 and 14 days. A single dish was also counted on day 1 to determine the number of adhering cells. All determinations were carried out in duplicate.

Immunoblot (Western Blot) Analysis. Preparation of protein extracts and their analysis with mAb PAb419, directed against TAg, were performed by standard procedures (26).

Immunofluorescence Analysis of Thymic Epithelial Cells. Cells grown on poly(L-lysine)-coated coverslips were stained with an antibody specific for keratin 8 (LE41; ref. 32) or an anti-TAg mAb, PAb412 (31).

RESULTS

Generation of *H-2K*^{b}-tsA58 Transgenic Mice. A hybrid construct containing the *H-2K*^{b} 5′ promoter sequences fused to the tsA58 early region gene, which encodes both TAg and the small tumor antigen (Fig. 1 *Upper*), was microinjected into fertilized oocytes from (CBA/Ca × C57BL/10) F_1 mice. After reimplantation, 88 mice were born, of which 34 carried one to five copies of the gene. RNA from a variety of tissues from one nontransgenic and three transgenic animals was analyzed by Northern blot analysis with an SV40 early region-specific probe (Fig. 1 *Lower*). RNA extracted from tissues of transgenic mice contained various amounts of a 2.5-kb RNA species, while no tsA58 TAg RNA was detected in tissues of the nontransgenic mouse; thymus and liver showed the highest level of expression, while brain showed the lowest.

Fibroblasts Derived from *H-2K*^{b}-tsA58 Transgenic Mice Are Conditionally Immortal. Skin fibroblasts from normal and founder transgenic animals 2–10 weeks old were placed in culture at 33°C, the permissive temperature for the tsA58 TAg, in the presence of IFN-γ (to increase expression from the *H-2K*^{b} promoter; refs. 21–23). Fibroblasts derived from nontransgenic mice stopped dividing *in vitro* within a small number of passages. This cessation of division, which has been termed both "senescence" and "crisis," occurs reproducibly in fibroblasts that do not express immortalizing genes. In contrast, fibroblasts derived from most transgenic mice continued to grow for as long as the cultures were maintained under appropriate conditions (see below).

Detailed analysis of skin fibroblast cultures for conditionality of growth revealed three families of cultures, depending upon the ability of cells to grow in fully permissive, semipermissive, and nonpermissive conditions. Permissive conditions were defined as growth at 33°C in the presence of IFN-γ; semipermissive conditions, growth at 33°C in the absence of IFN-γ or 39.5°C in the presence of IFN-γ; and nonpermissive conditions, growth at 39.5°C in the absence of IFN-γ (Fig. 2).

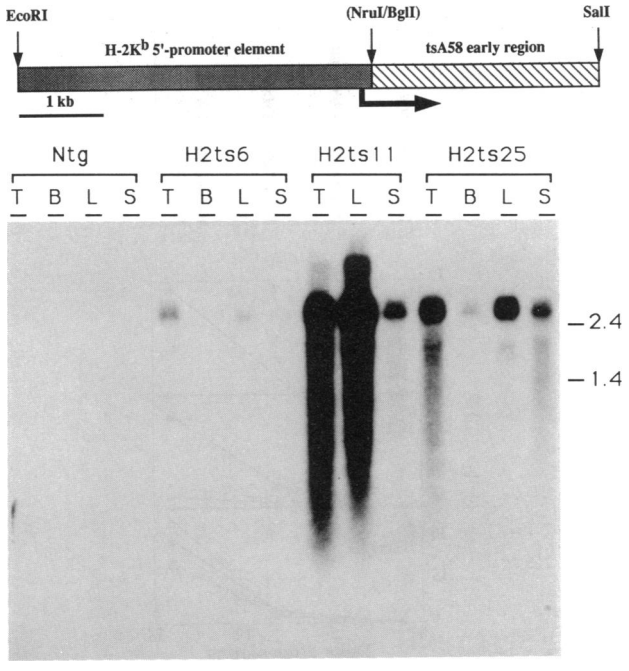

FIG. 1. (*Upper*) Schematic representation of the *H-2K*^{b}-tsA58 fragment. Size in kb is indicated. (*Lower*) Northern blot analysis shows TAg mRNA at various levels in thymus (lanes T), brain (lanes B), liver (lanes L), and skin (lanes S) of different *H-2K*^{b}-tsA58 transgenic mice. Loading of RNA was checked by hybridization of the same filter with an actin probe (not shown). Size in kb is indicated on the right. Ntg, nontransgenic.

In the first family of cultures, growth was fully conditional and only occurred under permissive conditions. If cells were grown at 39.5°C and/or were grown in the absence of IFN-γ, cell division did not occur either in standard growth assays or in colony-forming assays (Fig. 2). These fibroblasts thus behaved as expected from previous studies in which rat embryo fibroblasts were conditionally immortalized with tsA58 TAg by retroviral infection (15). All cultures derived from different individuals within this strain yielded identical results.

In a second family of cultures, optimal growth was obtained under fully permissive conditions, a lesser degree of growth was seen under semipermissive conditions, and no growth occurred under nonpermissive conditions. In the third family, cell growth did not completely cease even under nonpermissive conditions, although the best growth was seen under fully permissive conditions.

The conditionality of growth seen in fibroblasts derived from transgenic animals was correlated with the levels of tsA58 TAg (Fig. 2*e*). In all cultures, the level of tsA58 TAg was reduced by temperature increase and/or by removal of IFN-γ. Interestingly, when the most conditional cultures (those derived from progeny of mouse H2ts6) were grown at 33°C in the absence of IFN-γ, a condition where these cells did not grow, low levels of TAg were still detected (Fig. 2*e*).

Thymic Hyperplasia in *H-2K*^{b}-tsA58 Transgenic Mice. Enlarged thymuses occurred in all transgenic animals, a tissue-specific hyperplasia that previously has been observed in transgenic mice harboring wild-type TAg (33, 34); the time of onset of hyperplasia (2–20 weeks) was correlated with the levels of TAg mRNA (see Fig. 1 *Lower*). Despite the thymic enlargement, there was no evidence for malignant transformation of this tissue as judged by the following criteria: both lobes of the thymus were equally enlarged in all animals examined, and histological and immunohistochemical examination revealed

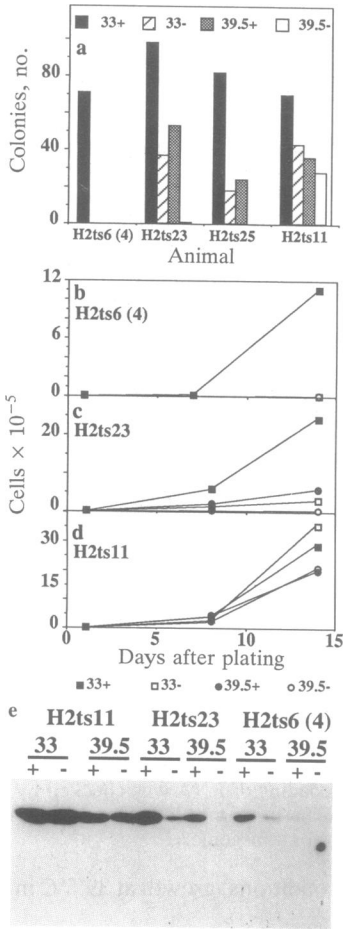

FIG. 2. (*a–d*) Cloning and proliferation analysis of skin fibroblasts from *H-2K*^{b}-tsA58 transgenic mice reveals three families of cells. In family 1 (mouse H2ts6-4), cloning and proliferation are fully conditional and only occur when cells are grown at 33°C in the presence of IFN-γ (33+) (*a* and *b*). In family 2 (mice H2ts23 and H2ts25), optimal results were obtained when cells were grown at 33°C in the presence of IFN-γ (33+), no growth occurred at 39.5°C in the absence of IFN-γ (39.5−), and intermediate levels of growth were seen in the semipermissive conditions of 33°C, IFN-γ$^-$ (33−) or 39.5°C, IFN-γ$^+$ (39.5+) (*a* and *c*). In family 3 (mouse H2ts11), growth occurred in all conditions but was most vigorous at 33°C in the presence of IFN-γ (*a* and *d*). A reduced cloning efficiency and rate of cell growth was seen in semipermissive conditions, and a still greater reduction was seen in fully nonpermissive conditions. (*e*) Western blot analysis of skin fibroblasts shows that the levels of TAg are correlated with the conditionality of *in vitro* growth. The most conditional cells (derived from progeny of H2ts6) contained the lowest levels of TAg, and the least conditional cells (derived from H2ts11) showed the highest levels of TAg. In all cases, the level of TAg present increased upon addition of IFN-γ to the cultures and decreased upon shift to 39.5°C.

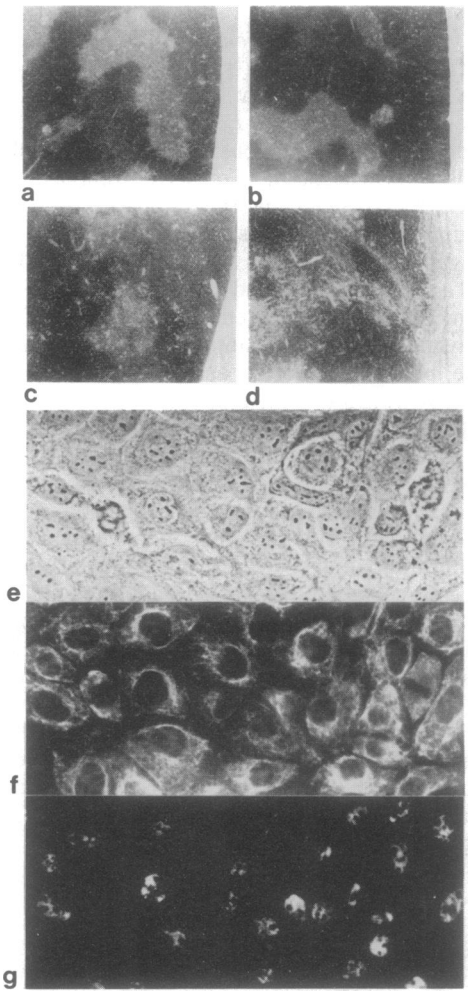

FIG. 3. (*a–d*) Histological analysis of thymic tissues from a nontransgenic mouse (*a*) and from H2ts6 mice 2 months (*b*), 4 months (*c*), and 6 months (*d*) old. The thymuses of H2ts6 mice up to 2 months old appeared to be identical to those of normal mice (*a* and *b*), exhibiting normal ratios between cortex (darkly stained tissue) and medulla (lightly stained tissue). The thymic architecture in 4-month-old mice (*c*) showed signs of disruption, while areas with cortical or medullary characteristics were still maintained. In thymic tissue of 6-month-old H2ts6 mice, extensive lightly staining areas were evident even in subcapsulary regions. However, even in these organs a clear demarcation between "cortical" and "medullary" areas was still maintained. (*e–g*) Immunofluorescent staining of thymic epithelial cells. Cells from an adherent cell line (7P) derived from the thymus of H2ts23 were photographed under: phase optics, showing flattened cells with tightly apposed borders (*e*); optics with fluorescein isothiocyanate, showing filamentous cytoplasmic staining characteristic of keratins (*f*); and optics with rhodamine isothiocyanate, indicating the presence and nuclear localization of TAg in virtually all cells (*g*). (×200.)

extensive growth of epithelial cells and the presence of apparently normal thymocyte populations, as determined by fluorometric cytometry (not shown). In addition, demarcation between cortical and medullary regions was still maintained even after prolonged hyperplastic growth (Fig. 3). Moreover, dissociated cells obtained from enlarged thymuses did not yield tumors in syngeneic recipients even when 10^7 cells were injected s.c. or i.p., and recipient animals were sacrificed after 3 months (unpublished observations). Finally, analysis of T-cell receptor β chain gene rearrangements by Southern blot of DNA from enlarged thymuses suggested polyclonal expansion of thymocyte populations (Y.T., unpublished observations), in contrast to the oligoclonal expansion observed in mice that harbor a hybrid *Thy-1-myc* gene (35). As it is possible that the large number of highly proliferative and hyperplastic cells in the thymus represents a target in which secondary cooperating mutations might occur, we cannot exclude the possibility that a very small number of cells within the hyperplastic thymus have undergone transformation.

In heterozygous progeny of one mouse (H2ts6), the thymus displayed normal development for extended periods, with the first histological appearance of hyperplasia seen at 4 months (Fig. 3). Homozygote offspring of H2ts6 developed thymic hyperplasia earlier (unpublished observations), in agreement with the view that the time of onset of this abnormality is correlated with TAg levels. Thymic hyperplasia was occasionally seen in conjunction with enlargement of peripheral lymphoid organs (spleen, lymph nodes), but these tissues

maintained their normal histological architecture. Macroscopic evidence of liver abnormalities was seen only in one animal even though levels of transgene expression in the liver were comparable to those in the thymus (Fig. 1 *Lower*).

Conditionally Immortal Lines of Cytokeratin$^+$ Thymic Epithelial Cells. Thymuses of transgenic mice readily yielded conditionally immortal cultures containing cells of both epithelial and fibroblastic morphologies, both of which could be readily cloned. Clones that exhibited epithelial-like morphologies expressed cytokeratin (Fig. 3). Both cytokeratin$^+$ and cytokeratin$^-$ clones showed conditional growth. Cells grew optimally in fully permissive conditions and did not grow in nonpermissive conditions (Fig. 4). Thus, we were able to readily derive conditionally immortal lines of epithelial cells and of fibroblasts from these mice.

Dose Dependence of Skin Fibroblasts Derived from H2ts6 to IFN-γ. The establishment of a colony of *H-2K^b*-tsA58 transgenic mice has allowed us to begin using these animals to study more detailed aspects of TAg function. In particular, observations that fibroblasts derived from progeny of H2ts6 showed a relatively low level of TAg expression at 33°C in the presence or absence of IFN-γ (although expression was clearly higher in the presence of IFN-γ; Fig. 2*e*) suggested that with this animal it might be possible to observe dramatic alterations in cell growth as a result of small changes in the level of this gene product.

Fibroblasts derived from progeny of H2ts6 mice showed promotion of cell growth by IFN-γ at levels as low as 1 unit/ml (Fig. 5). Analysis by colony formation and by cell number analysis showed that addition of IFN-γ at 100 units/ml to these cultures only increased the frequency of colony formation 3.5-fold in comparison with that seen in the presence of IFN-γ at 1 unit/ml and was only 40% increased over that achieved with IFN-γ at 10 units/ml. The difference in TAg levels at the different doses of IFN-γ was not large, with 1 unit/ml causing a 2.5-fold increase over basal levels of TAg and 100 units/ml causing an ≈6-fold increase over basal levels of TAg.

DISCUSSION

We have generated transgenic mice that have stably integrated the SV40 mutant strain tsA58 thermolabile TAg gene, a conditional immortalizing gene, under the control of the inducible 5′ flanking promoter of the mouse *H-2K^b* gene. The tsA58 TAg gene product is functional at the permissive temperature of 33°C but is rapidly degraded at the nonpermissive temperature of 39.5°C (13, 15). The *H-2K^b* promoter is active in a wide variety of tissues at various levels (18–21), and expression can be increased above basal levels in most cells by exposure to IFN (21–23). Fibroblasts and thymic stromal cells derived from the *H-2K^b*-tsA58 transgenic mice showed conditional proliferation that could be modulated both with temperature and by application of IFN-γ; cells from all mice grew optimally at 33°C in the presence of IFN-γ. Founder animal H2ts6, whose progeny yielded fibroblast cultures whose growth in cloning assays was completely dependent upon the permissive temperature and the presence of IFN-γ, has bred successfully to homozygosity to yield a strain of *H-2K^b*-tsA58 transgenic mice.

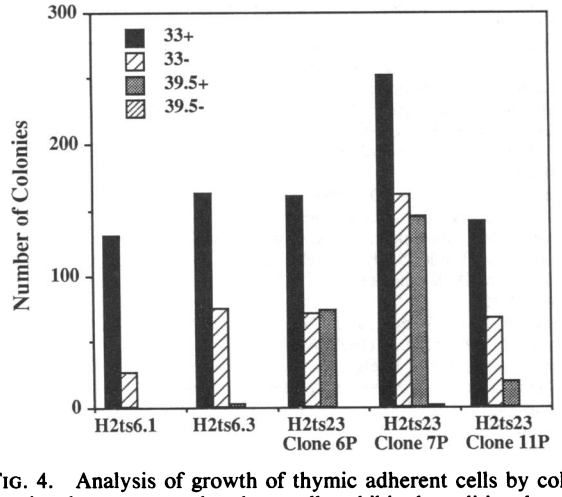

FIG. 4. Analysis of growth of thymic adherent cells by colony formation demonstrates that these cells exhibited conditional growth *in vitro*. Optimal growth occurred under the fully permissive condition of 33°C, IFN-γ^+ (33+) in all cases, and no colony formation occurred in nonpermissive conditions. Cultures of cells from H2ts6.1 and H2ts6.3 were derived from the thymuses of two separate progeny of founder mouse 6, and cultures of H2ts23 clones 6P, 7P, and 11P are three separate clonal cultures derived from the thymus cells of animal H2ts23; the clones 6P and 7P were morphologically epithelial, whereas clone 11P was fibroblastic. 33+, 33°C and IFN$^+$; 33−, 33°C and IFN-γ^-; 39.5+, 39.5°C and IFN-γ^+; 39.5−, 39.5°C and IFN-γ^-.

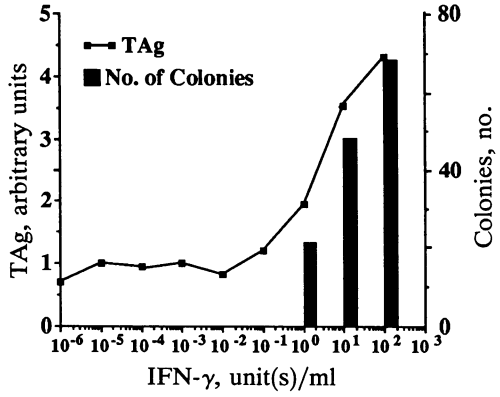

FIG. 5. Maintenance of growth of H2ts6-derived fibroblasts requires low levels of IFN-γ. No colonies were obtained in the absence of IFN-γ, but the presence of as little as 1 unit of IFN-γ per ml was sufficient to allow colony formation. Determination of the level of TAg by Western blot analysis coupled with densitometry showed that the increase in colony formation was associated with no more than a 2.5-fold increase in the level of TAg.

The assay system used to examine conditionality of immortalization was based on results of previous studies in which tsA58 TAg was introduced into fibroblasts by retroviral infection (15). These conditionally immortalized fibroblasts grew indefinitely when maintained at 33°C but rapidly ceased proliferation when switched to 39.5°C. Cells derived from *H-2K^b*-tsA58 transgenic mice behaved similarly. Skin cells from these mice grown *in vitro* at 33°C in the presence of IFN-γ readily yielded fibroblast cultures from all transgenic animals. Shift to semipermissive conditions of growth (i.e., 33°C/IFN-γ^- or 39.5°C/IFN-γ^+) was sufficient to eliminate growth of cells derived from the H2ts6 strain of mice. In all other cases, shift to semipermissive conditions was associated with a reduction in cell growth but not a cessation of growth. Cultures from almost all animals ceased growth when shifted to nonpermissive conditions—i.e., 39.5°C, IFN-γ^-. Moreover, in the cultures (family 3 in Fig. 3) in which growth occurred after temperature increase to 39.5°C in the absence of IFN-γ, this growth was still less vigorous than that seen in semipermissive conditions. It should be noted that all cultures established from the same founder mouse, or strain of mice, exhibited identical characteristics.

Determination of the amount of TAg present in different cultures by Western blot analysis showed a direct correlation between the amount of TAg present and the growth potential of the cells. Cells in which only small amounts of TAg were produced showed stringent growth regulation, while cultures expressing high levels of TAg showed poor growth regulation. It was also clear that only small increases of TAg were needed to maintain immortalization, in that we saw only a

2.5-fold difference in levels of TAg between untreated cultures and those grown in the presence of IFN-γ at 1 unit/ml, yet only the cultures receiving the IFN-γ were able to generate colonies in a limiting dilution assay.

Conditional immortalization and the ability to readily generate rapidly growing cultures were also seen with cells derived from thymuses of transgenic mice. As with skin fibroblasts, optimal growth of the thymic cultures occurred at 33°C in the presence of IFN-γ, was reduced in semipermissive conditions, and was reduced still further in nonpermissive conditions. Interestingly, thymic cells derived from H2ts6 animals did not grow at 39.5°C in the presence of IFN-γ but did grow at 33°C in the absence of IFN-γ. This pattern of growth may reflect a higher constitutive level of transcription from the *H-2K*^{b} promoter in the thymic cells and/or a greater sensitivity of thymic cells to the action of TAg as compared with fibroblasts. The probable relevance of the first explanation is supported by observations that *in vivo* expression of the transgene in the thymus was generally higher than in other organs, while the relevance of the second explanation is supported by observations that the liver—the one organ in which transgene expression was similar to that of the thymus—rarely showed abnormal growth. The different effects of the transgene on thymus and liver *in vivo* suggest that cell types can differ in their susceptibility to the action of TAg.

Long-term survival of the transgenic mice was correlated with the level of conditionality of growth of the *in vitro* cultures. The only visible cause of physical distress found repeatedly was thymic enlargement. This enlargement seemed to represent hyperplastic growth rather than malignancy because thymic histology, T-cell repertoire, and T-cell clonality were all normal, and cells derived from enlarged thymuses did not generate tumors in syngeneic recipients. Although all populations of the thymus were expanded *in vivo*, only adherent cell cultures were readily obtained in long-term culture, in contrast to cells derived from *Thy-1-myc* mice (35). The generalized hyperplasia of thymic populations we have observed is similar to that seen in transgenic mice when wild-type TAg gene expression was regulated by its own early region promoter (35) or by the promoter from growth hormone-releasing factor gene (34). It differs from the hyperplasia observed in mice where the *H-2K*^{b} promoter was used to drive expression of the *fos* oncogene in which expansion of the epithelial component, but not of the lymphoid component, was seen (36).

Although all animals eventually succumbed to thymic hyperplasia, thus indicating that the transgene was not fully inactivated *in vivo*, the H2ts6 heterozygotes survived to the age of 6 months and homozygotes survived to the age of 3 months. Both heterozygotes and homozygotes breed normally in brother/sister matings.

The presence of a viable strain of transgenic mice harboring the *H-2K*^{b}-tsA58 transgene will allow us to determine whether this approach to cell line production is applicable to tissues—including embryonic tissues—other than skin and thymus. As SV40 TAg can immortalize a wide range of cell types (4, 5, 7, 9, 11, 15–17) and IFN induces the expression of class I genes in a variety of tissues (21–23), the *H-2k*^{b}-tsA58 transgenic mice may allow direct derivation of cell lines from a wide variety of different tissues and cell types. Moreover, the ability to remove the immortalizing function of the tsA58 TAg in cells derived from these transgenic mice by temperature shift up may allow us to generate cell lines that are not only conditional in their growth but also may be capable of differentiating into different types of end-stage cells (see, e.g., refs. 4 and 16). Finally, as cells prepared from these transgenic mice are genetically homogeneous, can be prepared in large numbers, and can be synchronously exposed to interferon *in vitro*, these cells will allow study of the acute effects of SV40 TAg expression on division and differentiation in the absence of extensive *in vitro* growth and application of drug selection.

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