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Elevation of Cell Cycle Control Proteins During Spontaneous Immortalization of Human Keratinocytes

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A human line of *s*pontaneously *i*mmortalized *k*eratinocytes (SIK cells) has been derived from ostensibly normal epidermis and has proven useful in dissecting molecular changes associated with immortalization. The original cultures had a normal karyotype and a colony forming efficiency of ~3% through 10 passages. At passage 15, after which normal strains ordinarily senesce, these cells continued vigorous growth and gradually increased in colony forming efficiency, stabilizing at ~30% by passage 40. During the early stage of increasing colony forming efficiency, the cells acquired a single i(6p) chromosomal aberration and 5- to 10-fold increases in expression of the cell-cycle control proteins cyclin A, cyclin B, and $p34^{cdc2}$. Additional chromosomal aberrations accumulated at later passages (i(8q) and +7), but the i(6p) and the increased expression of cell-cycle proteins were maintained, raising the possibility that these features were important for immortalization. Regulation of cell growth and differentiation in the cultures appeared minimally altered compared with normal keratinocytes as judged by their microscopic appearance and generation of abortive colonies, sensitivity to growth suppression by transforming growth factor-β and tetradecanoylphorbol acetate, and dependence upon epidermal growth factor for progressive growth.

INTRODUCTION

Spontaneous immortalization of rodent cells has been observed routinely since the original derivation of 3T3 fibroblasts from mouse embryo by serial passage (Todaro and Green, 1963). This process also occurs in mouse epidermal cells (Yuspa *et al.*, 1980) and in keratinocytes and other epithelial cells of the rat (Heimann and Rice, 1983; Phillips and Rice, 1983). In rodent lines, immortalization presumably occurs as a consequence of spontaneous DNA damage that ultimately leads to visible chromosomal aberrations. The damage can start with point mutations, as observed in the Ha-*ras* gene of mouse keratinocyte primary cultures (Greenhalgh *et al.*, 1989).

In comparison with rodent cells, spontaneous immortalization of human epidermal cells in culture is a rare event, with only two cases having been reported (Baden *et al.*, 1987; Boukamp *et al.*, 1988). This species difference could reflect relatively low levels of DNA damage occurring in the original human tissue or upon cultivation. Alternatively, human cells may control cell proliferation and cell cycle events more rigorously than do rodent cells. It has been shown recently that cell cycle checkpoints, which normally prevent the onset of mitosis in the absence of a completely replicated genome and prevent exit from mitosis in the absence of a properly formed mitotic spindle, can be bypassed readily in certain rodent cells but not in human cells (e.g., Kung *et al.*, 1990; Yamashita *et al.*, 1990; Schlegel and Craig, 1991; Steinmann *et al.*, 1991). Compromised cell cycle control may be achieved more readily in rodent cells and could contribute to a loss of genetic stability and to an increase in spontaneous immortalization.

The cell cycle control proteins $p34^{cdc2}$, cyclin A, and cyclin B are required for the successful initiation of DNA replication and mitosis. Cyclin B and $p34^{cdc2}$ are, respectively, the regulatory and catalytic subunits of the

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histone H1 kinase commonly referred to as maturation promoting factor (Nurse, 1990). The activity of maturation promoting factor is controlled by the phosphorylation state of $p34^{cdc2}$ and its association with cyclin B (see Nishimoto *et al.*, 1992 for review). This kinase is essential for entry of mammalian cells into mitosis, as shown by antibody microinjection experiments (Riabowol *et al.*, 1989) and by a mouse cell line containing a temperature-sensitive *cdc2* gene product (Th'ng *et al.*, 1990). Evidence that $p34^{cdc2}$ also has an important role in the initiation of DNA replication includes the findings that antisense oligonucleotides to $p34^{cdc2}$ inhibit DNA synthesis in human T lymphocytes (Furukawa *et al.*, 1990), that the cyclin A/$p34^{cdc2}$ complex is sufficient to initiate SV40 DNA synthesis in vitro (D'Urso *et al.*, 1990), and that cyclin A/$p34^{cdc2}$ becomes activated as a kinase near the G_1/S boundary in human cells (Marraccino *et al.*, 1992). Cyclin A, which complexes with and activates $p34^{cdc2}$ and the related cyclin-dependent kinase cdk2 (Roy *et al.*, 1991; Marraccino *et al.*, 1992; Pagano *et al.*, 1992), has been shown by antibody microinjection and antisense studies to be required for the onset of both DNA replication and mitosis (Girard *et al.*, 1991; Pagano *et al.*, 1992). The present work raises the possibility that persistent elevation of such regulatory proteins can play a key role in immortalization of human keratinocytes.

MATERIALS AND METHODS

Cell Derivation and Culture

Cultures were derived from a sample of normal human foreskin obtained from the circumcision clinic of the Brigham and Women's Hospital (Boston, MA). After removal of subcutaneous tissue by dissection, the sample was disaggregated with trypsin, and released cells were propagated in the presence of lethally irradiated or mitomycin C-treated mouse 3T3 cells by standard techniques (Allen-Hoffman and Rheinwald, 1984). Cultures were passaged at weekly intervals at a dilution of 1:20. At passage 26, the cultures were noted to be contaminated by *Alcaligenes fecalis* (identified by the Diagnostic Microbiology Service of the University of California, Davis). The bacteria were eliminated without affecting keratinocyte growth by addition of tetracycline (50 μg/ml) and ceftizoxime (100 μg/ml) to the medium during passage 27. Cells at passage 35 tested negative for mycoplasma and bovine diarrhea virus (Diagnostic Microbiology Service, University of California, Davis). DNA prepared from passage 37 tested negative for E6/E7 immortalizing gene sequences of human papillomavirus types 16 and 18 (courtesy of Dr. Richard Schlegel).

The medium, changed twice weekly, was a mixture of Dulbecco's modified Eagle's and Ham's F-12 (3:1) supplemented with 5% fetal bovine serum, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 20 pM T_3, 0.18 mM adenine, 10 ng/ml cholera toxin, and 10 ng/ml epidermal growth factor (EGF). Colony forming efficiencies were measured in triplicate cultures containing 30–100 colonies each. Estimates of population doublings/passage were made based on these values, e.g., 9–10 up to passage 10 and 6–7 after passage 40. Human epidermal cells immortalized by papillomavirus E6/E7 genes (Barbosa and Schlegel, 1989) were cultured with 3T3 feeder layer support (as above) in this medium. In experiments without the 3T3 feeder layer, cells were grown in low calcium (0.15 mM Ca^{++}) serum free medium supplemented with EGF and pituitary extract (KGM; Clonetics, San Diego, CA) and, starting 3 d after inoculation, with transforming growth factor-β (TGF-β) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). EGF was purchased from Biomedical Technologies (Stoughton, MA), TPA from the Sigma Chemical (St. Louis, MO), and human TGF-β1 from RD Systems (Minneapolis, MN). Cell numbers were quantitated by electronic counting.

Chromosomal Analysis

Cultures in log phase growth were arrested in metaphase with 50 ng/ml colcemid, trypsinized, resuspended in hypotonic 75 mM KCl solution, treated with Carnoy's fixative, and air dried on glass slides. After aging for several weeks, the slides were treated briefly with trypsin and then stained with Giemsa (Seabright, 1971). In each sample, the chromosome identities and aberrations were determined in well-spread G-banded metaphases.

Immunoblotting

Spontaneously immortalized keratinocytes (SIK) cells were passaged and 3T3 feeder layers removed (as above) for cell cycle analysis. Adherent keratinocytes were solubilized for immunoblotting by heating at 100°C for 3 min in 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, 2% sodium dodecyl sulfate, and 10 mM dithiothreitol. Samples were adjusted to 5% in 2-mercaptoethanol and 10% in glycerol and submitted to denaturing polyacrylamide gel electrophoresis (Laemmli, 1970). Each lane contained 100 μg of protein as estimated by a modified Lowry protein assay (Peterson, 1977). After electrophoresis, proteins were transferred to nitrocellulose or immobilon-p in a tank blotting system.

Immunoblotting was performed with antiserum raised against the C-terminal peptide (LDNQIKKM) of the human $p34^{cdc2}$ protein (Lee and Nurse, 1987) or with human cyclin A or B antisera (Pines and Hunter, 1989). For $p34^{cdc2}$ immunoblotting (Gautier *et al.*, 1988), the concentration of dry milk in buffers was 5% and the concentration of ^{125}I-protein A was 0.75 μCi/ml. Immunoblotting of cyclins A and B (Pines and Hunter, 1989) was conducted with 0.5 μCi of ^{125}I-protein A/ml for detection of immune complexes, and 0.5% Nonidet-40 was added to the final three washes to reduce background. ^{125}I-labeled proteins were detected by indirect autoradiography with an intensifying screen and were quantitated on autoradiograms with a Millipore BioImage scanner (Millipore, Bedford, MA). Two autoradiograms with different exposure times were scanned to verify linearity of the signals.

RESULTS

Colony Forming Efficiency

In early passages, cultures had colony forming efficiencies of ~3% (Figure 1), indistinguishable from other samples derived from normal newborn epidermis. After 10 passages, by which time other normal epidermal strains had undergone senescence, SIK cells displayed continued vigorous growth. Increasing colony forming efficiencies became evident at passage 15. By passage 40, values reached ~30% and were maintained at this level for as long as the cells have been propagated (105 passages). The colony morphology was indistinguishable from normal by light microscopy. Most large colonies contained areas with numerous small cells mixed with squames, characteristic of normal cultures several passages from senescence.

When SIK cells were inoculated at low density and examined after 2 wk for colony forming efficiency, ~60% of the colonies were large and progressively growing, whereas ~30% were small and comprised of

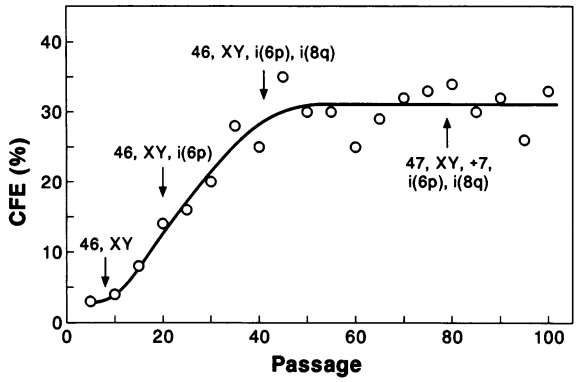

Figure 1. Colony forming efficiency (CFE) versus passage. At intervals of 5 passages, colony forming efficiencies were measured. Vertical arrows indicate cultures submitted to cytology and the observed karyotypes.

squames (abortive), as shown in Figure 2. A small fraction (~10%) were of medium size and not easily categorized as progressive or abortive. This ratio of colony types has been maintained through passage 105. In contrast, parallel cultures of immortalized human cell lines derived from squamous carcinomas (Rheinwald and Beckett, 1981) or by transformation with the papillomavirus type 16 E6/E7 genes (Barbosa and Schlegel, 1989) did not exhibit abortive colonies as seen in the present line. Similarly, normal epidermal cells immortalized by adenovirus E1A reportedly do not produce abortive colonies (Barrandon *et al.*, 1987).

The immortalization process appeared reproducible in mass cultures, since continued vigorous growth for at least 20 passages was obtained on two separate occasions. The frequency of immortalization within the population was tested by propagating individual clones isolated from early passages. From 144 single cells isolated from passages 2–5 by micropipet and cultured with feeder layer support (Barrandon and Green, 1987), 37 clones were obtained. From these, only one cell line survived three passages (colony forming efficiency = 2%) and now appears to have become established. By contrast, 48 single SIK cells of passage 46 resulted in 26 lines with vigorous growth and extended lifespan. To check whether immortalization of another cell type from this tissue sample could be demonstrated, fibroblasts were derived from a primary culture by omission of the 3T3 feeder layer and passaged at dilutions of 1:30 (early passages) or 1:10 (late passages). After an estimated 83 population doublings, no growth was evident for 2 mo.

Chromosomal Analysis

At two separate early passages (2 and 8), the chromosomal complement was indistinguishable from normal

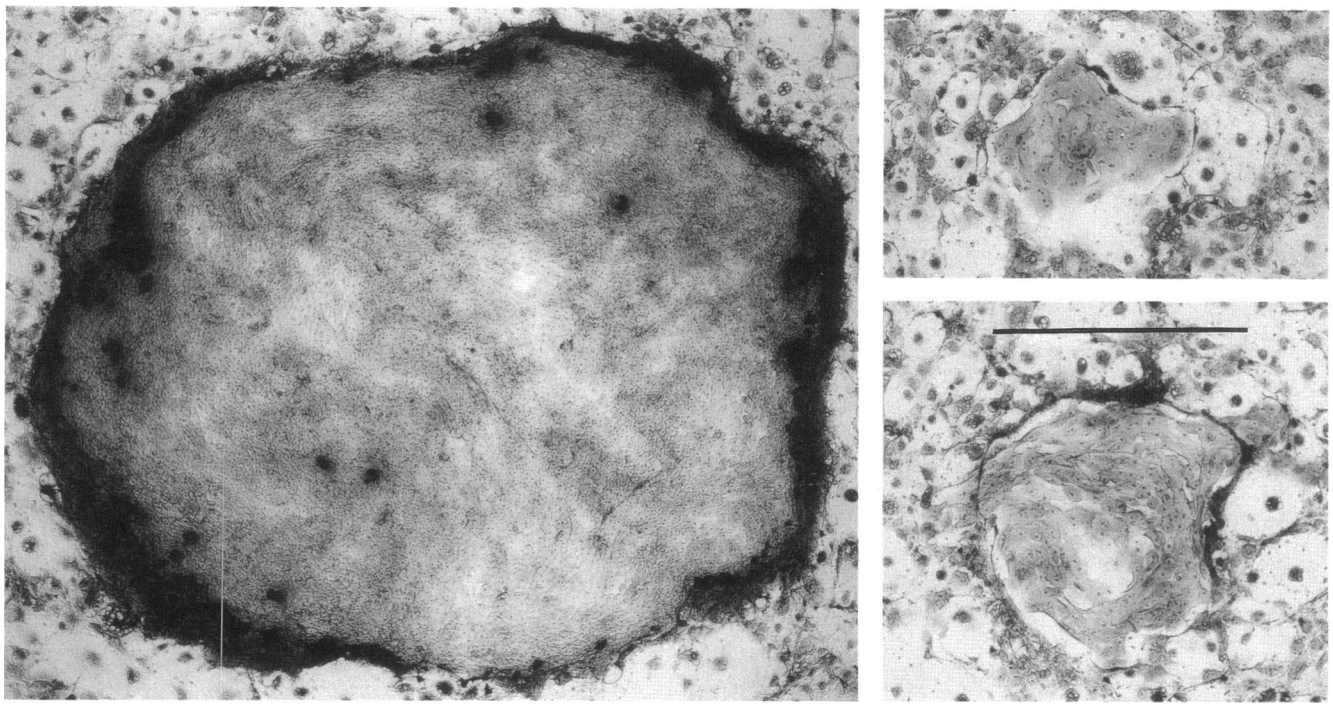

Figure 2. Colony types. (Left) Progressively growing colony with rounded borders and numerous small cells. (Right) Two abortive colonies with uneven borders, prominent squames and few if any small cells. These colonies, obtained from a single culture at passage 60, are all illustrated at the same magnification after fixing and staining. Bar, 1 mm.

(46,XY). By passage 78, however, several abnormalities were evident: isochromosomes 6p and 8q and an extra chromosome 7 (Figure 3). Examination of the karyotype at intermediate passages (summarized in Table 1) indicated that i(6p) was present in nearly all the cells (91%) by passage 20, whereas an additional i(8q) appeared in a majority (64%) by passage 41. Occasional aberrant chromosomes were also noted at these passages, but they did not become established with continued propagation. The extra chromosome 7 was observed only at the latest time sampled, passage 78, but it was found uniformly in the cells examined. The temporal sequence of these chromosomal aberrations with respect to increasing colony forming efficiency is shown in Figure 1.

Expression of Cell Cycle Control Proteins

Immunoblotting of whole-cell extracts revealed a sixfold increase in cyclin A protein between passages 10 and 16, whereas cyclin B protein levels increased fourfold during this period (Figure 4). A more gradual increase in $p34^{cdc2}$ levels occurred between passages 6 and 24, threefold by passage 16 and sevenfold by passage 24. Examination of SIK cells at passage 105, long after colony forming efficiency had reached a maximum of ~30%, and after several additional chromosomal aberrations had become established, showed that the levels of cyclin A, cyclin B, and $p34^{cdc2}$ remained at or above the levels seen at passage 16. The temporal changes in expression of these cell cycle regulatory proteins are summarized in Figure 4B. In contrast, the cells did not show altered levels of the early response kinases ERK1 and ERK2 or of the differentiation markers involucrin and keratinocyte transglutaminase (Rice and Schlegel, unpublished data).

The striking change in abundance of cell cycle control proteins cannot be accounted for by alterations in cell cycle distribution or growth rate, as illustrated in Figure 5. For example, the growth rates (~24 h doubling times) were indistinguishable for cells of passages 10 and 25.

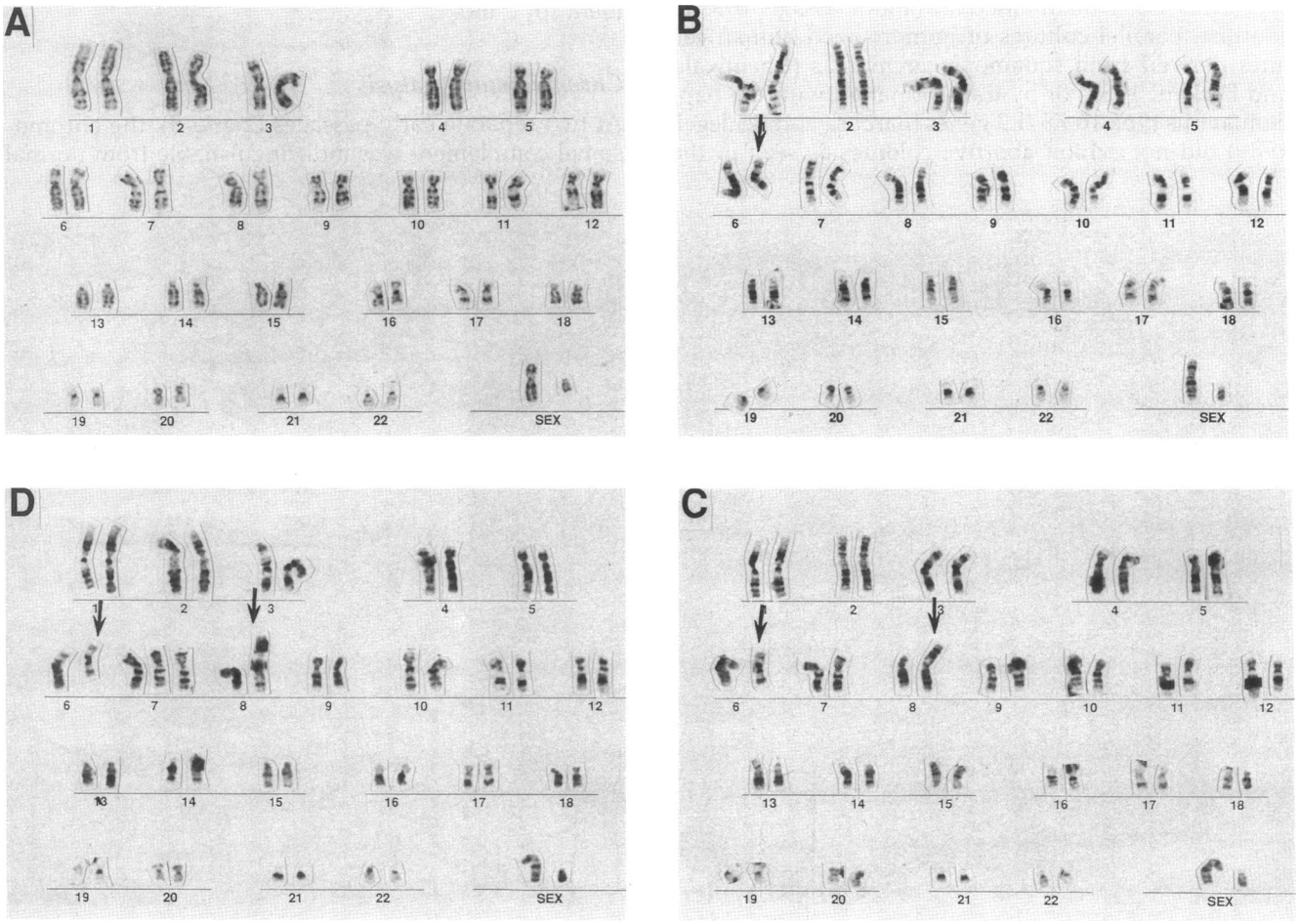

Figure 3. Karyotypes during immortalization. Illustrated are (A) 46,XY; (B) 46,XY,i(6p); (C) 46,XY,i(6p), i(8q); and (D) 47,XY,i(6p), +7, i(8q). The chromosomes for each karyotype were taken from a single spread. Arrows indicate isochromosomes.

Table 1. Accumulation of chromosomal aberrations with passage

		Chromosomal complement				
Passage	Spreads	46,XY	46,XY,i(6p)	46,XY,i(6p),i(8q)	47,XY,+7,i(6p),i(8q)	Other
2	40	40	0	0	0	0
8	13	13[a]	0	0	0	0
20	22	0	19	0	0	3[b]
31	25	0	24	0	0	1[c]
41	14	0	3	9	0	2[d]
78	20	0	0	0	20	0

[a] Includes 2 normal tetraploid spreads.

[b] Also seen (1 each) were 92,XXYY,2i(6p); 47,XY,+5,i(6p); 47,XY,+20,i(6p); and 48,XY,+2,−14,+2 ring chromosomes.

[c] One spread was tetraploid with this karotype lacking one Y chromosome.

[d] Also observed were 2 spreads with 46,XY,−2,−22,i(6p),+der(2),+der(22).

In addition, cultures of passage 24 were observed to have only a slightly elevated fraction of cells in the S and G2/M phases of the cell cycle (38 and 24%, respectively) compared with passage 8 (31 and 17%, respectively). The possibility that a small proportion of rapidly growing cells at passages 24 and 25 were responsible for the elevation of cell cycle regulatory proteins detected in mass culture is highly unlikely in view of the substantial magnitude of these changes. Moreover, since the degree of elevation was stable for up to 90 passages, the population was not observed to be overtaken by variants that produced even further elevations.

Growth Properties in Surface Culture

Because the immortalized cells resembled normal human epidermal colonies microscopically, SIK cells from passages 35–45 (estimated 270–330 population doublings) were examined for changes in response to several growth conditions. Like normal epidermal cells (Rheinwald and Beckett, 1980), SIK cells totally lost colony forming ability following suspension culture for 1 d and were unable to grow on plastic in the absence of feeder layer support at densities $< 10^5/6$ cm dish (Qin and Rice, unpublished data). In addition, SIK cells grew moderately well in serum free low calcium medium, in contrast to certain squamous carcinoma lines that grow poorly (Rollins *et al.*, 1989).

TGF-β and TPA are potent growth inhibitors of normal keratinocytes, but the effectiveness of these compounds is greatly reduced after transformation (e.g., Bascom *et al.*, 1989 for review; Parkinson *et al.*, 1983; Pietenpol *et al.*, 1990). Treatment of SIK cells with either TGF-β or TPA was highly effective in preventing growth in low calcium medium (Figures 6, A and B), showing a potency similar to that displayed toward normal keratinocytes (Rollins *et al.*, 1989). Sensitivity to TPA was also examined in the 3T3 feeder layer culture system, giving results comparable to those obtained in low calcium medium (deGraffenried and Rice, unpublished data).

Because normal epidermal cells require EGF for extended passage, the medium was supplemented with this growth factor for routine propagation. When EGF

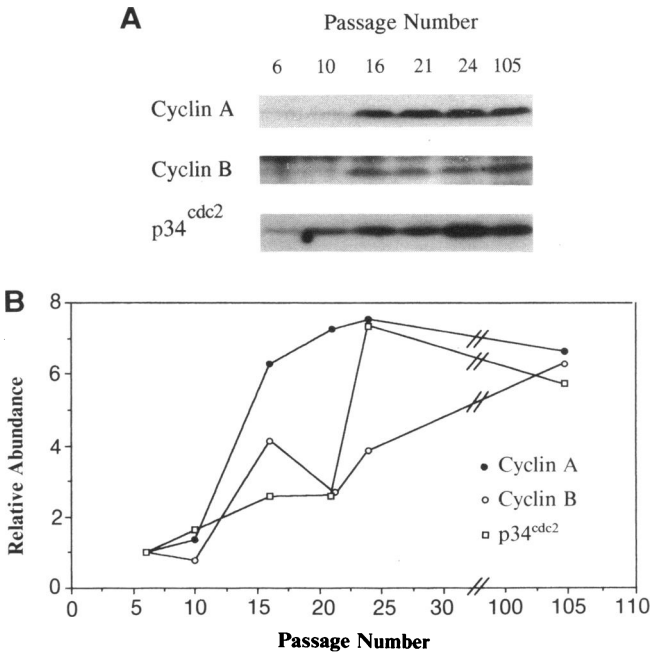

Figure 4. Changes in expression of cell cycle control proteins with passage. (A) Immunoblots of cyclin A, cyclin B, and $p34^{cdc2}$ in SIK cells before, during, and after immortalization. Equal aliquots of protein were analyzed at the indicated passages. (B) Relative abundances of cyclin A, cyclin B, and $p34^{cdc2}$ were calculated by densitometry of autoradiograms shown in (A).

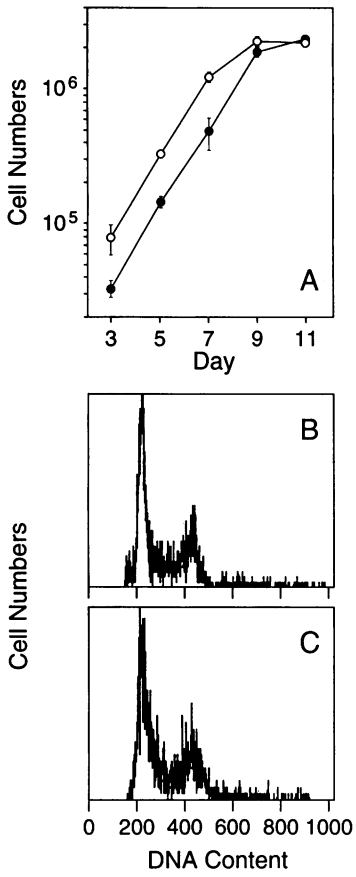

Figure 5. Comparison of growth and cell cycle parameters. (A) Parallel SIK cultures of passage 10 (○) and 25 (●) were inoculated with 2×10^4 cells and counted electronically in triplicate at 2-d intervals. Bars show standard errors around the mean values. For cell cycle analysis, subconfluent cultures of passage 8 (B) and 24 (C) SIK cells were trypsinized and passaged at a 1:3 dilution. Two days later, 3T3 feeder cells were removed by spraying with dilute EDTA (Sun and Green, 1976), and the adherent keratinocytes were collected by trypsinization. Cell cycle distribution was measured with a Becton-Dickinson FACS Scan with propidium iodide-treated cells and CellFit software. The relative integrated fluorescence was adjusted to a value of 220 for 2N chromosomes.

was omitted, growth of SIK cells at low colony or inoculation density nearly ceased (Figure 7). This dependence on EGF for growth was virtually identical to that originally demonstrated for normal epidermal cells (Rheinwald and Green, 1977). The effect of EGF deprivation for 20 d on colony forming efficiency was also measured. Under these conditions, the germinative capability of the cultures decreased to less than one-half, from 26 ± 1 to 10 ± 3(SD)% (two experiments, each in triplicate). Thus the ability to form large progressively growing colonies was substantially impaired. The dramatic dependence of growth on EGF was noted at all passages examined (35–45, 48, and 84).

DISCUSSION

Serial cultivation of keratinocytes from the present sample of ostensibly normal human skin led to spontaneous immortalization. The rise in colony forming efficiency detectable by passage 15 and accumulating chromosomal abnormalities testify to the selection of a series of variants which arose to dominate the population. Cells with high colony forming ability are unlikely to have existed in the tissue, since they should have become dominant in the mass culture much earlier. Despite the normal karyotype, however, some DNA damage might have been present in cells of the original tissue. The only attempt to demonstrate such damage directly, by sequencing the p53 gene, showed no evidence of mutation (Phillips and Rice, unpublished data) in exons 5–9, where damage is commonly observed in human tumors (Hollstein *et al.*, 1991). The finding that ≤3% of clones isolated from the population were capable of spontaneous immortalization suggests that the original damage, if present, was not sufficient by itself to prevent senescence.

A plausible model of keratinocyte growth and differentiation involves the occasional replication of stem cells with further population expansion through division of transitory amplifying cells (Potten, 1981). In culture, three categories of germinative cells have been identified, one of which resembles the hypothesized stem cell in its potential for sustained growth (Barrandon and Green, 1987). Upon aging, the latter are lost from the

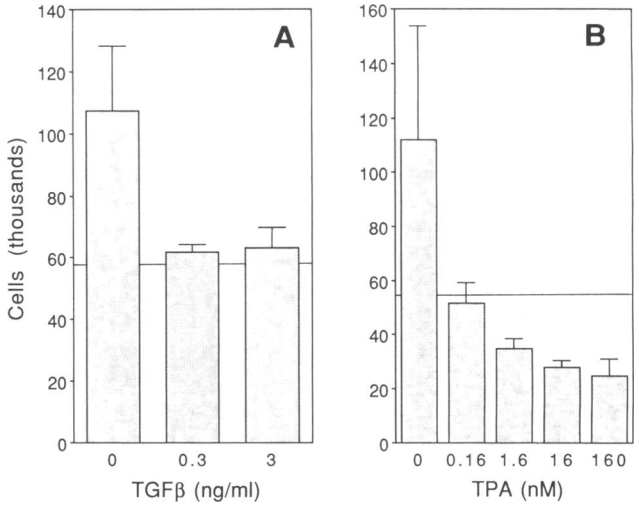

Figure 6. Response to TGF-β and TPA. Cells were inoculated at a density of 5×10^4/6-cm culture, treated starting on day 3, and trypsinized and counted 17 d later. Each experiment was performed 3 times with the same result, of which a representative trial with cells of passage 34 is illustrated. Error bars show the range of duplicate cell counts. Cell numbers present at the time of addition of (A) TGF-β or (B) TPA are indicated by a horizontal line.

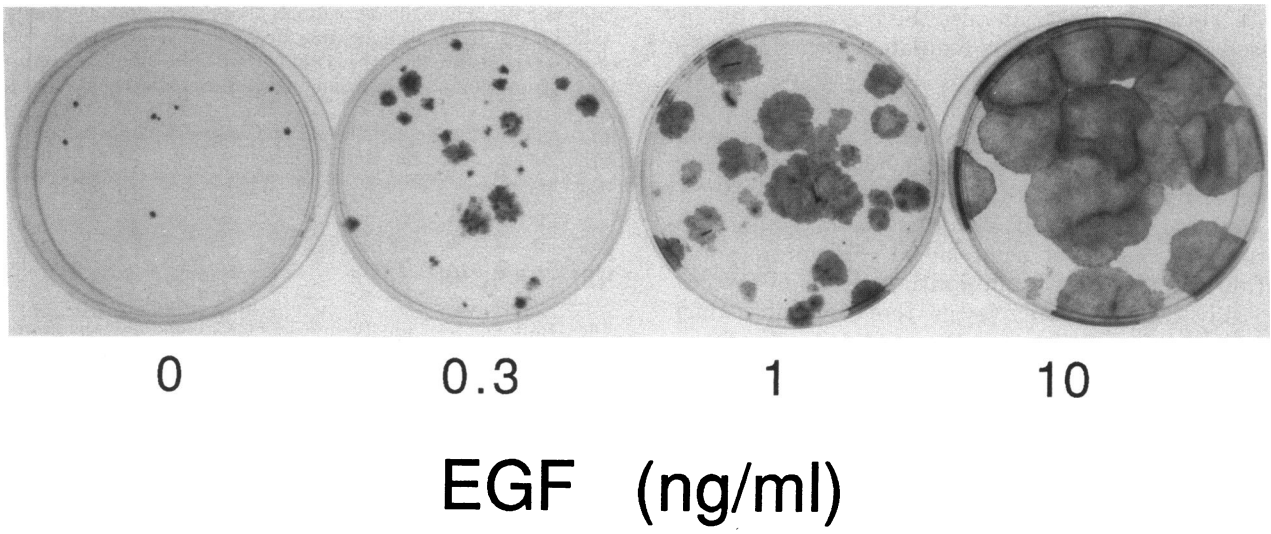

Figure 7. Dependence of growth on EGF. Cultures were inoculated with 300 cells of passage 37. Starting on day 4, they were treated with the indicated concentrations of EGF for 24 d and then fixed and stained.

population, resulting in an increasing proportion of abortive colonies. Thus the potential exists, as demonstrated in certain lymphocytes (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990), for immortalization to occur by preventing programmed loss of stem cells. In the present case, however, it is apparent (according to the model) that exit of at least some transitory amplifying cells from the mitotic pool into the terminal differentiation pathway must still occur. Moreover, regulation of major signal transduction pathways in SIK cells evidently is not grossly deranged, because effects of EGF, TGF-β, and TPA on growth are indistinguishable from normal. This finding is in contrast to immortalization by oncogenes from DNA tumor viruses, which have been reported to eliminate keratinocyte sensitivity to TGF-β and the observed dependence on EGF for clonal growth (Banks-Schlegel and Howley, 1983; Barbosa and Schlegel, 1989; Pietenpol *et al.*, 1990). Together with the absence of human papillomavirus E6/E7 expression, these results attest to the spontaneous nature of immortalization in SIK cells.

The most conspicuous changes associated with immortalization of SIK cells were the dramatically increased levels of cell cycle regulatory proteins and the aberrant i(6p) chromosome. Future experiments with the SIK mass population or with individual clones derived from early passage (in progress) may pinpoint more precisely the temporal and perhaps mechanistic relationship between these events. In related work, rare chromosomal aberrations that may contribute to extended lifespan can be observed in human epidermal cultures grown in low calcium medium (Weaver *et al.*, 1991). The observed partial trisomies appear to originate by amplification of adjacent chromosomal material, suggesting that triplication of certain regions can delay cell senescence (Rosenberg *et al.*, 1991). The i(6p) aberration in immortalized SIK cells and the trisomy 8 in NM1 cells and i(8q) in its subclones (Goldaber *et al.*, 1990) are compatible with this suggestion. Coincidentally, SIK cells display an i(8q) aberration, but its appearance many passages after the immortalization process was well under way indicate it is unlikely to have been an important early factor. Isochromosome 6p is a common feature of retinoblastoma tumors, appearing in ~60% of the cases (Squire *et al.*, 1984). A dominant effect of extra (or damaged) 6p material is consistent with its presence in these and certain other infrequent human cancers, generally leukemias (Nagarajan *et al.*, 1986) and with the neoplastic consequences of excess expression of the *pim*-1 oncogene (located on human chromosome 6p) in the mouse (van Lohuizen *et al.*, 1989). However, the loss of 6q material from such chromosomes may be of equal or greater consequence. Damage to or loss of 6q, which has tumor suppressor activity, is frequent in melanomas and certain other common human tumors (cf., Millikin *et al.*, 1991).

Cells that are senescent or committed to terminal differentiation suppress expression and activity of cell cycle control proteins. For example, senescent human diploid fibroblasts stimulated with serum express little or no CDC2, cyclin A or cyclin B mRNA (Stein *et al.*, 1991), even though they can express other genes associated with serum stimulation of nonsenescent cells, such as c-*myc*, c-*jun*, c-Ha-*ras*, thymidine kinase, and histone H3 (Rittling *et al.*, 1986; Seshadri and Campisi, 1990). In addition, chemically induced differentiation of murine erythroleukemia cells suppresses cyclin A expression and histone H1 kinase activity (Kiyokawa *et al.*,

1992). These observations raise the possibility that increased expression of these regulatory proteins at the time of immortalization plays a role in rescuing SIK cells from senescence. Cyclin A, cyclin B, and $p34^{cdc2}$ are not located on chromosome 6p (Blanquet *et al.*, 1990; Carson *et al.*, 1991; Nazarenko *et al.*, 1991; Sartor *et al.*, 1992), suggesting that increased expression of these proteins results from downstream events initiated by the i(6p) aberration or from events unrelated to this chromosomal aberration, perhaps by mutations not detectable microscopically. Because protein levels were elevated as much as eightfold above normal with only minor differences in growth rate and cell cycle distribution between normal parental cells and immortal SIK cells, increased expression was not simply an indirect consequence of more rapid cell growth or of a greater proportion of cells in S and G_2/M phases of the cell cycle, where cyclins A and B are maximally expressed (Pines and Hunter, 1989, 1990).

Perturbations in cyclin expression have also been linked to human cancers. In a human hepatocellular carcinoma, a single hepatitis B integration site was found in the cyclin A gene, resulting in an increase in both mRNA expression and protein stability of a hepatitis B virus/cyclin A fusion protein (Wang *et al.*, 1990, 1992). It is interesting to note that cyclin A displayed the largest increase among the cell cycle proteins examined in SIK cells. A human parathyroid tumor greatly overexpresses cyclin D_1, a G_1 cyclin, as a result of a chromosomal rearrangement that positioned the cyclin D_1 gene near the enhancer of the parathyroid hormone gene (Motokura *et al.*, 1991). Cyclin D_1 has also been implicated in human B lymphocyte malignancy. This disease is associated with a t(11;14)(q13;q32) translocation that activates the putative oncogene *bcl*-1. Cyclin D_1 is overexpressed in these cells and has been tentatively identified as the *bcl*-1 gene (Withers *et al.*, 1991; Seto *et al.*, 1992). The related cyclin D_3 has recently been cloned and localized to chromosome 6p (Inaba *et al.*, 1992; Xiong *et al.*, 1992; Motokura *et al.*, 1992). Experiments are underway to test whether the i(6p) aberration in immortalized SIK cells alters cyclin D_3 expression. Although there is currently no direct evidence that any of the cyclins can transform cells, mounting evidence suggests that aberrant regulation of cell cycle regulatory proteins is important in the etiology of some human cancers. In the present case, this phenomenon could contribute to the observed immortalization and accumulation of chromosomal aberrations with continued passage.

ACKNOWLEDGMENTS

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