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This grant continues to study the nature of the synergistic interaction of the growth factor TGFα and the nuclear protooncogene product c-myc for bitransgenic mouse mammary tumorigenesis. We found evidence of a multifactoral interaction of the two genes, involving cooperative stimulation of proliferation, anchorage inqependent colony formation, and suppression of cell death (apoptosis). In addition, we observed that the TGFα-related growth factor amphiregulin was likely to have tumor promoting effects similar to TGFα in breast cancer. Ongoing studies are now addressing the detailed molecular basis for the TGFα/myc interaction and the possible interaction of the two gene products in a novel paracrine mammary system *in vivo*.

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FOREWORD

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Robert B. Dix 9/15/97

PI - Signature Date

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Introduction

This project continues to focus on characterization of the potent tumorigenic interaction of c-Myc (a nuclear oncogene) and transforming growth factor α (TGFα, a growth factor) in the mammary gland. Both genes are commonly overexpressed in human breast cancer, and we have established a novel bitransgenic mouse model for mechanistic studies of their co-overexpression (1). Tumors are characterized for expression of the transgenes, state of differentiation, and expression of several other malignancy-associated genes. Detailed biochemical and molecular studies are then to assess regulation of apoptosis (programmed cell death) and cell cycle aberrations as potential mechanisms of transgene interaction. Finally, xenograft transplantation of mammary cells expressing Myc are to be combined in variable proportions with cells expressing no transgene or TGFα and implanted in recipient mammary fatpads from non-transgenic or TGFα transgenic mice to assess TGFα-Myc paracrine interactions *in vivo*.

Body

The goals of the research in the third year of our project (Task 3) focused on mRNA and protein analysis of relevant growth regulatory molecules in our bitransgenic TGFα-Myc model. The growth regulatory molecules of interest included TGFα, Myc, EGF receptor, Myn, and cyclins.

Table I presents information concerning mammary tumor frequency and latency in our bitransgenic mouse model. Tumors arose in all males and virgin females containing the two transgenes with a latency of 66 days in each sex. In contrast, in virgin females, the mean tumor latency in single transgenic, Myc-bearing animals was nearly one year, and no tumors were ever seen in single transgenic TGFα-bearing animals. Table II presents the histopathologic characterization of all mammary lesions: bitransgenic tumors were adenocarcinomas. We observed that although bitransgenic mammary (and salivary) tumors arose in male and female bitransgenic animals very rapidly and independent of pregnancy, that the mammary tumors contained moderate levels of receptors for estrogen and progesterone. However, growth regulation of tumor cells *in vivo* and *in vitro* was independent of these steroids. In addition, the tumors were of epithelial morphology and expressed cytokeratins as detected with a pan-cytokeratin antibody. Tumors contained multiple copies of the expected transgenes, but were not amplified for genes encoding EGF receptor, Myn, or cyclins. These results were reported in the scientific literature (1, see Appendix).

We next set out to examine mRNA and protein expression in bitransgenic tumors for the same genes. Interestingly, we observed that co-overexpression of the two transgenes resulted in their expression at the mRNA level which was clearly in excess of either transgene expression in single transgenic, long latency control tumors (Figure 1). This strongly suggested a selective advantage for transgene co-overexpression. Northern blot data were confirmed by *in situ* hybridization data in mammary and salivary gland tumors. When we examined Myn and Cyclin D_1 mRNA expression, they were also observed to be highly expressed at the mRNA level consistent with a high degree of proliferation and malignant transformation of bitransgenic tumors. The EGF receptor (Figure 2) was expressed at moderate levels in bitransgenic and single transgenic tumors (1, see Appendix).

We were able to establish cell lines from the single and bitransgenic tumors, in order to further address mechanisms of interaction. We observed that compared to single transgenic tumor cells, the bitransgenic tumor cells proliferated more rapidly and more readily grew in anchorage independent colonies. In addition, c-Myc single transgenic tumor cells exhibited a high degree of apoptosis (programmed cell death) compared to TGFα-expressing single and bitransgenic tumor lines.

The absence of the Myc transgene suppressed apoptosis to the assay background level. Apoptosis was suppressed *in vitro* in c-Myc cells by addition of TGFα, EGF, FGF-2, and IGF-1; the EGF receptor-mediated effects were selectively blocked with an EGF receptor-selective tyrosine kinase inhibitor. Finally, we confirmed the high level of c-Myc-associated apoptosis *in vivo*, selectively in single transgenic tumors using several assays. These results have just been published (2, see Appendix). As a followup, we observed that TGFα induction of the survival factor induced Bcl-X_L was associated with suppression of apoptosis in c-Myc-overexpressing mouse mammary tumor cells *in vitro* and *in vivo* (3). Next, we completed and submitted for publication a paper which establishes that the action of Myc on the mammary epithelial cell cycle is to shorten the G_1 phase. This appears to be due to diminished p21, activation of cdk2, and phosphorylation of Rb (4, see Appendix). This may result in chromosomal instability (5).

A continuing goal of the study is to evaluate paracrine interactions of the two transgenes (Task 1b) In the first year of our work, we encountered a probable immunologic barrier to our cross-transplantation experiments due to different backgrounds of Myc and TGFα transgenic mice. However, Charles River breeders has now backcrossed their Myc mice into the FVB strain (identical to the TGFα background). Using skin grafts, in year 2, we confirmed that Myc tissue is compatable when transplanted to the TGFα strain and *vice versa.* These paracrine transplantation experiments are all well underway, as indicated in Chart 1, below.

Chart 1 - Ongoing Paracrine Interaction Studies

Experiment	Epithelial Source	Fatpad Source	N	Status
1A	Myc (Tissue)	NT (cleared)	7	4/97 begun
	Myc (Tissue)	TGFα (cleared)	6	No tumors yet
1B	Myc (Tissue)	NT (cleared)	7	5/97 begun
	Myc (Tissue)	TGFα (cleared)	3	No tumors yet
2	Myc/TGFα (Tissue)	NT (cleared)	14	10/1 planned
3	Myc (Tissue)	NT (not cleared)	10	10/15 planned
	Myc (Tissue)	TGFα (not cleared)	10	10/15 planned
4	Myc (cells) + TGFα (cells) (1:1)	NT (cleared)	14	11/1 planned
5	Myc (cells) + TGFα (cells) (1:10)	NT (cleared)	14	11/15 planned

* NT = Non transgenic

The purpose of these experiements is to first test whether TGFα, released by stromal cells in the mammary fatpad, is sufficient to drive tumorigenesis of transgenic, Myc-overexpressing mammary epithelium. The first of these experiments (1A and 1B) have now been set up for several months. No palpable tumors have been detected to date. The experiment will proceed for several more months, ending in detailed histopathologic analysis. Experiment 2 is a positive control, designed to ensure that bitransgenic Myc/TGFα mammary tissue is able to form tumors in the same experimental design. We already know that Myc/TGFα mammary epithelium, even at 3-4 weeks of age, is histologically cancerous and is able to form tumors if transplanted into a nude mouse (1). The purpose of experiment 3 will be to determine if the transgenic, Myc-overexpressing epithelium can form tumors if it encounters transgenic TGFα-overexpressing epithelium in the context of ductal morphogenesis. The purpose of experiment 4 will be to determine if the transgenic, Myc-overexpressing epithelial cells can form tumors if they are exposed to transgenic TGFα-overexpressing cells in a contiguous developmental, paracrine context at a ratio of 1:1. Experiment 5 is similar to experiment 4, but the ratio of Myc:TGFα-overexpressing cells is altered to more strongly favor production of TGFα.

Additional progress was made on studies closely related to our specific aims and acknowledging support of the PI by this grant. We observed that Myc and TGFα single transgenic tumors express aberrantly processed forms of the EGF family members amphiregulin, and cripto 1 (6). Amphiregulin and cripto 1 were each capable of causing preneoplastic growth of the mouse mammary gland (7, 8). In addition, we observed that in analogy to TGFα, amphiregulin is inducable in human breast cancer cells, by both estrogen and by phorbol ester activators of protein kinase C (9, 10). We also collaborated with another laboratory using our TGFα transgenic mice and observed that these mice exhibited preneoplastic-appearing female reproductive tract lesions. However, these lesions were non-progressing; DES induced fully malignant lesions and positively interacted in this respect with TGFα (11, 12). Finally, we published six review articles on various aspects of the subject of this grant (13-18).

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%)[a]	Mean tumor onset time (days)	Tumor incidence
TGFα/c-*myc*	23 (15)	66 ± 12	100%[b]
TGFα	24 (15)	NA	0%
c-*myc*	39 (25)	298 ± 55	50%
Wild type	71 (45)	NA	0%

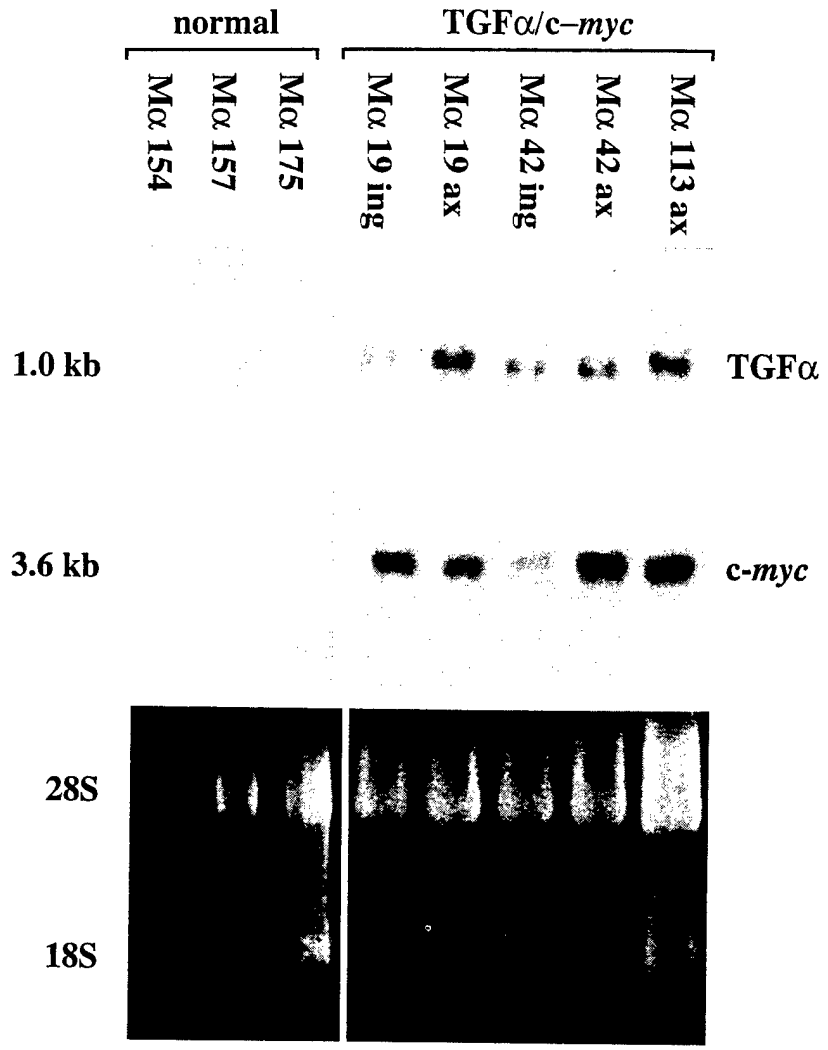
[a] Total offspring number is 157.
[b] One hundred % equals 20/20 animals; 3 animals died at very young ages.
NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGFα/c-*myc*	Multiple adenocarcinomas (types A and B) in females and males[a]	NA
TGFα	Normal	Cystic ducts
c-*myc*	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

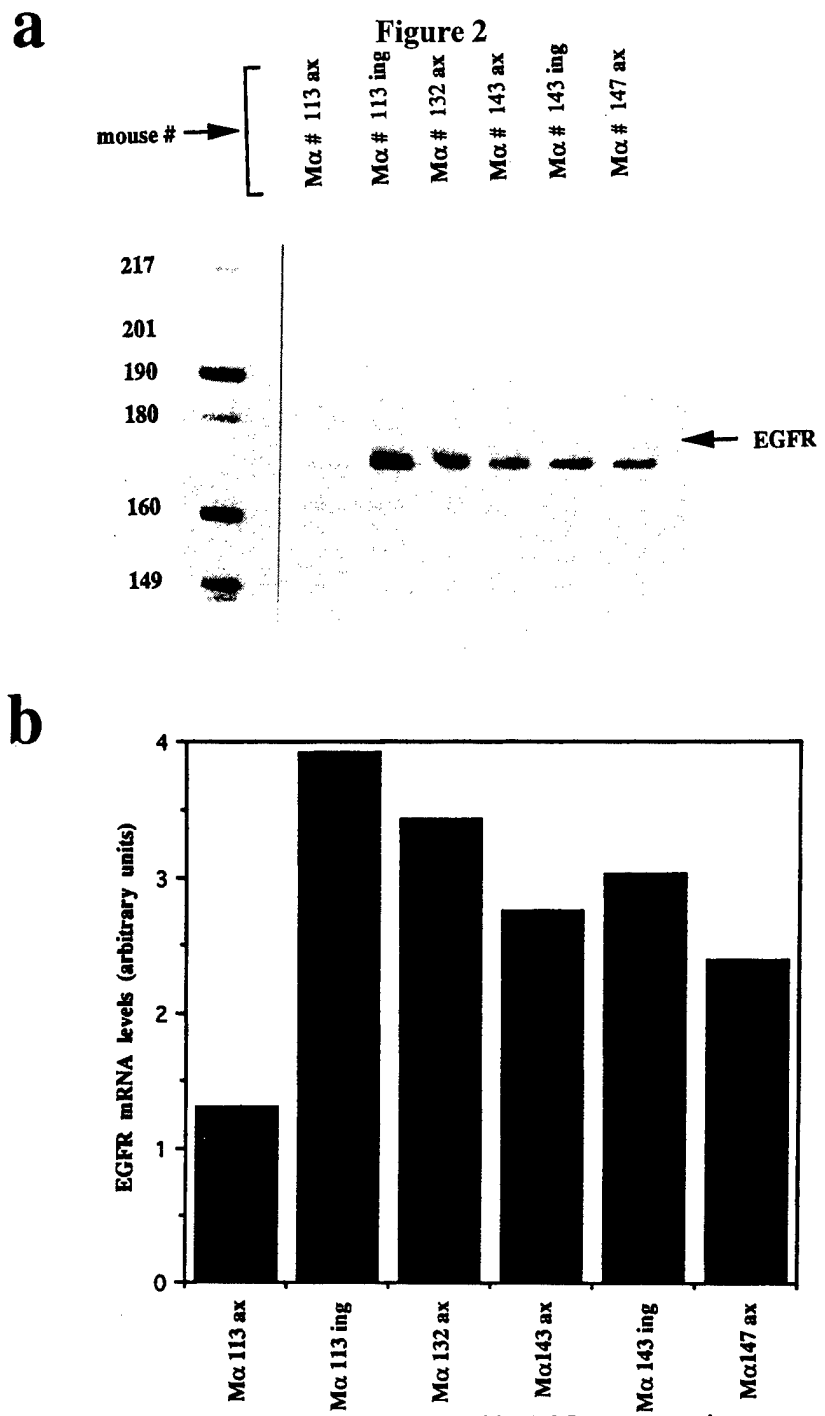
[a] Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

Figure 1



Northern analysis showing expression of the TGFα and *c-myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGFα transcript is 1.0 kb, and the *c-myc* transcript is 3.6 kb. Note that the endogenous 2.3-kb *c-myc* transcript is not seen here. Loading controls are the 18S and 28S ribosomal RNA bands. Mα 19 through 175 denotes the number of each animal used here. *Ax*, axillary gland tumor; *ing*, inguinal gland tumor.

Figure 2



Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. Mα113 through Mα147 denotes the number of each double transgenic mouse used in the assay. *Ax*, axillary gland tumor; *ing*, inguinal gland tumor.

Conclusions

1. Bitransgenic TGFα/Myc tumors are derived from mammary epithelial cells and contain estrogen, progesterone, and EGF receptors.

2. While multiple copies of the two transgenes were detected, other related growth control genes were not amplified in the bitransgenic tumors.

3. Bitransgenic tumors provide a strong selection for further overexpression at the mRNA level of transgenes and expression of high levels of Myn and Cyclin D_1.

4. The mechanisms of the tumorigenic interaction of TGFα and Myc included cooperative stimulation of proliferation, anchorage independent colony formation, and suppression of apoptosis.

5. One aspect of TGFα-suppression of apoptosis involved induction of the survival-promoting Bcl-X_L gene.

6. Another important aspect of Myc-initiated tumors involved shortening of the G_1 phase of the cell cycle; this appeared due to p21 down-regulation, cdk-2 activation, and Rb phosphorylation. Both Myc and Myc/TGFα tumors exhibited chromosomal instability.

7. Transgenic tumors also expressed the TGFα-family growth factors amphiregulin, and cripto 1, but in a variety of unusual isoforms.

8. Amphiregulin and cripto-1, like TGF α, were capable of induction of preneoplastic outgrowths of the mouse mammary gland.

9. Amphiregulin mRNA and protein synthesis are upregulated by estrogen and by protein kinase C in human breast cancer cells, in analogy to the previously-described regulation of TGFα.

10. Female TGFα transgenic mice are susceptible to reproductive tract abnormalities and to DES-induced neoplasias, in addition to Myc-induced mammary tumors.

11. In summary, these results strongly support the initial premises of the grant; that the Myc transcription factor and the TGFα family of growth factors are each potent modulators of mammary tumor onset and progression. Binary interaction of Myc and TGFα in the mouse mammary gland allows rapid tumor development due to a synergistic, multifactorial interaction.

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Appendix

Amundadottir, L.T, Johnson, M.D., Merlino, G.T., Smith, G.H., and Dickson, R.B.: Synergistic interaction of transforming growth factor α and c-myc in mouse mammary and salivary gland tumorigenesis. Cell Growth and Different, 6:737 -748 1995.

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Synergistic Interaction of Transforming Growth Factor α and c-*myc* in Mouse Mammary and Salivary Gland Tumorigenesis[1]

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Abstract

The c-*myc* oncogene is commonly amplified in breast cancer and is known to interact synergistically with transforming growth factor α (TGFα) *in vitro* to promote phenotypic transformation of mammary epithelial cells. In addition, both genes are under sex steroid hormone regulation in breast cancer. We have used a bitransgenic mouse approach to test the relevance of Myc-TGFα interaction in mammary gland tumorigenesis of virgin animals *in vivo*. We mated single transgenic TGFα and c-*myc* mouse strains to yield double transgenic offspring for TGFα and c-*myc*. All (20 of 20) double transgenic TGFα/c-*myc* animals developed synchronous mammary tumors at a mean age of 66 days. An unexpected finding was that tumor latency and frequency in males and virgin females were identical. Thus, two gene products that are known to be coinduced in breast cancer by the sex hormones estrogen and progesterone strongly synergize to induce synchronous mammary tumors, independent of sex. The tumors, despite being estrogen receptor positive, were readily transplanted as highly malignant s.c. cancers in ovariectomized nude mice. Although approximately one-half of single transgenic c-*myc* virgin females also eventually developed mammary gland tumors, these were stochastic and arose after a long latency period of 9–12 months. Single transgenic virgin TGFα females and males, c-*myc* males, and transgene-negative littermates did not develop tumors (ages up to 15 months).

The salivary glands of double transgenic animals also coexpress the two transgenes and show pathological abnormalities ranging from hyperplasias to frank adenocarcinomas. In contrast, the salivary glands of single transgenic and wild-type animals showed only mild hyperplasias or metaplasias, but tumors were not observed.

***In situ* hybridization analysis of mammary and salivary glands revealed that hyperplastic and tumorous areas colocalize with regions that overexpress both the TGFα and c-*myc* transgenes. This indicates that there is a requirement for the presence of both proteins for transformation of these glands. In summary, TGFα and c-Myc synergize in an extremely powerful way to cause breast and salivary gland tumorigenesis in males and virgin females without a requirement for pregnancies.**

Introduction

Gene amplification and/or deregulated expression of a number of genes are frequent findings in human breast cancer. Among these are the genes for c-*myc* and TGFα.[3] The protein product of the c-*myc* gene is a nuclear phosphoprotein involved in transcriptional regulation, and TGFα is a member of the EGF family of mitogens, which bind to and activate the EGF receptor (1, 2). The c-*myc* proto-oncogene is amplified in 25 to 30% of breast cancer cases and is overexpressed (without gene amplification) in many more (3–6). Furthermore, amplification of the c-*myc* gene has been shown to correlate with poor prognosis of the disease (3, 7, 8). Although the TGFα gene is not found amplified in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (9–12). The EGFR is also found expressed in about 30–50% of human breast cancers with high expression associated with poor prognosis and high a degree of invasiveness (13).

Expression of both genes is induced during estrogen and progesterone treatment of hormone-responsive breast cancer cells *in vitro* (10, 14–18). In addition, treatment with antisense oligonucleotides to either TGFα or c-*myc* inhibits estrogen-induced expression of these genes and estrogen-stimulated growth *in vitro*, indicating that they are important mediators of estrogenic effects on cell growth (19, 20).

Transgenic mouse models have provided insight into the roles of both genes in mammary gland development and malignant progression *in vivo*. Overexpression of TGFα in the mammary gland from the mouse metallothionein promoter or the MMTV promoter/enhancer caused the appearance of mammary carcinomas after a relatively long latency period of 7–12 months. Tumors were stochastic and arose predominantly in female mice that had undergone multiple pregnancies (21–23). Transgenic mice with MMTV-*myc* constructs directing expression to the mammary gland also develop clonal tumors after a long latency period of 7–14 months, again with a requirement for multiple pregnancies (24).

Long latency times in transgenic mice are consistent with the hypothesis that oncogenesis is a multistage process composed of a series of genetic events (25, 26). Thus, although one proto-oncogene is overexpressed in a given

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[3] The abbreviations used are: TGFα, transforming growth factor α; EGF, epidermal growth factor; EGFR, EGF receptor; MMTV, mouse mammary tumor virus; MT, metallothionein; LTR, long terminal repeat.

organ of a transgenic mouse, the occurrence of additional events is necessary before cancer can arise. We chose to study the interaction of TGFα with c-*myc*, since *in vitro* studies had suggested possible cooperative interactions. In various cell types *in vitro*, overexpression of c-*myc* resulted in an increased responsiveness to the effects of mitogenic growth factors. For example, rodent fibroblasts and human and mouse mammary epithelial cell lines transfected with *myc* constructs showed transformed behavior, in many cases, only in the presence of TGFα or fibroblast growth factor family members (27–30). High levels of *myc* expression may, therefore, permit a tumorigenic transformation by a TGFα/EGFR autocrine growth mechanism, or it may sensitize cells to such a mechanism.

The interaction of TGFα and c-Myc has not been studied in mammary gland transformation *in vivo*, but two recent studies investigated their interaction in the liver and pancreas with bitransgenic mouse models. Animals expressing both transgenes from liver- and pancreatic-specific promoters formed tumors in these organs at an elevated rate compared to single transgenic animals, suggesting a synergistic interaction (31, 32).

We show here that TGFα and c-Myc cooperate in an extremely powerful, synergistic manner in mouse mammary and salivary gland tumorigenesis. Double transgenic male and virgin female mice develop synchronous mammary tumors in all glands at about 2 months of age, whereas single transgenic animals develop clonal tumors at about 12 months of age or not at all. In addition, epithelial rudiments from 3-week-old TGFα/c-*myc* double transgenic animals could be established in nude mice, indicating that the mammary gland is transformed right at the start of its development. Tumors were also found in salivary glands of double transgenic animals at 3 months of age, whereas single transgenic and wild-type mice have not been observed to develop tumors. Expression of the TGFα and c-*myc* transgenes was associated with hyperplastic and tumorous areas in mammary and salivary glands, indicating a requirement for the presence of both gene products for malignant transformation.

Results

Generation of TGFα/c-*myc* Double Transgenic Mice. Heterozygous mice transgenic for TGFα (MT-TGFα MT100 strain) (21) and c-*myc* (MMTV-c-*myc* M strain) (24) were mated to yield offspring of four possible groups: double transgenic TGFα/c-*myc* mice; single transgenic TGFα mice; single transgenic c-*myc* mice; and mice negative for transgenes (wild type). Since the parental mice are of FVB/N (TGFα) and CD-1 × C57BL/6J (c-*myc*) backgrounds, offspring mice of all groups are of the following genetic background: FVBN/CD-1 × C57BL6J. Mice of the two single transgenic groups and of the wild-type group serve as controls in a similar genetic background as the double transgenic TGFα/c-*myc* mice.

At 3 weeks of age, offspring were weaned, and DNA was extracted from tail biopsies. Screening for transgenes was performed by Southern blot analysis and/or PCR (data not shown). According to Mendelian rules, when mating animals heterozygous for two traits, 25% of offspring should fall into each of four possible genotype groups. However, of 157 offspring, 45% were wild type; 15 and 25% were single transgenic for TGFα and c-*myc*, respectively; and 15% were double transgenic TGFα/c-*myc* (Table 1). Thus, there

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%)[a]	Mean tumor onset time (days)	Tumor incidence
TGFα/c-*myc*	23 (15)	66 ± 12	100%[b]
TGFα	24 (15)	NA	0%
c-*myc*	39 (25)	298 ± 55	50%
Wild type	71 (45)	NA	0%

[a] Total offspring number is 157.
[b] One hundred % equals 20/20 animals; 3 animals died at very young ages.
NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGFα/c-*myc*	Multiple adenocarcinomas (types A and B) in females and males[a]	NA
TGFα	Normal	Cystic ducts
c-*myc*	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

[a] Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

appeared to be a selection bias against mice positive for the TGFα transgene. A reduced body weight at weaning was not associated with the lower frequency genotypes (data not shown), in contrast to observations of Luetteke *et al.* (33) for a different strain of MT-TGFα transgenic mice. However, we have noted that TGFα-positive mice consistently die at younger ages than c-*myc* single transgenic and wild-type mice and show signs of malnutrition. This is probably due to the effects of the TGFα transgene product on the stomach, as described previously (34). Offspring of each genotype group were approximately equally divided between females and males.

Synergistic Induction of Mammary Gland Tumors in TGFα/c-*myc* Double Transgenic Mice. Of 23 double transgenic TGFα/c-*myc* animals, 20 developed multiple mammary tumors at a mean age of 66 ± 12 days, and three mice died from other causes at very young ages. We can, therefore, conclude that all mice of the TGFα/c-*myc* genotype that reached an age of about 2 months developed mammary gland cancers. An additional striking finding was that tumors arose in both virgin female and male animals with the same latency and frequency. Frank tumors (*i.e.*, palpable) arose first in axillary mammary glands (glands nos. 1, 2, and 3) and then subsequently in inguinal glands (nos. 4 and 5). The average number of palpable tumors at time of necropsy were 2.5/mouse. In addition, pathological diagnoses of hematoxylin/eosin-stained sections revealed the presence of adenocarcinomas in glands without frank palpable tumors, thus showing that every mammary gland from double transgenic animals was cancerous. Surprisingly, no normal tissue was found adjacent to mammary gland tumors in double transgenic animals; therefore, the whole gland could be characterized as malignant. Even a very young (5 weeks old) TGFα/c-*myc*-positive female was diagnosed as having

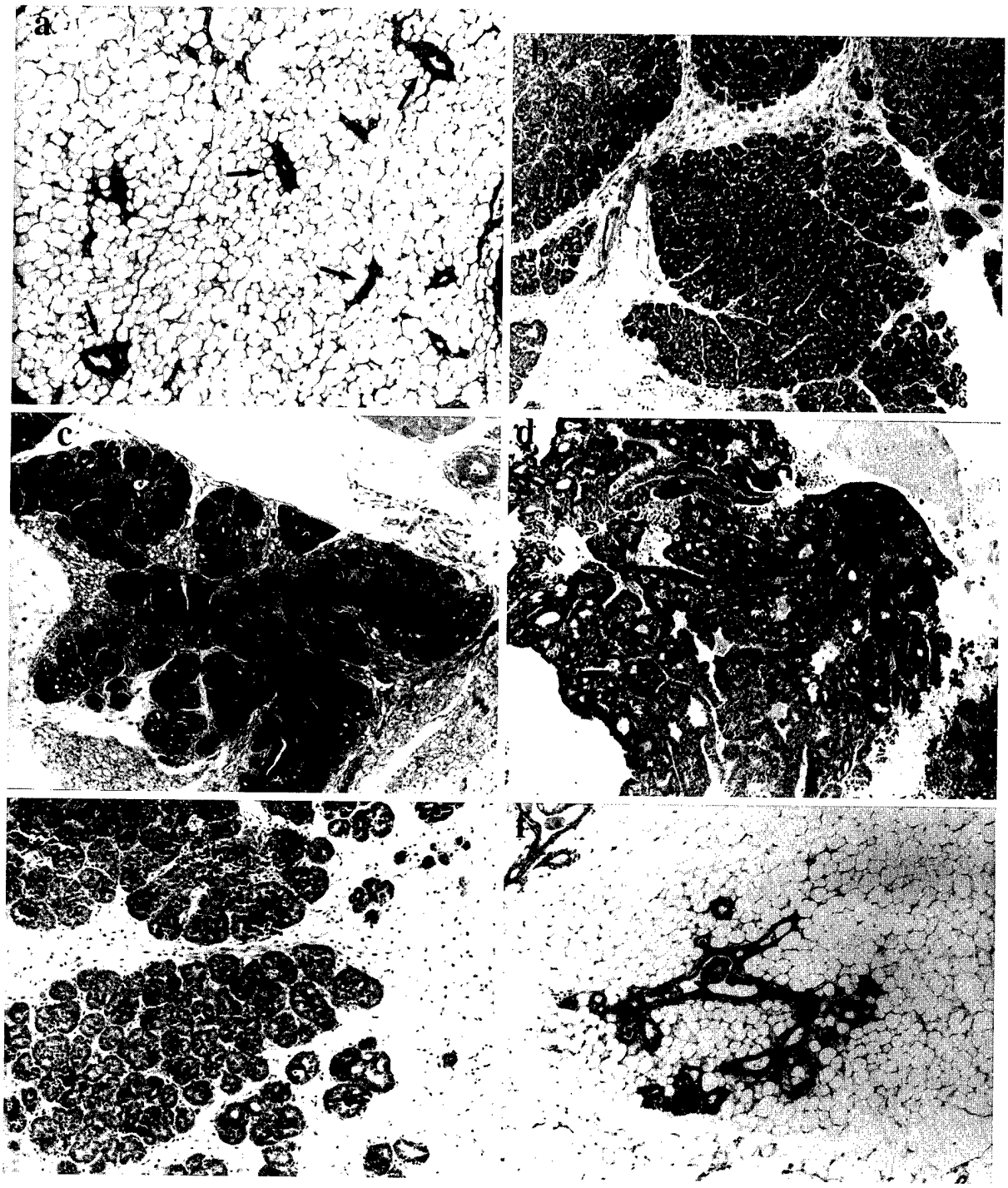

Fig. 1. Hematoxylin & eosin-stained sections of mammary glands. *a*, a normal virgin gland at 3 months of age. It is representative of TGFα and c-*myc* single transgenic animals and of nontransgenic littermates at this age. *Arrows*, epithelial ducts. Panels *b–d*, mammary gland tumors from double transgenic TGFα/c-*myc* animals at 3 months of age. *e*, a section of an inguinal mammary gland from a 5-week-old TGFα/c-*myc* female. *f*, an atypical hyperplastic gland from a c-*myc* single transgenic virgin female at 9 months of age. ×100.

mammary gland adenocarcinoma based on histopathology. This is striking since, at this age, the gland is not fully developed. Our results suggest that, in this model, overexpression of TGFα and c-*myc* is sufficient to cause a complete tumorigenic transformation of the mouse mammary gland.

Mean tumor onset times and frequency are shown in Table 1. Pathological diagnosis of mammary glands from

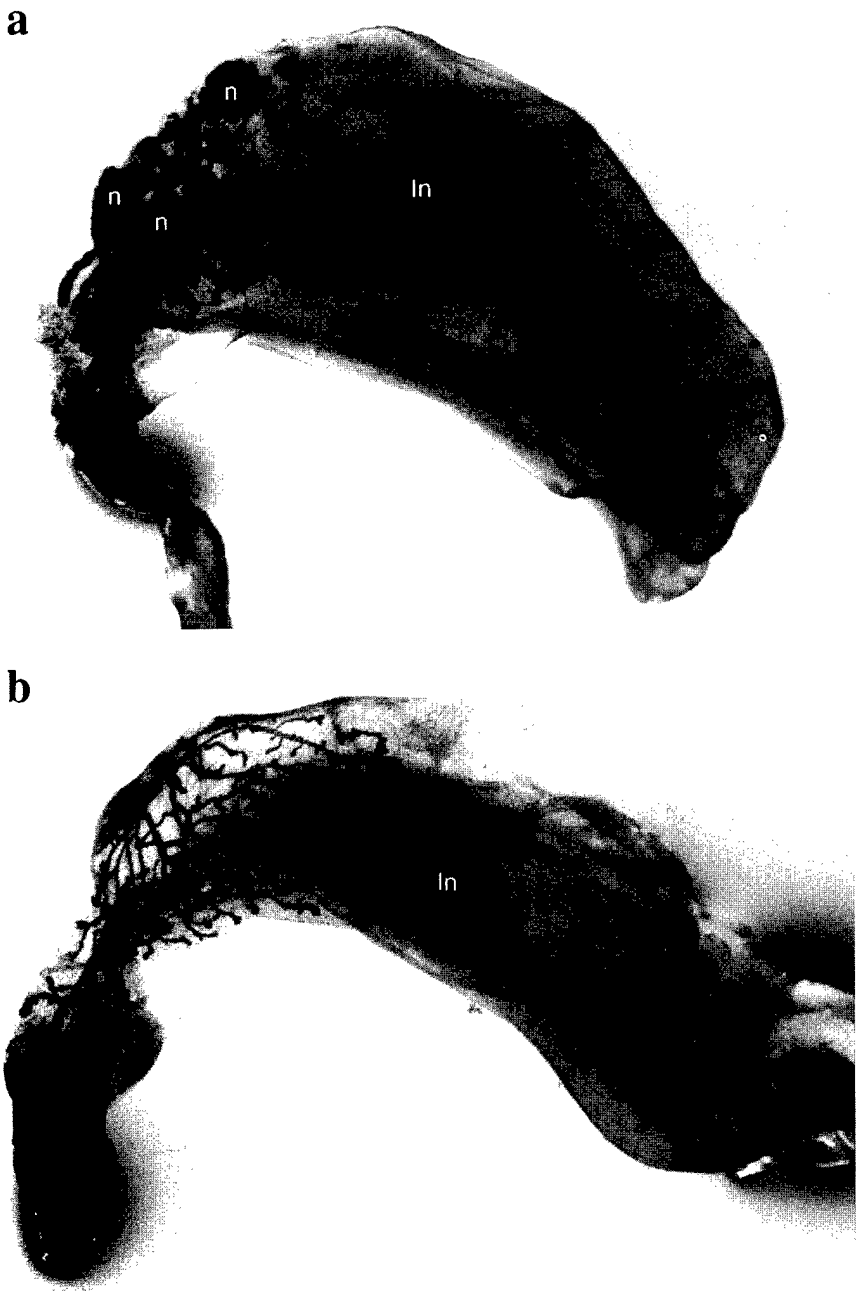

Fig. 2. Whole-mount staining of mammary glands from a TGFα/c-*myc* animal (*a*) and from a wild-type animal (*b*). The carmine alum stain reveals the epithelial network of the gland and the lymph node (*ln*). The animal in *a* was 24 days old, and the one in *b* was 28 days old. Note tumorous nodules (*n*) in the mammary gland from the TGFα/c-*myc* animal (*a*) and normal ductal pattern in the wild-type animal (*b*).

transgenic mice of each genotype group, at 3 and 7 months of age, is shown in Table 2. Representative hematoxylin/eosin-stained sections are shown in Fig. 1. The normal virgin gland at 3 months of age is mostly composed of adipose tissue, with scattered epithelial ducts consisting of two layers of cells (Fig. 1*a*). It is representative of pathology from the two single transgenic groups and the wild-type group at 3 months of age. In contrast, every mammary gland from double transgenic animals (virgin females and males) is tumorous at the same age (Fig. 1, *b–d*). Tumors were classified as adenocarcinomas of types A and B. A type tumors are fairly well differentiated, with the acinar structure of the gland prominent and two layers of epithelial cells seen surrounding lumens (Fig. 1*b*). Type B is less organized and locally invasive (Fig. 1*d*). When tumor sections were stained with periodic acid-Schiff stain, the basement membrane was seen intact in type A tumors but was often disrupted in type B tumors (data not shown). No distant metastases have been found to date. Fig. 1*e* shows adenocarcinoma from a 5-week-old double transgenic virgin female animal. At this age, the epithelial tree has not fully penetrated the mammary fat pad.

About 50% single transgenic c-*myc* virgin females also developed mammary gland tumors, but these were stochas-

tic and arose only after a very long latency period of 298 ± 55 days. The remainder had mild atypical hyperplasias and cystic ducts (Fig. 1*f*). Single transgenic virgin TGFα mice and transgene-negative littermates have not developed tumors to this date (ages up to 15 months). In the case of single transgenic males, we observed atypical hyperplastic areas in mammary glands of a 14-month-old single transgenic c-*myc* male (data not shown). Mammary glands from single transgenic TGFα males and wild-type males at the same ages were normal.

A whole organ staining (termed whole-mount staining) of mammary glands from virgin double transgenic animals at 24 days of age revealed multiple nodules in each gland that appeared tumorous (Fig. 2*a*). These were successfully established in nude mice, indicating that the gland is transformed from the start of its development. In comparison, whole-mount staining of mammary glands from wild-type virgin animals at 28 days of age revealed only the normal ductal pattern (Fig. 2*b*).

The observation that mammary gland tumors arose in double transgenic TGFα/c-*myc* males as well as in virgin females suggested that they might be estrogen independent. Estrogen receptor ligand binding assays revealed that tumors from males and females contained from 13–30 fmol/mg protein of the receptor, and are, therefore, considered estrogen receptor positive (data not shown). Control tumors (MCF-7 or MKL-4 cells grown as tumors in nude mice) contained about 3-fold higher levels of receptor. The ovariectomizing of TGFα/c-*myc* females ($n = 2$) at the time of weaning did not result in a significantly delayed tumor onset (69 *versus* 66 days). In addition, both axillary and inguinal mammary gland tumors could be successfully transplanted into ovariectomized nude mice (data not shown). Together, these data indicate that, although relatively low levels of the estrogen receptor are present in mammary gland tumors as measured by binding to ligand, they are not dependent on estrogen for growth.

Expression of Transgenes and the Epidermal Growth Factor Receptor in Mammary Gland Tumors. We have used Northern analysis, RNase protection assays, *in situ* hybridization analysis, and immunohistochemistry to examine the expression of transgenes and that of the EGFR gene in mammary gland tumors from double transgenic TGFα/c-*myc* animals. RNA expression was compared between axillary mammary gland tumors (frank tumors or lumps) and inguinal gland tumors (carcinoma revealed by histopathology) of TGFα/c-*myc* animals.

Transgenes were expressed in all mammary gland tumors from double transgenic animals but were not detectable in normal glands from single transgenic animals at 3 months of age (Fig. 3). There was about a 5-fold difference in the expression of the c-*myc* transgene, and about 7-fold for the TGFα transgene between the lowest- and highest-expressing tumor. An association of transgene expression at the RNA level and pathological diagnosis (adenocarcinoma type A *versus* B), location (axillary *versus* inguinal glands), tumor size, or sex was not observed. Expression of TGFα and c-*myc* was not detected in normal glands by this method. Transgene expression was also examined by *in situ* hybridization analysis to establish the pattern of transgene expression in the tumors (Fig. 4, *a*, *c*, and *e*). Sequential tumor sections from double transgenic TGFα/c-*myc* animals were hybridized to ^{35}S-labeled TGFα and c-*myc* probes. We observed a very strong and uniform expression of c-*myc* mRNA in mammary gland tumors from double

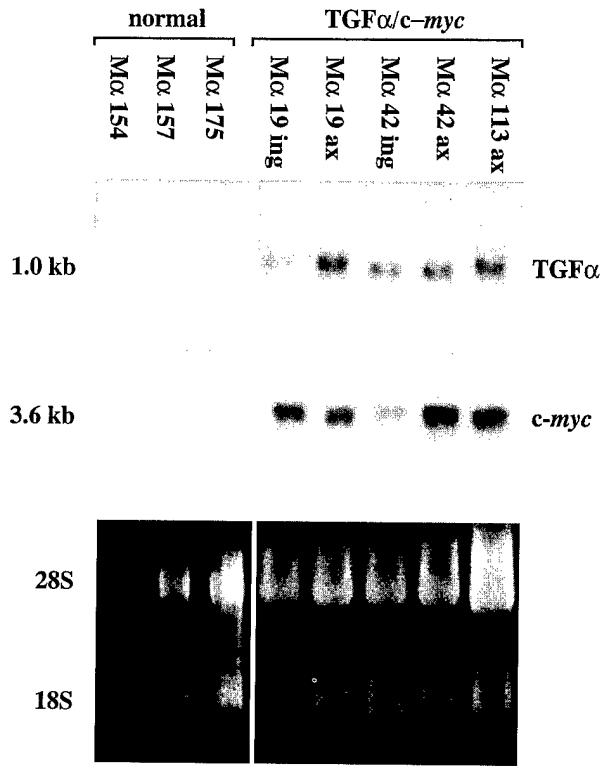

Fig. 3. Northern analysis showing expression of the TGFα and c-*myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGFα transcript is 1.0 kb, and the c-*myc* transcript is 3.6 kb. Note that the endogenous 2.3-kb c-*myc* transcript is not seen here. Loading controls are the 18S and 28S ribosomal RNA bands. Mα 19 through 175 denotes the number of each animal used here. *Ax*, axillary gland tumor; *ing*, inguinal gland tumor.

transgenic animals at 3 weeks of age and higher. A scattered expression of TGFα mRNA was noted in most areas of the mammary glands by this method. An immunohistochemical evaluation of TGFα protein levels in bitransgenic tumors with a TGFα-specific antibody also revealed a scattered pattern of strong expression but no association of enhanced staining with a more aggressive phenotype (Fig. 5, *a* and *b*). We also measured endogenous EGFR mRNA levels in tumors from double transgenic animals by RNase protection assays. As seen in Fig. 6, EGFR mRNA levels were comparable in all but one mammary gland tumor (axillary tumor from TGFα/c-*myc* animal no. 113). An association of EGFR mRNA levels with pathological diagnosis, location, tumor size, or sex was not seen.

Synergistic Induction of Salivary Gland Tumors by TGFα and c-*myc*. The MT promoter is active in most epithelial tissues, whereas the MMTV promoter is restricted to only a few tissues. Therefore, the MMTV promoter limits coexpression of the transgenes to mammary glands, salivary glands, and some reproductive organs. An interaction between TGFα and c-*myc* was not observed in reproductive organs, but a positive interaction was noted in the salivary glands. Ductule hyperplasia (sometimes with atypia) was seen in all salivary glands of double transgenic TGFα/c-*myc* virgin female and male animals at 3 months of age. In some cases, squamous metaplasia was observed in the sublingual gland,

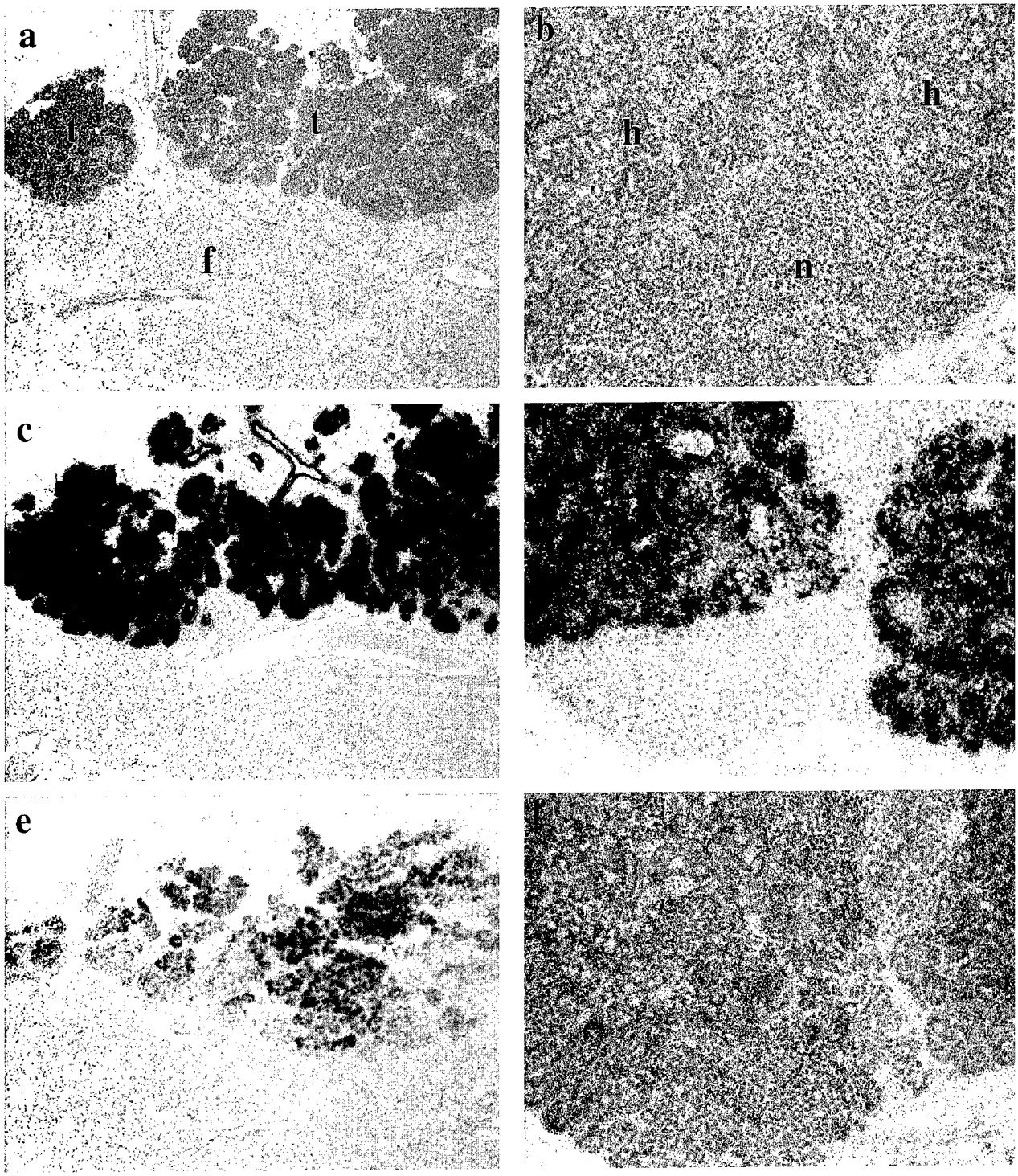

Fig. 4. *In situ* hybridization analysis of mammary and salivary glands from TGFα/c-*myc* animals. Sequential paraffin-embedded tissue sections were hybridized to ^{35}S-labeled riboprobes generated against the TGFα and c-*myc* transgenes. *a* and *b*, control sections hybridized to sense riboprobes. *c* and *d*, sections hybridized to c-*myc* antisense riboprobes. *e* and *f*, sections hybridized to TGFα antisense riboprobes. *a*, *c*, and *e*, from mammary glands; *b*, *d*, and *f*, from salivary glands. Note a near uniform expression of the transgenes in a mammary gland tumor from this 3-week-old double transgenic animal. Observe a patchy expression of c-*myc* and scattered distribution of TGFα in this 6-week-old double transgenic animal. Also note that where both transgenes are expressed, the glands appear hyperplastic and tumorous. *t*, tumor; *f*, fatty tissue; *h*, hyperplastic; *n*, normal. All panels are brightfield photographs. *a*, *c*, and *e*, ×50; *b*, *d*, and *f*, ×200.

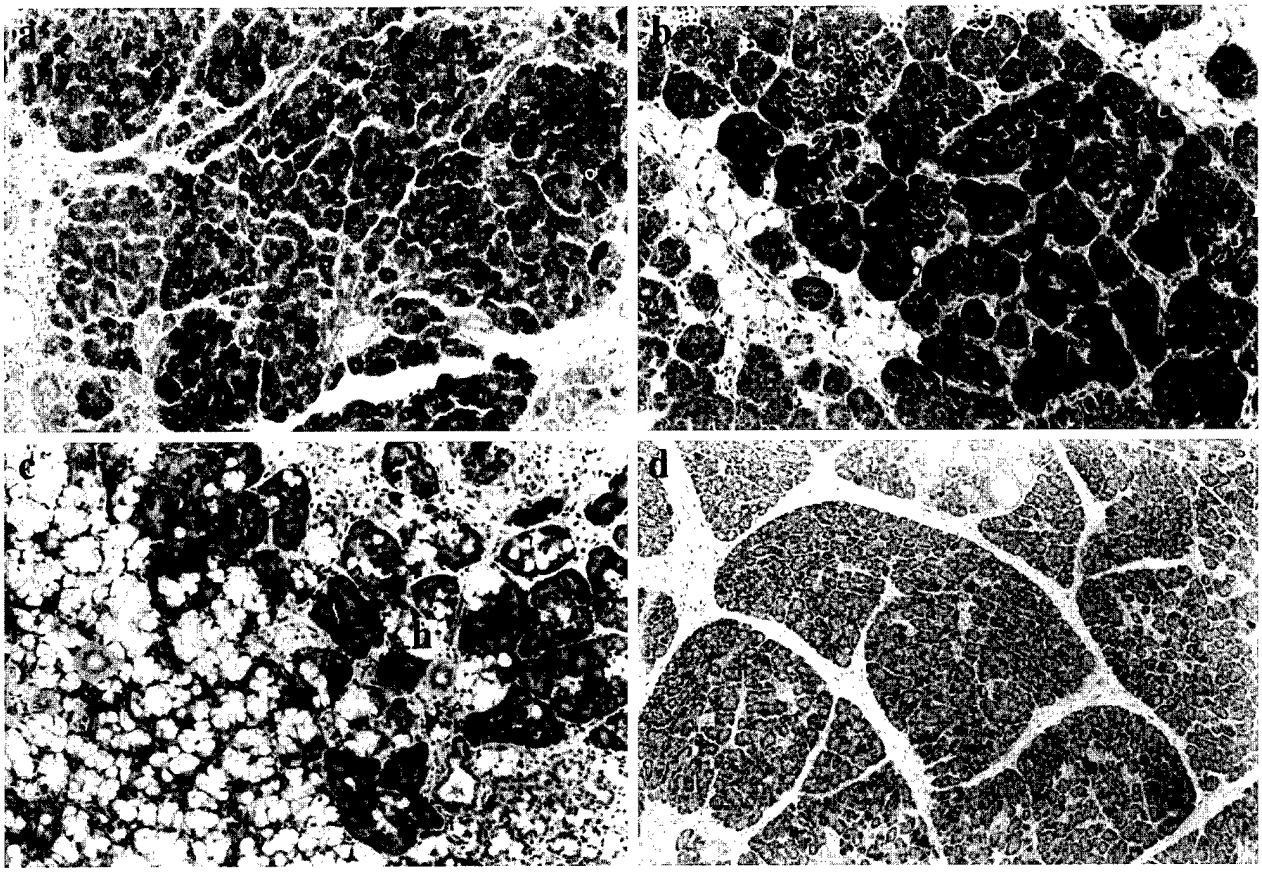

Fig. 5. Immunohistochemical staining of mammary and salivary glands from TGFα/c-*myc* double transgenic animals with an anti-TGFα polyclonal antiserum. *a*, a mammary gland tumor from a 3-month-old animal and *b*, from a 5-week-old animal. Both have a scattered pattern of strong TGFα staining. *c*, an immunohistochemical staining of a bitransgenic salivary gland (sublingual) with a premalignant atypical hyperplastic lesion (*h*) that stains strongly for TGFα, while the surrounding normal areas (*n*) appear negative. *d*, a negative control without a primary antibody.

and adenoma and adenocarcinoma in the parotid gland at the same age. Salivary glands of single transgenic TGFα animals showed minimal ductule hyperplasia, but single transgenic c-*myc* mice and transgene-negative littermates were free of pathological abnormalities at 3 months of age (Table 3; Fig 7).

At 7 months of age, histopathology of salivary glands from single transgenic animals revealed minimal serous metaplasia in the sublingual glands of c-*myc* mice, and mild duct(ule) squamous metaplasia and hyperplasia in the sublingual and submandibular glands of TGFα animals. Wild-type mice at 7 months of age had no apparent abnormalities. No tumors were ever observed in the salivary glands of single transgenic or wild-type mice (up to 10 months for TGFα mice and 15 months for c-*myc* mice).

To obtain information about the localization of expression of both transgenes within the gland, *in situ* hybridization analysis was performed on sequential sections of salivary glands from TGFα/c-*myc* animals (Fig. 4, *b, d,* and *f*). It revealed a very patchy pattern of expression of the c-*myc* transgene and scattered expression of the TGFα transgene. Expression was quite different from what we observed in the mammary glands in that only about 5% of salivary gland tissue was positive for both transgenes. Interestingly, areas where expression of both transgenes was detected appeared hyperplastic and atypical, whereas areas with only one transgene expressed looked quite normal. These areas might represent premalignant areas within the salivary glands, indicating that only when both transgenes are expressed does malignant conversion occur. Immunohistochemical staining of salivary glands from TGFα/c-*myc* animals was performed with a polyclonal antiserum that recognizes both the endogenous mouse TGFα and the transgene-derived human TGFα. In agreement with *in situ* hybridization data, we observed a scattered distribution of TGFα expression and an association of an intense staining with premalignant hyperplastic atypical nodules (as seen in the sublingual gland in Fig. 5*c*), whereas surrounding areas of normal or hyperplastic salivary gland had little or no TGFα staining. Finally, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) on salivary gland sections revealed a strong staining in areas that coexpressed the transgenes, indicating that DNA synthesis was occurring (Fig. 8).

Discussion

In this study, matings of MT-TGFα and MMTV-c-*myc* transgenic strains were carried out to investigate the *in vivo*

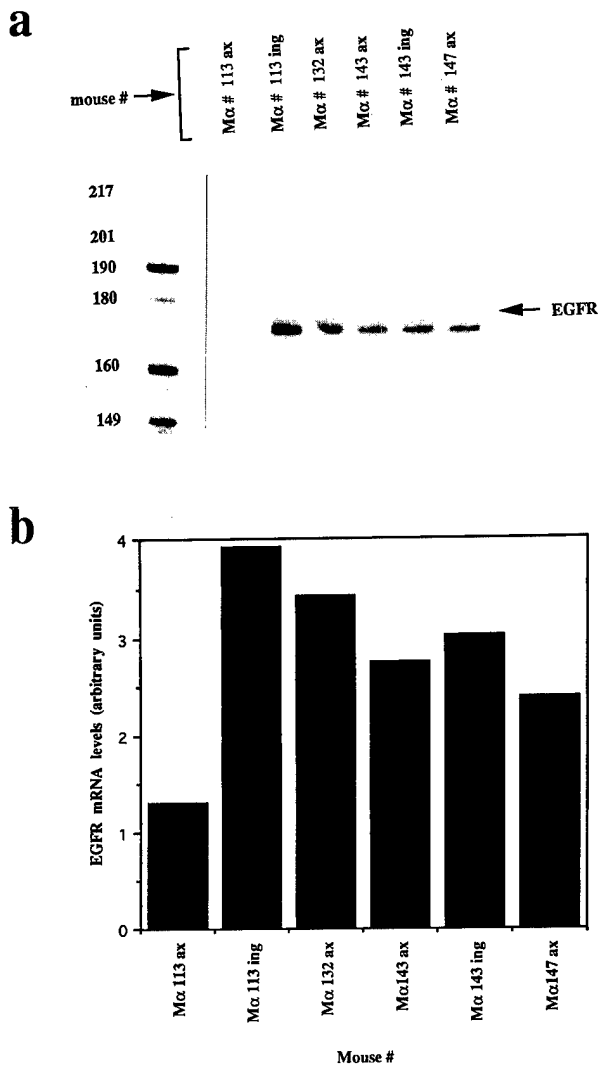

Fig. 6. Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. Mα113 through Mα147 denotes the number of each double transgenic mouse used in the assay. *ax*, axillary gland tumor; *ing*, inguinal gland tumor.

interaction of TGFα and c-Myc in mouse mammary gland transformation and confirm our previous *in vitro* studies that showed cooperation between the two. We found that tumorigenesis in mammary glands of double transgenic TGFα/c-*myc* virgin females and males is strikingly different from single transgenic and wild-type animals and also from multiparous single transgenic TGFα and c-*myc* mice. In previous studies, both MT-TGFα and MMTV-c-*myc* single transgenic mice developed polyclonal mammary gland tumors only after a long latency period and multiple pregnancies (21, 24, 35). In contrast, our studies show that both virgin females and males harboring both transgenes develop multiple mammary gland tumors after a mean latency period of only 66 days. All of 20 double transgenic virgin females and males developed rapidly growing mammary gland tumors that could be established in nude mice in the absence of estrogens. Single transgenic virgin TGFα and

Table 3 Summary of histopathological findings in salivary glands of transgenic animals at 3 and 7 months of age

All data represent both male and female animals.

Genotype	3 mos.	7 mos.
TGFα/c-*myc*	Hyperplasia with atypia, squamous metaplasia and adenoma, and adenocarcinoma	NA[a]
TGFα	Ductule hyperplasia	Ductule hyperplasia and squamous metaplasia
c-*myc*	Normal	Serous metaplasia
Wild type	Normal	Normal

[a] NA, not available (all animals of this genotype are deceased at this time point).

wild-type animals of both sexes did not develop any tumors, whereas about one-half single transgenic c-*myc* virgin females developed stochastic mammary gland tumors after a long latency period of about 8–12 months. The early onset and multiple tumor formation in double transgenic TGFα/c-*myc* animals suggests that very few, if any, additional genetic events are necessary for tumorigenesis in our model. In fact, at 3 weeks of age, when the glandular tissue has just started to penetrate the fat pad, the mammary gland is already tumorous.

It is also quite interesting that tumors form in a synchronous manner in our model, so that normal mammary gland tissue is not found at all. Two previous studies have described transgenic models with synchronous tumorigenesis of mammary glands. In the first one, an activated rat *neu* oncogene was expressed from the MMTV-LTR promoter/enhancer, and in the second, the polyoma middle T oncogene was expressed from the same promoter (36, 37). An extremely high level of transgene expression observed in the former strain might have contributed to the phenotype, since MMTV-*neu* transgenic mice made by another group developed only stochastic mammary gland tumors (38). However, the study has been repeated using the same transgene construct with similar results (39). Our model is comparable to the effects of a mutated growth factor receptor, Neu, or the powerful viral protein product of the polyomavirus middle T oncogene that mediates cellular transformation by targeting a number of intracellular signaling pathways (40–43). The fact that overexpression of two normal proteins in the mammary gland of transgenic mice has a similar effect on tumorigenesis in this organ as a mutated, highly active growth factor receptor and a strong viral oncoprotein further emphasizes the cooperative effect of TGFα and c-Myc.

An intriguing finding from our studies was that double transgenic TGFα/c-*myc* males developed mammary cancer in a manner indistinguishable from virgin females. Mammary gland cancer has been described previously in transgenic male mice of MMTV-*neu*, MMTV-v-Ha-*ras*, MMTV-*int*-1, MMTV-*int*-3, and MMTV-polyomavirus middle T strains. However, tumor onset is typically delayed compared to female mice (35–37, 44, 45). Both TGFα and c-*myc* are estrogen-inducible genes, and each has been shown to be responsible, at least in part, for estrogen-mediated growth *in vitro* (19, 20). It is, therefore, possible that when both genes are overexpressed *in vivo*, they induce growth of the male mammary gland in the absence of

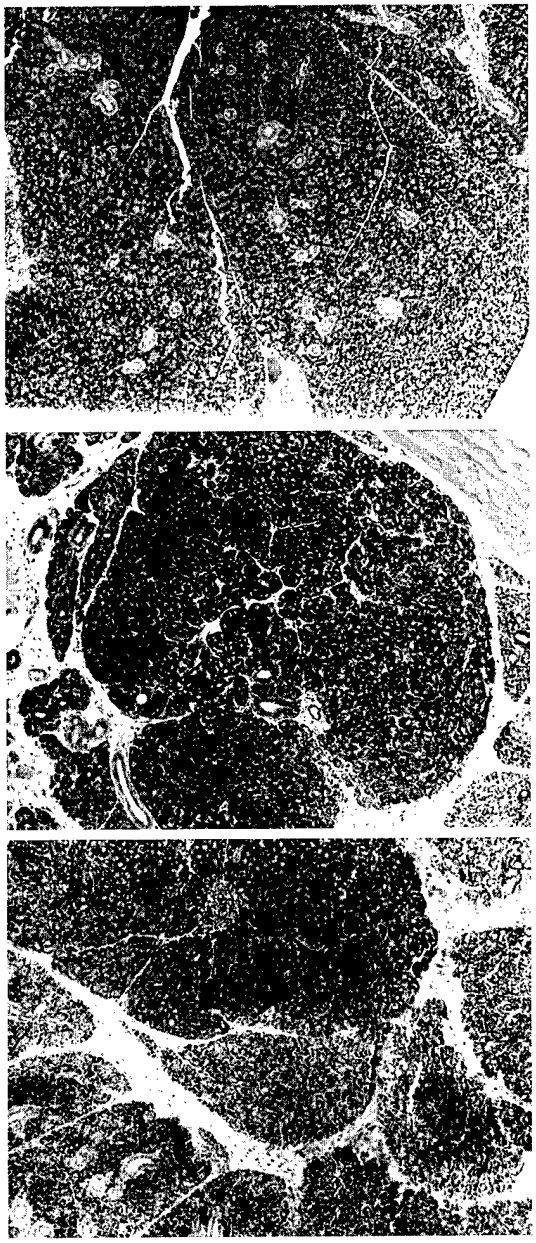

Fig. 7. Hematoxylin & eosin-stained sections of salivary glands. *a*, a normal parotid gland from a wild-type mouse. *b*, a parotid adenoma and *c*, an adenocarcinoma from TGFα/c-*myc* double transgenic animals. All animals are about 3 months old. ×100.

estrogens. Breast cancer in human males is extremely rare, but in the presence of exogenously applied estrogens, males can develop mammary gland hyperplasias (gynecomastia), thus showing that estrogens can cause proliferation in the male mammary gland in the presence of androgens. It is remarkable that overexpression of two estrogen-induced genes can stimulate the growth and malignant transformation of the male mammary gland as we have seen here. It encourages further studies to dissect the role of, and interaction between, mediators responsible for hormone action on the normal and malignant development of the mammary gland.

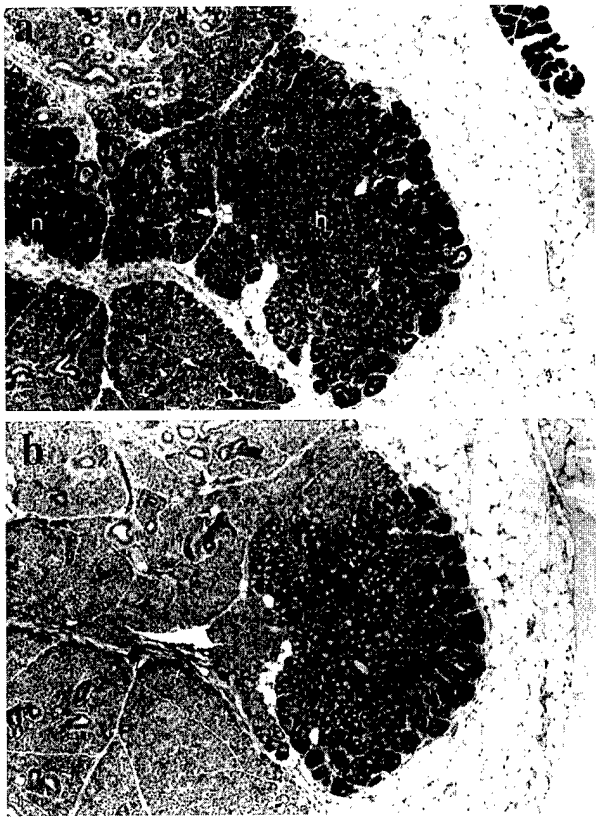

Fig. 8. Immunohistochemical staining of proliferating cell nuclear antigen in salivary glands from a 6-week-old double transgenic TGFα/c-*myc* animal. *a*, a hematoxylin & eosin-stained section of the salivary gland and *b*, a sequential section stained for proliferating cell nuclear antigen. Note intense proliferating cell nuclear antigen staining in hyperplastic areas of this field. This area in the parotid gland coexpressed the TGFα and c-*myc* transgenes. *h*, hyperplastic; *n*, normal. ×100.

A cooperative interaction between TGFα and c-Myc also exists in the salivary glands, although malignancies were somewhat less prominent than in mammary glands. In contrast to mammary glands of bitransgenic mice where normal tissue was not found, the salivary glands of double transgenic animals contained normal tissue juxtaposed with hyperplastic areas and frank tumors. However, single transgenic and wild-type animals did not develop salivary gland tumors, whereas they developed mammary gland tumors after a long latency (MMTV-c-*myc* female mice). Therefore, we conclude that a strong cooperative interaction also exists in the salivary glands.

In situ hybridization analysis revealed that expression of transgenes was more uniform in mammary glands than salivary glands. In fact, only about 5–10% of salivary gland tissue expressed detectable levels of c-Myc (mainly in the parotid gland). Interestingly, areas that expressed both transgenes appeared hyperplastic (salivary glands at 5 weeks) or tumorous (mammary glands at 3 weeks), indicating a requirement for both TGFα and c-Myc in tumorigenesis. In the salivary gland, this was quite clear since areas were found that expressed either TGFα, c-*myc*, both transgenes, or no transgenes. A progressive tumor onset was associated with a patchy expression of transgenes in the salivary glands. On the other hand, an extremely rapid

tumor onset was associated with a near uniform expression of transgenes in the mammary glands. This might suggest that additional events must occur in the course of the slower tumorigenesis in salivary glands. In the mammary glands, TGFα and c-Myc appear to be sufficient to mediate transformation, although additional events cannot be ruled out. In both glands, there appears to be a selective advantage to express increasing levels of the TGFα transgene in the course of malignant progression. The mechanism of this effect is not known, but an apparently similar phenomenon was observed previously in mouse skin carcinogenesis in transgenic TGFα mice (46).

In summary, TGFα and c-*myc* are extremely powerful, synergistic-acting genes in breast and salivary gland carcinogenesis in the mouse strains described here. Since TGFα and c-Myc cause uniform transformation of the mammary gland of transgenic mice, this model provides an ideal system to examine possible secondary events for malignant progression/metastasis and characterize the relevance of a deregulated TGFα/EGF receptor pathway in mammary tumorigenesis.

Materials and Methods

Transgenic Mice. The MMTV-c-*myc* mice used in this study were obtained from Charles River Laboratories (Wilmington, MA); experiments were carried out under a breeding license agreement with Du Pont Medical Products (Wilmington, DE). All mice were rederived and were free of adventious agents. Line MT100 has a mouse metallothionein I (MT) promoter driving expression of a human TGFα cDNA transgene in an FVB/N inbred genetic background (21). The MMTV-c-*myc* M line harbors a mouse c-*myc* gene driven by the mouse mammary tumor virus long terminal repeat promoter/enhancer (MMTV-LTR) in a CD-1 × C57BL/6J background (24). Both strains have been shown to form stochastic mammary gland tumors after a long latency period and multiple pregnancies. Double transgenic mice were generated by mating the MT-TGFα MT100 line to the MMTV-c-*myc* M line. Offspring were maintained on 50 mM $ZnCl_2$ drinking water from the time of weaning (3–4 weeks of age) to induce maximal expression of the TGFα transgene from the metallothionein promoter.

Genotyping of Offspring for Transgenes. DNA was isolated from 1-cm tail biopsies by an overnight proteinase K digestion at 55°C, followed by phenol/chloroform extractions and ethanol precipitation. For Southern analysis, 10 μg of tail DNA was digested overnight at 37°C with the following restriction enzymes: *Bgl*II for TGFα; and *Bam*HI and *Cla*I for c-*myc*. After electrophoresis through 0.8% agarose gels and transfer to nitrocellulose, blots were probed with random-primed ^{32}P-labeled cDNA probes for human TGFα and mouse c-*myc*. The TGFα probe was a 925-bp *Eco*RI fragment from the plasmid pTGFα, kindly provided by Dr. Francis Kern (Georgetown University, Washington, DC; Ref. 47). The c-*myc* probe was a 2400-bp *Eco*RI-*Xba*I fragment from the plasmid fpGV-1, generously provided by Dr. MaryLou Cutler (NIH, Bethesda, MD; Ref. 48).

For PCR, 3 μg DNA from tail biopsies was used as a template to amplify transgenes. 3′ primers were complementary to sequences in the TGFα and c-*myc* transgenes, and 5′ primers to sequences in the metallothionein (MT) and MMTV promoters, respectively: MT-TGFα 5′ primer, 5′-TCG TCC CCG AGC CAG TCG-3′; MT-TGFα 3′ primer, 5′-GTC CGT CTC TTT GCA GTT CTT-3′; MMTV-c-*myc* 5′ primer, 5′-CCC AAG GCT TAA GTA AGT TTT TGG-3′; and MMTV-c-*myc* 3′ primer, 5′-GGG CAT AAG CAC AGA TAA AAC ACT-3′. Primers were made by the Lombardi Cancer Center Macromolecular Synthesis and Sequencing Core facility (Georgetown University, Washington DC). PCR was performed using the Perkin Elmer Taq polymerase kit (Perkin Elmer, Norwalk, CT). The TGFα and c-*myc* transgenes were detected with Southern analysis and/or PCR.

Tumors and Histopathology. Mice were palpated biweekly for tumors and sacrificed before tumor sizes reached 10% of body weight. Location and size of each tumor were determined. Tumors were fixed in Bouin's solution for 5–12 h, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined to determine histopathological diagnoses. Transplantation of tumors into ovariectomized female NCR *nu/nu* mice was performed as follows. Tumor-bearing mice were anesthetized with methofane, and tumors were excised aseptically. Tumors were cut into about 1-mm^2 pieces and inserted s.c. (between nipples nos. 2 and 3) of nude mice under anesthesia.

Whole-Mount Staining. Animals were sacrificed, and the inguinal mammary glands were removed and fixed in 25% glacial acetic acid and 75% ethanol for 60 min at room temperature. After staining overnight in carmine alum solution [1 g carmine and 2.5 g aluminium potassium sulfate (both from Sigma Chemical Co.) in 500 ml water] glands were dehydrated in a series of ethanol washes and finally cleared in toluene. Glands were stored and photographed in methyl salicylate.

Estrogen Receptor Binding Assay. Frozen tumor samples (50–100 mg) were pulverized in liquid nitrogen and homogenized at 0°C in TEDG [10 mM Tris-OH (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10% glycerol] plus 0.5 M NaCl and a cocktail of proteolysis inhibitors (leupeptin at 1 mg/ml, aprotinin at 77 μg/ml, and pepstatin A at 1 μg/ml). Homogenates were centrifuged at 105,000 × *g* at 4°C for 30 min to yield a whole-cell lysate, which was then adjusted to 2 mg/ml protein. Lysates were incubated with 10 nM [^{3}H]17β-estradiol with or without a 100-fold excess of unlabeled estradiol for 16 h at 4°C. Binding was assayed by adding dextran-coated charcoal to adsorb free hormone. After centrifugation, aliquots of supernatant were removed and counted in 10 ml of liquid scintillation fluid in a Beckman liquid scintillation counter. Estrogen receptor-positive control tumors were MCF-7 and MKL-4 breast cancer cell lines grown in nude mice (49). They were generously provided by Dr. Sandy McLeskey (Georgetown University, Washington DC).

RNA Isolation and Analysis. Total RNA was isolated by pulverizing frozen tumors in liquid nitrogen, followed by homogenization in guanidine thiocyanate, acid phenol extraction, and precipitation with isopropanol. TGFα and c-*myc* transgene expression was assessed by Northern blot hybridization; 15 μg total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL), and probed with a ^{32}P-labeled random-primed probes. The c-*myc* probe was generated from the plasmid fpGV-1 (as described above) and the TGFα probe from the plasmid pTGFα-RP as described previously (50).

EGFR expression was determined using RNase protection assays in which ^{32}P-labeled antisense riboprobes (cRNA) were synthesized *in vitro* from the plasmid pME2.0 for the

EGFR, by linearizing with *Hin*dIII and transcribing with SP6 polymerase (51). It yields a 170-bp protected EGFR fragment. This plasmid was kindly provided by Dr. M. Rosner (University of Chicago, Chicago, IL). Total RNA (30 μg; EGFR) was hybridized for 12–16 h at 42°C to the ^{32}P-labeled cRNA probe and treated with RNase A for 30 min at 25°C. The radiolabeled riboprobes protected by total RNA were run on a 6% polyacrylamide/7M urea gel, which was subsequently dried and exposed to autoradiography.

***In Situ* Hybridization Analysis.** To detect localization of transgene expression, *in situ* hybridization analysis was performed on mammary and salivary glands from 3; 6; and 10-week old double transgenic TGFα/c-*myc* animals. Animals were sacrificed, and glands were fixed in 4% paraformaldehyde in PBS for 24 h. *In situ* hybridization analysis was performed by Molecular Histology, Inc. (Gaithersburg, MD Ref. 52). Probes were generated from plasmids; p.c.-*myc*20 was generously provided by Dr. S. Thorgeirsson (NIH, Bethesda, MD). For the antisense riboprobe, this plasmid was linearized with *Eco*RI and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was linearized with *Hin*dIII and transcribed with SP6 polymerase. Plasmid pTGFα-pGem3Z was used to detect the TGFα transgene. For an antisense riboprobe, this plasmid was linearized with *Hin*dIII and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was digested with *Eco*RI and transcribed with SP6 polymerase.

Immunostaining. Tissues were fixed in Bouin's solution for 5–12 h, embedded in paraffin, and sectioned. After treatment with 0.02% trypsin for 15 min, sections were incubated overnight at room temperature with a 1:20,000 dilution of a rabbit polyclonal antiserum generated against a rat pro-TGFα intracellular peptide (residues 137–159). The antibody was kindly provided by Dr. Larry Gentry (Medical College of Ohio, Toledo, OH). TGFα was localized using the Vectastain Rabbit Elite kit (Vector Laboratories, Burlingame, CA), as described previously (50). PCNA immunostaining was performed as described previously (53).

Acknowledgments

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Cooperation of TGFα and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis

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We have previously shown that TGFα and c-Myc interact in a strong, synergistic fashion to induce mammary gland tumors in double transgenic mice. Here we show this interaction can be explained, at least in part, by a cooperative growth stimulus by the two proteins, and by TGFα-mediated inhibition of c-Myc-induced apoptosis. We initially compared rapidly progressing mammary tumors from double transgenic mice to long latency tumors from single transgenic mice and observed a striking difference in the occurrence of apoptosis among the three groups. Tumors exhibiting apoptosis were derived exclusively from mice that expressed the c-*myc* transgene in the absence of the TGFα transgene, indicating that TGFα might protect c-Myc-overexpressing cells from programmed cell death. Cell lines were derived from single and double transgenic mammary tumors to examine further the mechanism underlying the cooperative interaction between the two gene products. In accordance with our *in vivo* data, apoptosis was only detected when the c-*myc* transgene was expressed without the TGFα transgene. Furthermore, exogenous addition of TGFα inhibited apoptosis in cells overexpressing c-Myc alone. In addition, tumor-derived cells that overexpressed both TGFα and c-Myc exhibited faster growth rates *in vitro* and *in vivo* and were less sensitive to the inhibitory effects of TGFβ *in vitro* compared to cell lines expressing only one of the transgenes. Based on our findings we propose that TGFα acts both as a proliferative and a survival factor for c-Myc-expressing tumor cells. Our results indicate that TGFα and c-Myc cooperate in tumorigenesis *via* a dual mechanism: TGFα can inhibit c-Myc-induced apoptosis and both proteins provide a growth stimulus.

Keywords: c-Myc; TGFα; apoptosis; mammary tumorigenesis

Introduction

It is well documented that overexpression of the proto-oncogene c-*myc* can induce proliferation, transformation, and apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Kato and Dang, 1992; Khazaie *et al.*, 1991; Marcu *et al.*, 1992; Meichle *et al.*, 1992; Telang *et al.*, 1990; Valverius *et al.*, 1990). It has also been reported by a number of investigators that c-Myc can cooperate with growth factors such as transforming growth factor alpha (TGFα) or epidermal growth factor (EGF) to promote a transformed phenotype *in vitro* (Khazaie *et al.*, 1991; Stern *et al.*, 1986; Telang *et al.*, 1990; Valverius *et al.*, 1990). We and others have recently shown that c-Myc and TGFα synergize in an extremely strong way to induce mouse mammary gland tumors in transgenic mice *in vivo* as well (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). In order to understand the mechanisms responsible for this interaction, we were interested in examining proliferation, anchorage independent growth, and apoptosis as possible points of interaction between TGFα and c-Myc that may enhance tumorigenesis in the mammary gland.

Both gene products have been implicated in the genesis of many human cancers, including breast tumors. The c-*myc* gene is frequently found amplified and/or overexpressed in human breast cancer (Bonilla *et al.*, 1988; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Costantini *et al.*, 1988). Although TGFα is not amplified at the gene level in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (Artega *et al.*, 1988; Bates *et al.*, 1988; Derynck *et al.*, 1987; Perroteau *et al.*, 1986; Travers, 1988). In addition, various groups have reported a tumorigenic action of these genes when overexpressed in the mammary gland of transgenic mice (Jhappan *et al.*, 1990; Leder *et al.*, 1986; Matsui *et al.*, 1990; Sandgren *et al.*, 1990; Schoenenberger *et al.*, 1988; Steward *et al.*, 1984).

Apoptosis is an active process whereby the cell is programmed to carry out a series of events that eventually lead to its auto-destruction. When apoptosis is initiated, cells undergo various biochemical and morphological changes which result in the degradation of genomic DNA and fragmentation of the cell into apoptotic bodies (Bellamy *et al.*, 1995). Apoptosis occurs during development as well as in adult organisms and can be activated or inhibited by specific agents, such as hormones or growth factors (Schwartzman and Cidlowski, 1993). Inhibition of apoptosis can also contribute to tumorigenesis.

The c-Myc protein has been implicated in the regulation of apoptosis. When c-Myc expression is deregulated, cells are prone to enter an apoptotic pathway, depending on the cell environment (Askew *et al.*, 1991; Evan *et al.*, 1992). A number of survival factors which protect cells from c-Myc-mediated

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The first two authors (LT Amundadottir and SJ Nass) made equal contributions to the writing of this manuscript. RTA established and characterized the 3 cell lines in the study and was responsible for Figures 1, 2ab, 3ab, 5a and 6 while SJN contributed Figures 1, 2c, 3ac, 4, 5ab and 6.
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apoptosis have been identified (Askew *et al.*, 1991; Harrington *et al.*, 1994). In mouse fibroblasts for example, apoptosis induced by c-Myc was inhibited by various growth factors, such as insulin-like growth factors and PDGF, whereas EGF and bFGF were ineffective. In the current study, we show that TGFα can function as a survival factor for mammary epithelial cells which overexpress c-Myc. We observed that apoptosis occurred in mouse mammary gland tumors and in their derived epithelial cell lines only in cases where c-Myc was overexpressed in the absence of TGFα overexpression. Furthermore, when c-Myc overexpressing cells were treated with TGFα *in vitro*, apoptosis was greatly decreased. Our results suggest an explanation for the cooperative interaction between TGFα and c-Myc in tumorigenesis: both factors stimulate anchorage dependent proliferation and anchorage independent growth, and in addition, TGFα suppresses c-Myc-induced apoptosis.

Results

*Programmed cell death occurs only in mammary gland tumors from c-*myc *single transgenic mice*

To examine whether apoptosis was a factor in the cooperation between TGFα and c-Myc in tumorigenesis, we measured apoptosis in five mammary gland tumors from each of the three transgenic mouse strains (double transgenic TGFα/c-*myc* mice, and single transgenic TGFα and c-*myc* mice). *In situ* nick end-labeling of nucleosomal fragments by Klenow DNA polymerase I revealed that apoptosis was occurring in mammary gland tumors from c-*myc* transgenic animals and not in mammary tumors from single transgenic TGFα animals or double transgenic TGFα/c-*myc* animals. As shown in Figure 1c, only tumors from c-*myc* mice exhibited scattered cells with positive staining. When apoptosis was quantitated by counting apoptotic cells in 20 random fields of each tumor type (400 × magnification) we observed that tumors from c-*myc* mice had 23.0 ± 2.8 apoptotic cells per field whereas tumors from TGFα and TGFα/c-myc animals had 0.3 ± 0.2 and 1.2 ± 0.5 labeled cells per field respectively.

Generation of cell lines from mammary gland tumors

We have generated three cell lines from mammary gland tumors arising in double and single transgenic mice. Our intent was to use them to verify our findings in mammary gland tumors *in vivo* and examine further the molecular mechanisms underlying the cooperation between TGFα and c-Myc. The following nonclonal cell lines were generated: TGFα/Myc#75 was derived from a tumor arising in a double transgenic virgin female (TGFα/c-*myc* animal number 75), Myc#83 was derived from a c-*myc* virgin single transgenic female (c-*myc* animal number 83) and TGFα#13 from a multiparous TGFα single transgenic female (TGFα animal number 13). Additional cell lines from tumors arising in c-*myc* and TGFα/c-*myc* animals have recently been isolated and were used where noted to confirm our findings with the first three cell lines.

Expression of cytokeratins and morphology of cell lines grown as subcutaneous tumors in nude mice was used to verify epithelial origins of the cell lines. The single transgenic cell lines TGFα#13 and Myc#83 were positive for keratin 14 at the mRNA level (not shown). TGFα/Myc#75 cells apparently did not express keratin 14, but positive immunofluroescent signal was observed in these cells with a pan-keratin antibody (not shown). All three lines also gave rise to tumors in nude mice that had a very distinct epithelial morphology. We therefore conclude that all three lines are epithelial, but that line TGFα/Myc#75 has probably lost expression of some of its keratins. This is not without precedent, since human breast

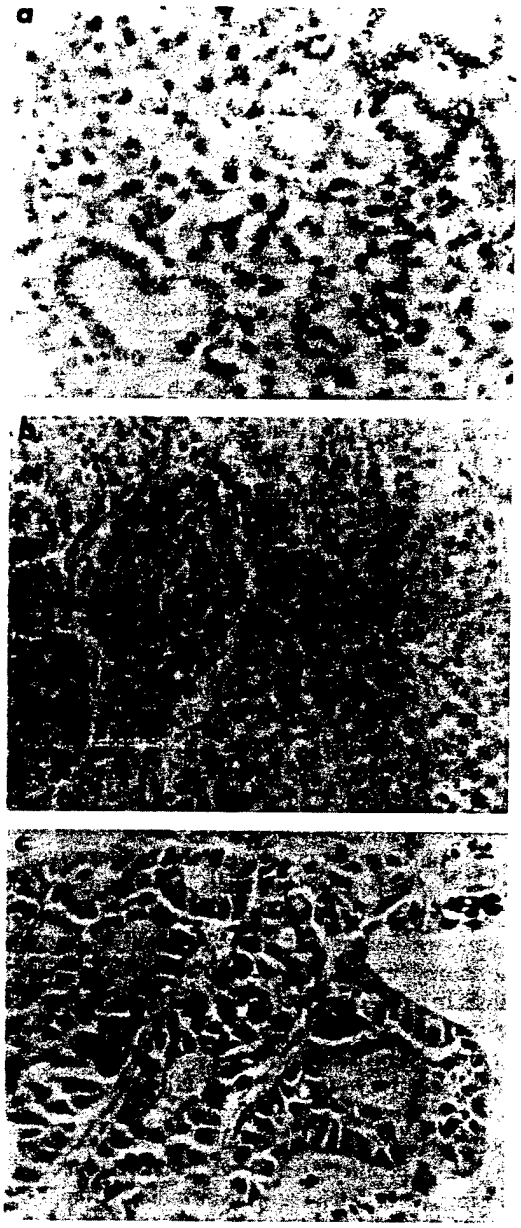

Figure 1 Detection of apoptosis in mammary gland tumors from transgenic mice. Tumor sections were analysed by *in situ* nick end-labeling of DNA fragments. (**a**) shows a tumor from a double transgenic virgin TGFα/c-*myc* mouse, (**b**) a tumor from a single transgenic multiparous TGFα mouse and (**c**) a tumor from a single transgenic c-*myc* mouse. Note cytoplasmic staining in scattered cells of the tumor from a c-*myc* single transgenic animal (arrows, **c**) indicating DNA fragmentation

carcinoma cell lines, especially hormone independent lines, have been described to do the same (Sommers *et al.*, 1989, 1992).

Expression of the TGFα and c-*myc* transgenes was also assessed by Northern analysis (not shown). The single transgenic lines, TGFα#13 and Myc#83, expressed only the TGFα or c-*myc* transgenes, respectively. In contrast, the double transgenic cell line, TGFα/Myc#75, expressed both transgenes. Therefore, all three cell lines expressed the expected transgenes at the mRNA level. In addition, expression of the TGFα transgene was upregulated by ZnC12 and CdC12 as expected, since the TGFα transgene is expressed from the heavy metal-inducible metallothionein promoter (Jhappan *et al.*, 1990). The endogenous c-*myc* gene was downregulated in the two cell lines expressing the c-*myc* transgene (TGFα/Myc#75 and Myc#83), consistent with a negative autoregulation of the c-Myc protein on its own promoter, as has been described previously (Penn *et al.*, 1990).

In order to assess ploidy of the cell lines, cells were stained with propidium iodide and analysed by FACS. Each cell line was tested at two or three different timepoints (between passages 6 and 31). Only the double transgenic line TGFα/Myc#75 was found to be aneuploid. It was tetraploid at all three timepoints tested. In contrast, the two single transgenic lines were diploid at the time points tested (not shown).

Apoptosis occurs in c-Myc overexpressing mammary tumor cells in vitro

Tumor derived cell lines were tested for their ability to undergo apoptosis by two independent methods. For the first method, cytoplasmic DNA fragments were isolated and run on agarose gels. In this assay, the Myc#83 line was positive with a characteristic nucleosomal ladder whereas both TGFα#13 and TGFα/Myc#75 were negative (Figure 2a). Those results were confirmed by an ELISA apoptosis assay that is based on detecting histone-associated DNA in cytoplasmic cell lysates via a peroxidase catalyzed color change (A_{405}). CEM cells (a T cell leukemia cell line) treated with 10^{-7} M dexamethasone served as a positive control (Catchpoole and Stewart, 1993; not shown). The Myc#83 line showed a high degree of apoptosis, whereas the other two had levels close to background (Figure 2b). Five additional cell lines derived from Myc-single transgenic tumors also showed a propensity to undergo apoptosis as determined by the apoptosis ELISA (not shown). The appearance of apoptotic Myc#83 cells under conditions of EGF deprivation or TGFβ1 treatment was further confirmed by observing morphological changes which are characteristic of apoptosis (Figure 2c). The cells displayed prominent apoptotic bodies with concomitant reduction of cytoplasm and altered nuclear morphology.

When the Myc#83 line was treated with the growth factors TGFα, EGF, IGF-I or bFGF, apoptosis was inhibited up to 75% as measured by the ELISA assay (Figure 3a). In contrast, treatment with TGFβ1 resulted in elevated levels of apoptotic DNA, even in the presence of EGF. The effects of both TGFα and TGFβ1 on apoptosis were concentration dependent (Figure 3b and c), with maximal responses at 10 ng/ml and 100 pM, respectively.

Additional evidence for the importance of the TGFα/EGF receptor system was provided by using a synthetic inhibitor of EGF receptor tyrosine kinase activity (PD153035). PD153035 has been shown to

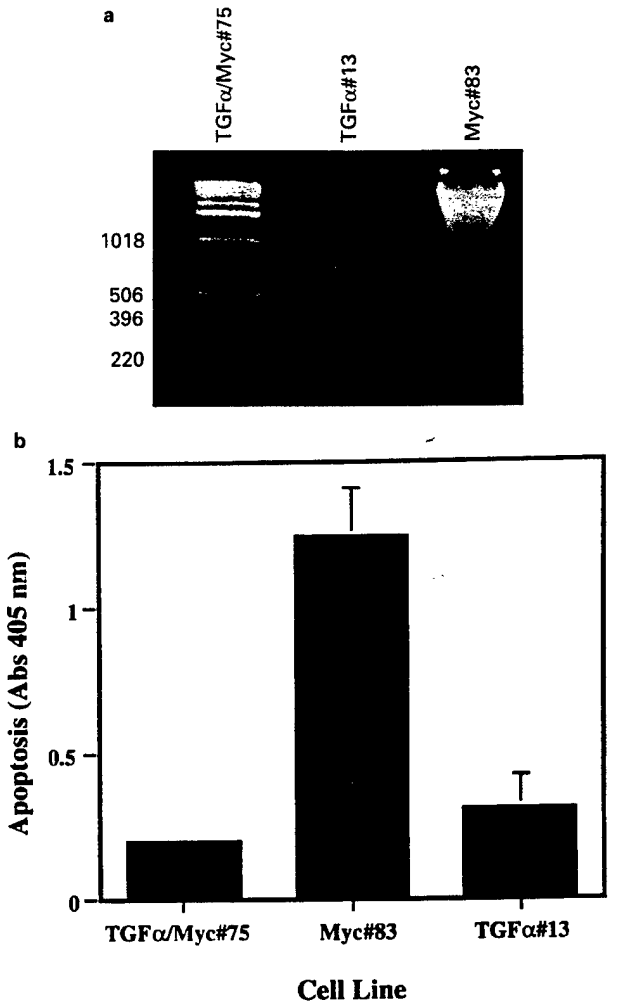

Figure 2 Detection of apoptosis in tumor-derived cell lines. (**a**): Nucleosomal ladder. DNA was isolated from cytoplasmic fractions of transgenic tumor cell lines and run on a 1.8% agarose gel which was stained with ethidium bromide. Note characteristic nucleosomal DNA with a size interval of approximately 180 bp. (**b**): ELISA-apoptosis assay. Apoptotic DNA fragments were detected in cytoplasmic lysates *via* a histone-DNA ELISA. Peroxidase substrate conversion was quantitated by measuring absorbance at 405 nm. (**c**): Morphological changes in apoptotic Myc#83 cells. Cells on the left side were grown in media containing EGF. The right side is representative of cells which have become apoptotic via EGF deprivation or TGFβ1 treatment, for 24 h. Note the characteristic reduction in cell size due to cytoplasmic blebbing (apoptotic bodies)

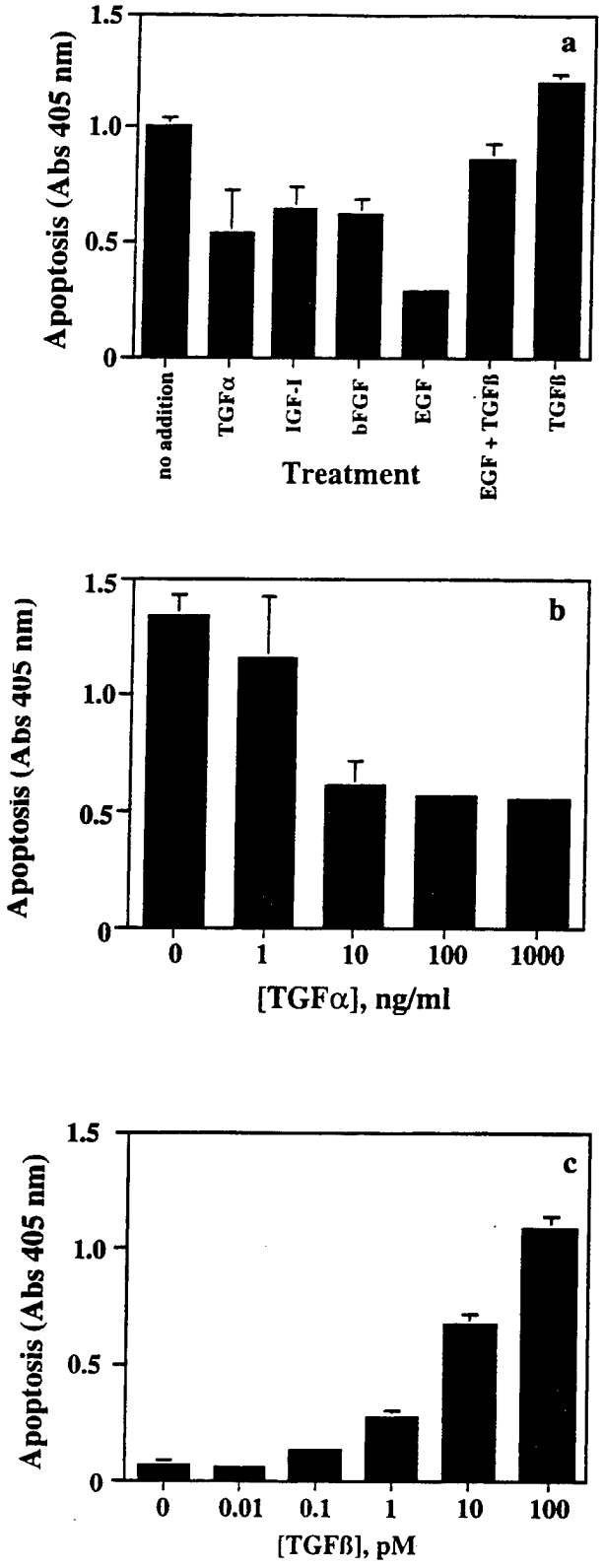

Figure 3 Effects of growth factors on apoptosis in the Myc#83 line. (a) shows the effects of TGFα (5 ng/ml), IGF-I (50 mM), bFGF (8 ng/ml), EGF (10 ng/ml), TGFβ1 (100 pM) or EGF + TGFβ on apoptosis. (b) shows that the inhibitory effect of TGFα on apoptosis in the Myc#83 cell line is concentration dependent. (c) demonstrates that the stimulatory effect of TGFβ1 on apoptosis is also concentration dependent. In all three panels, cells were treated for 24 h prior to harvest for apoptosis ELISA. Each point represents the mean (±SE) of two determinations

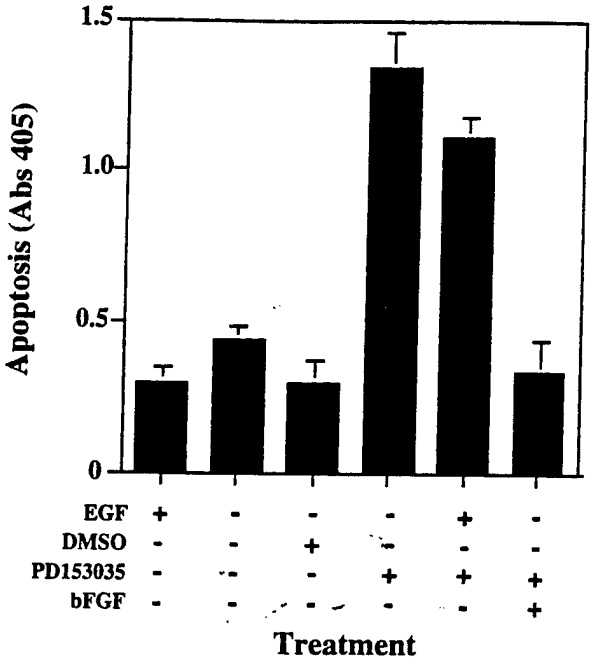

Figure 4 Induction of apoptosis in the TGFα/Myc#75 line by PD153035, a specific inhibitor of EGF receptor tyrosine kinase activity. Cells were incubated for 24 h with the indicated additions (10 ng/ml EGF or bFGF, 10 μM PD153035, 1 μl/ml DMSO as a control) and apoptosis was measured via ELISA assay. $n=4$ (±SE)

specifically downregulate the tyrosine phosphorylation status of the EGFR (Fry *et al.*, 1994). We verified this in our cell system by an anti-phosphotyrosine Western blot which showed that a 170 kb species was reduced greater than 90% in cells treated with the compound, while other phosphotyrosine bands remained constant (not shown). The TGFα/Myc#75 cells became apoptotic when exposed to PD153035 for 24 h (Figure 4). Removing EGF from the growth media of these cells did not affect viability, but exposure to PD153035 in either the presence or absence of EGF induced apoptosis. In contrast, bFGF, which acts through a different receptor tyrosine kinase, could rescue the cells from the effects of the drug.

Anchorage dependent (ADG) and anchorage independent (AIG) growth analysis of tumor derived cell lines

The TGFα/Myc#75 double transgenic cell line had the fastest ADG growth rate *in vitro* under normal growth conditions (doubling time of 16.7 h ± 0.4 h). Myc#83 and TGFα#13 had much longer doubling times of 33.4 h (±1.7) and 35.0 h (±0.82) respectively when grown in normal media containing EGF (Figure 6a). Growth rates of all three cell lines were similar when EGF was replaced with TGFα (not shown). The two cell lines that overexpress TGFα (TGFα/Myc#75 and TGFα#13) were able to grow in the absence of exogenous EGF, but with a significantly reduced growth rate (Figure 5a). Two additional TGFα/Myc cell lines also exhibited relatively fast growth rates and were not dependent on exogenous EGF for growth or survival, similar to TGFα/Myc#75 (not shown). In

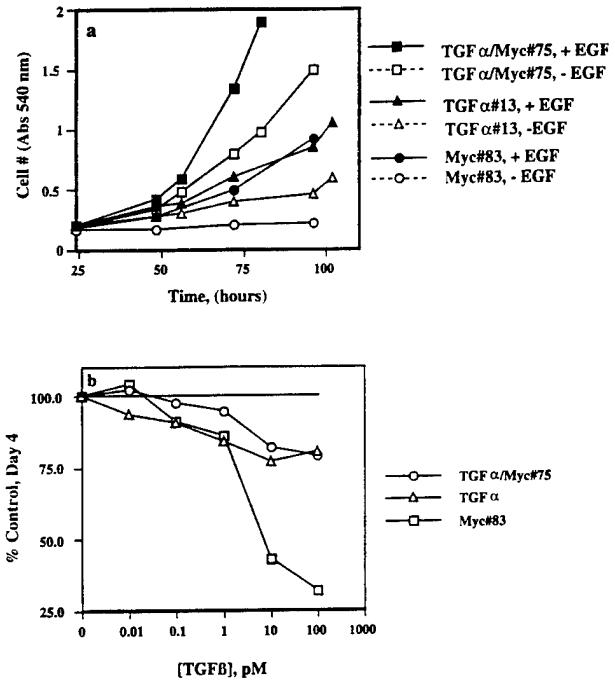

Figure 5 Co-expression of TGFα and Myc results in cooperative growth stimulus under anchorage dependent conditions. (**a**): Cells were grown in the presence or absence of EGF in 96 well plates for the indicated times and stained with crystal violet. (**b**): Cells were grown in the presence of EGF and increasing concentrations of TGFβ1 for 3 (TGFα/Myc#75) or 4 (Myc#83, TGFα#13) days and then stained with crystal violet. For (**a**) and (**b**), $n=8$ (±SE)

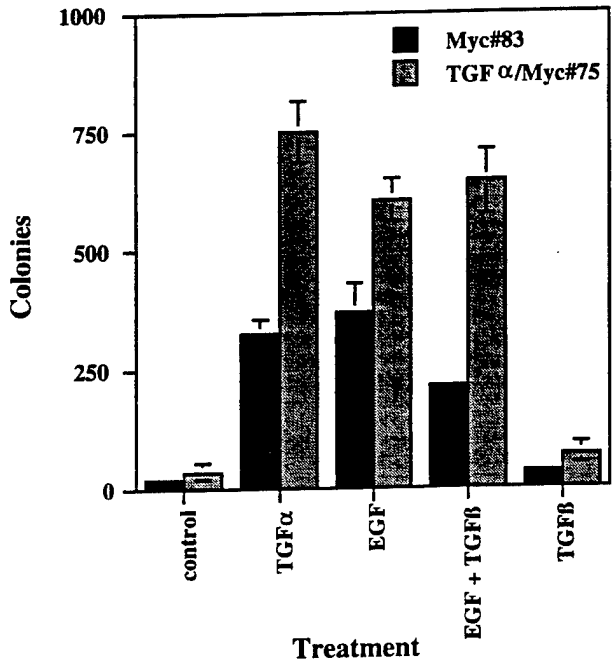

Figure 6 Co-expression of TGFα and Myc results in cooperative growth stimulus under anchorage independent conditions. Cells were suspended in 0.3% agar with 10% FCS and the following additions: TGFα (5 ng/ml), EGF (10 ng/ml), TGFβ1 (100 pM) or EGF + TGFβ1. Colonies were counted on day 7 (TGFα/Myc#75) or day 10 (Myc#83). $n=3$ (±SD). Results for TGFα#13 are not shown since that cell line did not grow well in soft agar under any of the above conditions

contrast, Myc#83 cells were completely dependent on exogenous EGF and showed no significant growth in its absence. However, FACS analysis demonstrated that Myc#83 cells were not arrested in G0/G1 when deprived of EGF (not shown). Those results are consistent with the hypothesis that Myc overexpressing cells are unable to withdraw from the cell cycle and undergo apoptosis in the absence of EGF.

All three cell lines exhibited concentration dependent inhibition by TGFβ1 under ADG conditions, but sensitivity to the growth factor varied (Figure 5b). At the highest concentrations (10–100 pM), TGFα/Myc#75 and TGFα#13 were marginally responsive to TGFβ, with about 20% fewer cells in treated wells than in untreated controls after 3–4 days in culture. In contrast, Myc#83 cells were quite sensitive to high concentrations of TGFβ1, reflecting the observation that TGFβ induces apoptosis in these cells (Figure 3a,c).

TGFα/Myc#75 and Myc#83 cells both grew well under AIG conditions in the presence of exogenous EGF or TGFα (Figure 6), whereas TGFα#13 cells grew poorly in soft agar. The effects of those growth factors on Myc#83 cells are similar to published results for other Myc-overexpressing breast cells (Telang *et al.*, 1990; Valverius *et al.*, 1990). The rate of colony formation and growth was much higher for the TGFαMyc#75 cells, and a dose response curve showed that those cells were extremely sensitive to TGFα, with optimal induction by only 0.1 ng/ml of the growth factor. Maximal colony formation by the Myc#83 line occurred with 10–30 ng/ml TGFα (not shown). Addition of TGFβ1 significantly reduced the number of Myc#83 colonies stimulated by addition of EGF, but had no effect on TGFα/Myc#75 colony formation (Figure 6).

Tumorigenicity of tumor derived cell lines

Cells were injected into female *nu/nu* mice to establish their *in vivo* tumorigenicity and growth rate. All three cell lines grew readily in nude mice, but with different latency times. The double transgenic line TGFα/Myc#75 formed tumors with a latency period of only 4 weeks, while the single transgenic TGFα#13 and Myc#83 lines formed tumors with a latency period of about 9 weeks. None of the cell lines appeared to have metastatic capabilities over the period of time the tumors were allowed to grow (About 2 months for TGFα/Myc#75 and 3 months for TGFα#13 and Myc#83).

Discussion

Myc overexpression (achieved by gene amplification, translocations and other means) has been strongly implicated in the genesis of many types of human tumors including breast cancer (Bonilla *et al.*, 1988; Cole, 1986; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Constantini *et al.*, 1988). However, since deregulated c-Myc expression can promote cell death *via* apoptosis, it is likely that the apoptotic pathway(s) induced by c-Myc must be inhibited or inactivated to achieve aggressive tumor formation. That may be accomplished, directly or indirectly, by secondary

events which alter the cell environment (such as growth factor secretion) or gene expression (such as mutations in downstream effectors). In accordance with that hypothesis, we detected apoptosis only in mammary gland tumors that expressed the c-*myc* transgene alone without the TGFα transgene. Tumors from TGFα single transgenic or TGFα/c-*myc* double transgenic mice did not contain apoptotic cells. Van Dyke and co-workers have proposed a similar 'multi-hit' hypothesis of tumor formation based on studies of SV40 T antigen-induced brain tumors (Symonds *et al.*, 1994). In that system they found that wild type T antigen induced rapidly growing, aggressive tumors, whereas a mutated form of the protein which only interfered with pRb function produced very slow growing tumors which displayed a high percentage of apoptotic cells. In contrast, expression of the mutant T antigen in a p53-null background resulted in tumors which were indistinguishable from those induced by the wild type protein. Taken together, the results suggest that the first event in cancer initiation stimulates both proliferation and apoptosis and that a secondary event which blocks apoptosis is necessary for aggressive tumor formation.

Cell lines derived from the tumors provided an *in vitro* confirmation of our *in vivo* observations. Apoptosis was observed only in the cell line derived from a Myc single transgenic animal (Myc#83), while the two cell lines overexpressing TGFα (TGFα/Myc#75 and TGFα#13) did not undergo apoptosis under normal culture conditions. TGFα/Myc#75 cells only became apoptotic when exposed to a specific inhibitor of EGF receptor tyrosine kinase activity (PD153035), suggesting that these cells were dependent on autocrine stimulation by TGFα for survival. Analogously, exogenous TGFα inhibited apoptosis in the Myc#83 cells. Results from both mammary gland tumors and their derived cell lines are therefore in good agreement and mirror previous studies which have shown that apoptosis was induced when c-Myc was overexpressed (Askew *et al.*, 1991; Evan *et al.*, 1992).

Our findings could provide at least a partial explanation for why TGFα and c-Myc cooperate in mammary gland tumorigenesis in the powerful way we described previously (Amundadottir *et al.*, 1995). In that study, single transgenic virgin c-Myc mice developed mammary gland tumors at around 10 months of age and virgin single transgenic TGFα animals never developed mammary gland tumors. In contrast, double transgenic TGFα/c-*myc* mice exhibited a tumor latency that was shortened to only 66 days, and mammary gland tissue from mice as young as 3 weeks grew readily as a tumor in nude mice. A similar synergism was observed in a WAP-TGFα × WAP-Myc double transgenic model (Sandgren *et al.*, 1995). In that report, as well as a study involving the MT100TGFα strain used in our model (Smith *et al.*, 1995), it was also observed that TGFα overexpression inhibited post-lactational involution, a process dependent on apoptosis. Those oberservations lend further credence to the hypothesis that TGFα can act as a survival factor in the mammary gland.

TGFα has not been shown previously to inhibit c-Myc-mediated apoptosis but insulin like growth factors (IGF-I and IGF-II) and platelet derived growth factor (PDGF) acted as survival factors for Rat-1 fibroblasts which overexpressed c-Myc (Harrington *et al.*, 1994). Interestingly, EGF could not inhibit c-Myc-induced apoptosis in those cells, even though they expressed functional EGF receptor. Although the reasons for this discrepancy are not known, it is most likely the result of cell type specificity. That assumption is supported by the observation that EGF could act as a survival factor for nontransformed mammary epithelial cells which were serum starved or grown to confluency (Merlo *et al.*, 1995). Our data from the Myc#83 tumor cell line indicate that EGF, bFGF and IGF-I can also inhibit c-Myc-mediated apoptosis, suggesting that they could potentially cooperate with c-Myc in mammary tumorigenesis as well.

Mutations in the p53 gene may also cooperate with c-Myc in tumorigenesis, since p53 has been shown to be required for Myc-mediated apoptosis in some, but not all cases (Sakamuro *et al.*, 1995; Hsu *et al.*, 1995; Hermeking and Eick, 1994). In addition, upregulation of *bcl*-2 gene expression (a death suppressor), downregulation of *bax* gene expression (a death promotor) or abrogated expression of other proteins involved in Myc-induced apoptosis might be involved. Bcl-2 has been shown to cooperate with c-Myc in transformation *in vitro* and *in vivo* (Bissonette *et al.*, 1992; Fanidi *et al.*, 1992; Strasser *et al.*, 1990; Wagner *et al.*, 1993), and Bax gene expression may be regulated by c-Myc, since its promotor contains several putative Myc binding sites (Miyashita and Reed, 1995). However it is not known whether TGFα can directly influence the apoptotic machinery of the cells. The effects of TGFα and TGFβ1 on expression of p53, Bcl-2, Bax and other proteins involved in apoptosis are currently being investigated.

A cooperative growth stimulus also appears to contribute to the synergism between TGFα and c-Myc in mammary tumorigenesis. The doubling time of TGFα/Myc#75 cells was approximately half that of cells expressing only one of the transgenes, and their growth in soft agar and nude mice was much more aggressive than the other two cell lines. Taken together, the data suggest that one aspect of the positive interaction between TGFα and c-Myc in tumorigenesis might be via upregulation of genes that control progression through the cell cycle. Collectively, these gene products might account for high growth rates and malignant progression. Potentially, coexpression of TGFα and c-Myc could also alleviate negative control on growth and transformation.

In vitro studies with the tumor-derived cell lines suggest that may be the case. TGFβ1 inhibits the growth of most epithelial cells, including mammary epithelial cells (Daniel *et al.*, 1989; Jhappan *et al.*, 1993; Pierce *et al.*, 1993; Silberstein and Daniel, 1987; Valverius *et al.*, 1989; Zugmaier *et al.*, 1989). However, the TGFα/Myc#75 line was only marginally responsive to TGFβ1 in ADG assays and was insensitive to the growth factor under anchorage independent conditions. In contrast, Myc#83 cells grown on plastic were quite sensitive to TGFβ, and their rate of colony formation in soft agar was significantly reduced in the presence of TGFβ. Apoptosis assays revealed that Myc#83 cells were not merely growth-inhibited by TGFβ, but rather they were stimulated to undergo apoptosis, even in the presence of a survival factor (EGF). Induction of apoptosis by TGFβ has previously been reported for

some other cell types, including normal and malignant ovarian epithelial cells (Havrilesky *et al.*, 1995), endometrial cells (Rotello *et al.*, 1991), rat prostate cells (Martikianen *et al.*, 1990), normal and transformed hepatocytes (Oberhammer *et al.*, 1992), and leukemia cells (Selvakumaran *et al.*, 1994a,b; Taetle *et al.*, 1993). Furthermore, mammary glands from pregnant WAP-TGFβ transgenic mice showed high levels of apoptosis with a subsequent lack of secretory lobule development (Korden *et al.*, 1995). Since TGFβ expression is elevated in human tumor cells compared to normal mammary tissue and protein levels are positively correlated with disease progression (Gorsch *et al.*, 1992), breast tumor cells must develop the ability to grow in the presence of relatively high concentrations of TGFβ. The results from our *in vitro* studies indicate that cells which overexpress only c-Myc would not have that ability.

Our results suggest a new role for TGFα as a survival factor in breast cancer. We therefore conclude that the strong synergism of TGFα and Myc in mammary gland tumorigenesis is in fact due not only to a dual growth stimulus, but to the ability of TGFα to suppress a negative aspect of Myc overexpression.

Materials and methods

Transgenic animals

Transgenic mice were generated as described previously by mating the MT100 TGFα strain to the MMTV-c-*myc* M strain (Amundadottir *et al.*, 1995). The four resulting genotypes were: TGFα/c-*myc* double transgenic mice, TGFα single transgenic mice, c-*myc* single transgenic mice, and wild type mice. Tumors were observed to form in each strain as follows: in TGFα/c-*myc* virgin females and males with a latency of about 66 days; in multiparous single transgenic TGFα females with a latency of about 10 months; and in virgin or multiparous single transgenic c-*myc* females with a latency of about 10 months.

Detection of apoptosis in tumors

The occurrence of apoptosis in mammary gland tumors was detected by *in situ* nick end-labeling of nucleosomal DNA fragments (Ansari *et al.*, 1993). Paraffin embedded tumor sections were deparafinized in a series of xylene and ethanol washes. This was followed by a 0.3% H_2O_2 treatment for 30 min to inactivate endogenous peroxidases, after which slides were immersed in buffer A for 5 min (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.76 mM 2-mercaptoethanol and 0.005% BSA). Subsequently, slides were incubated with Klenow enzyme (50 U/ml, Boehringer Mannheim, Indianapolis, IN), 5 μM biotinylated dUTP (Boehringer Mannheim) and 2 μM dATP, dGTP and dCTP (Promega, Madison, WI) in buffer A for 60 min at 37°C. After washing slides in PBS, they were incubated with solution AB (ABC kit, Biomeda, Foster City, CA), rewashed in PBS and stained with diamino benzidin (DAB, Sigma, St. Louis, MO). Finally, the slides were counterstained with aqueous methyl green (Sigma), dehydrated and mounted.

Primary cultures from tumors

Tumor bearing transgenic animals were sacrificed and tumors were excised aseptically. Tumors were then cut into about 1 mm^3 pieces and digested overnight at 37°C in DMEM-F12 (Biofluids, Rockville, MD) with 10% fetal calf serum (FCS, Biofluids), 5 ng/ml EGF (Upstate Biotechnology Incorporated[UBI], Lake Placid NY), 10 μg/ml insulin (Biofluids) supplemented with 1 mg/ml collagenase type 1A (Sigma), antibiotics and fungizone (Biofluids). The following day cells were pelleted by centrifugation and washed three times in growth media (DMEM-F12 with 2.5% FCS, 5 ng/ml EGF, 10 μg/ml insulin and antibiotics). Cells were plated at $1-2\times10^6$ cells per T75 flask in growth medium. Fungizone was used in the cell medium for the first 2–3 weeks to prevent fungal contamination. Media were changed every 2–3 days and fibroblast overgrowth was prevented by differential trypsination of cultures until fibroblasts were no longer observed (based on morphology). When epithelial cells were about 60–70% confluent (after 2–3 months of growth), the cultures were passed at 1:2 dilutions with dispase (Boehringer Mannheim). At later passages cells were split with trypsin (Gibco BRL, Gaithersburg, MD) twice a week at 1:5 to 1:50, depending on the line.

RNA isolation and analysis

Cultured cells were harvested by rocking plates with guanidine thiocyanate for 5–10 min. RNA was extracted with acid phenol and precipitated with isopropanol. Ten μg total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL) and probed with ^{32}P-labeled riboprobes generated with the Riboprobe II Core System (Promega) from the following plasmids: pTGFα-pGEM3Z linearized with HindIII and transcribed with T7; c-*myc*-pGEM4Z linearized with EcoRI and transcribed with T7; and pmK14-pGEM3 (mouse keratin#14) linearized with HindIII and transcribed with SP6 polymerase. Labeled pBluescript polylinker was hybridized with the 28S RNA as an internal loading control for Northern analysis (Witkiewicz *et al.*, 1993).

Growth assays

Anchorage dependent growth assays were performed in 96-well plates (Costar, Cambridge, MA). Cells were plated at a density of 1500 cells per well and were cultured in normal growth media, with or without EGF (10 ng/ml). At various time points (two per day for 4 days), the plates were stained with crystal violet (Sigma, 0.5% in 30% MeOH), rinsed with water and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc). Doubling times were calculated from the slope of the line generated by plotting log (absorbance) vs time. In order to test the sensitivity of the cells to TGFβ1, cells were also plated in normal growth media with EGF plus TGFβ1 (0.01–100 pM [R&D Systems, Minneapolis, MN]). When TGFβ was used as a treatment, the media were changed every other day and cell number was measured on day 3 (TGFα/Myc#75) or 4 (TGFα#13 and Myc#83).

The cell lines were also tested for their ability to grow under anchorage independent conditions. Cells (10^4) were suspended in 0.3% Bactoagar (Difco, Detroit MI) and seeded into 35 mm dishes over a 0.8% agar base layer in IMEM plus 10% FCS with the following additions: TGFα (10 ng/ml, UBI), EGF (10 ng/ml), TGFβ1 (100 pM), or TGFβ1 and EGF together. Every other day, 300 μl of media with growth factors was added to each plate. After 7–10 days, colonies larger than 40 μm in diameter were counted with an Omnicron 3600 image analysis system (Artek Systems Corp., Farmingdale NY).

Tumorigenicity of cell lines

Cell lines were injected into female NCR *nu/nu* mice in order to determine whether they retained tumorigenic potential. About 10^6 cells were injected subcutaneously (between nipples number 2 and 3, and 4 and 5) into nude mice under anesthesia. Two to four sites were injected per animal.

Detection of apoptosis in cell lines

Apoptosis in the cell lines was detected by an apoptotic cell death ELISA assay (Boehringer Mannheim) and by visualization of nucleosomal laddering in cytoplasmic fractions of cells (Kamesaki *et al.*, 1993). The ELISA detects cytoplasmic nucleosomal DNA fragments with antibodies directed against histones and DNA. Cells were plated in 6-well plates (1.7×10^5 cells/well) and treated 24 h later. Treatments consisted of normal growth media without EGF plus the following additions: TGFα (1–1000 ng/ml), bFGF (10 ng/ml, UBI), IGF-I (50 mM, UBI), EGF (10 ng/ml), or EGF plus TGFβ (10 ng/ml and 0.01–100 pM, respectively). The TGFα/Myc#75 cells were also treated with PD 153035 (10 μM, Park Davis), a specific inhibitor of EGF receptor tyrosine kinase activity (Fry *et al.*, 1994). Treatment with DMSO (μl/ml) served as a negative control since the stock drug was suspended in DMSO. Twenty-four hours later, cytoplasmic lysates were prepared from the cells. The ELISA plate was coated overnight (4°C) with the first antibody (anti-histone) and then incubated with lysis buffer for 30 min at room temperature (RT). The wells were washed three times and then incubated with 100 μl cytoplasmic lysate for 90 min (RT). Wells were washed again and incubated with the peroxidase-linked second antibody (anti-DNA). Following the final wash, ABTS peroxidase substrate was added and color development was detected by measuring absorbance at 410 nm. CEM cells (T cell leukemia cell line) treated with dexamethasone (Catchpoole and Steward, 1993) served as a positive control.

Internucleosomal cleavage of the DNA is a hallmark of apoptosis and can be observed as a 'ladder' in agarose gels. DNA was isolated from cytoplasmic fractions of the cells and was run out on 1.8% agarose gel which was stained with ethidium bromide to visualize the DNA ladder (Kamesaki *et al.*, 1993). Cells that are undergoing apoptosis show a characteristic DNA 'ladder' in this assay whereas other cells do not contain DNA in their cytoplasm and are therefore negative.

FACS analysis

Cell nuclei were analysed by the detergent-trypsin method (Vindelov *et al.*, 1983) with a Fluorescent Activated Cell Sorter (FACS) to obtain cell cycle histograms and to determine ploidy. Approximately 10^6 cells were pelleted and resuspended in 100 μl of citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 0.05% v/v DMSO, pH 7.6) and stored at −70°C before analysis. For cell cycle analysis, cells were plated in normal growth media with EGF. After 24 h, the cells were switched to media without EGF and then harvested at various time points to determine whether the cells were growth arrested in G_0/G_1. For ploidy analysis, tumor cells (passages 6–31) were analysed alone and also mixed with normal primary mouse fibroblast cultures (passage 2). The normal fibroblasts served as a control to establish a diploid mouse histogram.

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ALTERED CELL CYCLE REGULATION IN MAMMARY EPITHELIAL CELLS WHICH OVEREXPRESS c-MYC[1]

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[4]Abbreviations: MEC, mammary epithelial cell; EGF, epidermal growth factor; TGFα, transforming growth factor α; TGFß, transforming growth factor ß; cdk, cyclin dependent kinase

Abstract

The c-*myc* gene is commonly amplified and/or overexpressed in primary human breast cancers, and mouse transgenic models have demonstrated that c-*myc* expression can play an important role in mammary tumorigenesis. We investigated cell cycle control in mammary epithelial cells with constitutive c-*myc* expression. In both mouse and human cells, c-*myc* overexpression decreased the doubling time by about 6 h compared to parental lines. The decrease was not due to a change in growth factor sensitivity, but rather to a shortening of the G_1 phase of the cell cycle. Rb was constitiutively hyperphosphorylated in cells with exogenous Myc expression, in contrast to the parental cells which exhibited a typical phosphorylation shift as they traversed G_1. The abnormal phosphorylation status of Rb in c-*myc*-overexpressing cells was associated with premature activation of cdk2 kinase activity as a result of reduced p27 expression and elevated cyclin E expression.

Introduction

The proto-oncogene c-*myc* encodes a highly conserved nuclear phosphoprotein which contains a leucine zipper and a basic-helix-loop-helix motif common to many transcription factors (1-6). When bound to its heterodimeric partner Max, Myc protein binds specifically to DNA and can activate transcription. However, the specific targets of *myc* regulation are not well characterized, and thus its mode of action is poorly understood, despite intense investigation.

Myc has been implicated in the regulation of cell proliferation, differentiation, and death by apoptosis (reviewed in 1-6). Since aberration of any of those normal processes can contribute to tumorigenesis, it is not surprising that deregulated expression of the c-*myc* gene is often associated with neoplasia. *In vitro*, c-*myc* overexpression can cooperate with other oncogenes such as Ras to transform cells. Additionally, the ability of inappropriately expressed Myc to promote tumorigenesis *in vivo* has been clearly demonstrated by transgenic mouse models (7)

Classified as an immediate early gene, c-*myc* expression is tightly regulated and correlated with the proliferative state of the cell (8). In normal quiescent cells, Myc protein levels are very low and its expression is strongly induced following mitogen stimulation. Similarly, expression decreases as cells become growth arrested or undergo differentiation. A reduction in c-*myc* levels due to disruption of one copy of the gene results in a lengthened G_1 cell cycle phase (9), while inhibition of c-*myc* expression blocks cell cycle progression and leads to G_1 arrest (10-11). Conversely, when c-*myc* expression is deregulated, cells may grow at a faster rate and are often unable to withdraw from the cell cycle when signaled to do so (12-14).

Based on the above observations, c-*myc* has long been thought to control key aspects of the proliferative response. Since passage through the cell cycle is orchestrated by the cyclins and their associated cyclin dependent kinases (cdks[4], reviewed in 15), those regulatory proteins would be logical targets for such a proposed action of Myc. Normally, expression of the various cyclins is tightly regulated and is characteristic of specific stages of the cell cycle. Several studies in fibroblasts and hematopoietic cells in fact suggest that expression or activity of some cyclins (ie.

cyclins D1, E, and A) and cdks (ie. cdks 1 and 2) may be altered by c-*myc* expression (9, 16-21). In addition to the activating cyclin subunits, cdk activity can be modulated by cdk inhibitors as well as by a number of kinases and phosphatases (reviewed in 22), some of which have also implicated as targets of Myc (21 [p27]; 23 [Cdc25A]). However, with the exception of the cdc25A gene, the genes in question lack Myc-Max consensus binding sites in the promoter region, indicating that their regulation by Myc is indirect.

Although some mechanistic details of the action of Myc have emerged from studies with rodent fibroblasts, there is considerable interest in further elucidating the mechanisms(s) of malignant transformation by Myc in human epithelial and hematological malignancies. Overexpression of c-*myc* is thought to play a role in the development of breast cancer since it is commonly amplified and/or overexpressed in human breast tumors (reviewed in 24). Amplification of the c-*myc* gene is often associated with highly proliferative tumors and poor prognosis. In addition, Myc confers tumorigenicity when overexpressed in the mammary gland of transgenic mice. Recent results from our laboratory (25) and others (26) showed that overexpression of TGFα can strongly synergize with c-*myc* in transgenic mice to promote mammary tumor development, confirming previous *in vitro* observations that Myc can cooperate with growth factors such as TGFα or EGF to transform mammary epithelial cells (MECs) (27, 28). The contribution of TGFα may be due, at least in part, to the suppression of Myc-induced apoptosis *via* increased expression of Bcl-x_L (29, 30). However, tumors and cell lines derived from the double transgenic mice also showed an accelerated growth rate compared to those from single transgenic mice (25, 29). Since Myc has been implicated in cell cycle regulation of fibroblasts, the tumorigenic action of constitutive c-*myc* expression in the mammary gland may also be due to aberrant cell cycle progression. Although a variety of changes in the expression of cell cycle regulators have been identified in human breast cancer cell lines and primary tumors (reviewed in 31), little is known about the causes or consequences of cell cycle deregulation in breast cancer. Thus, the purpose of this study was to identify changes in cell cycle regulation in MECs which overexpress c-*myc*, and to examine the impact of EGF on those cells.

Materials and Methods

Cell lines

A pair of human mammary epithelial cell lines (184A1N4, 184A1N4-myc) were used to study the effects of c-*myc* overexpression on cell cycle regulation. The parental cell line, A1N4, was derived from normal mammary tissue obtained by reduction mammoplasty and was immortalized with benzo(a)pyrene (32). The A1N4-myc line (28) was established *via* retroviral infection of A1N4 cells with a construct containing mouse c-*myc* under the control of the Moloney mouse leukemia virus long terminal repeat (MMLV LTR). Both cells lines were maintained in IMEM (Gibco-BRL, Gaithersburg, MD) containing 0.5% FCS, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin (Biofluids, Rockville, MD), and 10 ng/ml EGF (Upstate Biotechnology Incorporated [UBI], Lake Placid, NY). The cells arrest in G_1 in the absence of EGF (33).

A pair of mouse mammary cell lines (HC14 and HC14-myc) was also used in preliminary experiments. The HC14 line was established from a mid-pregnant mammary gland and was transfected with a c-*myc* expression construct driven by the MMLV LTR (34). Both cell lines were routinely grown in IMEM with 10% FCS.

Growth assays

Cells were plated in 96-well plates (Costar, Cambridge, MA) at a density of 1000-2000 cells/well. At various time points, plates were stained with crystal violet (Sigma, 0.5% in 30% MeOH), rinsed with water and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc.). Doubling times were calculated from the slope of the line generated by plotting log(absorbance) vs time.

FACS Analysis

Cells were plated ($5x10^5$ cells/plate) in 10 cm dishes (Falcon 3003, VWR Scientific, Philadelphia, PA) in normal growth media containing EGF. The next day the cells were changed to EGF-free media to arrest them in G_1. After 48 hours, the cells were restimulated with EGF (10

ng/ml) and cells were harvested at 3 h intervals. Nuclei were isolated and stained with propidium iodide for cell cycle analysis according to the method of Vindelov et al. (35).

Western Analysis

Cells were plated, arrested, and restimulated with EGF as described for FACS analysis. At 1.5 or 3 hour intervals following EGF stimulation, total cell lysates were prepared. Cells were washed with cold PBS and then scraped into cold lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton x-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3O_4V, 100 mM NaF, 10 mM pyrophosphate, 10 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). After a 10 minute incubation on ice, lysates were spun for 10 minutes in a cold microcentrifuge to remove cellular debris and were frozen at -70 °C. Twenty µg of protein from each sample were separated by SDS-PAGE and transferred to either nitrocellulose or PVDF membranes. Acrylamide concentrations varied depending on the target protein as follows; Rb, 6%; cyclin D1, cyclin E, cdk2, and cdk4, 10%; p21 and p27, 14%. Blots were blocked in 4% milk, 1% BSA in Tris buffered saline with Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween-20) for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (1 µg/ml): Rb (Pharmingen, San Diego, CA), cyclin D1 and E (UBI), cdk2 and cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or p27 (Santa Cruz). Proteins were visualized with an HRP-linked second antibody (1/2000 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce, Rockford, IL). Amido black or India ink staining of the membranes demonstrated equal loading and transfer of the samples. Since appropriate antibody for Cdc25A was not commercially available, we chose to examine its expression at the RNA level only (see below).

Kinase assays

Cell lysates (100 µg) were incubated with 1 µg anti-cdk2 antibody for 2 h (4°C) prior to precipitation with Agarose A beads (Santa Cruz). Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 1 mM DTT, 0.3 mM ß-glycerophosphate, 1 mM Na_3O_4V, 10 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). The beads were then resuspended in 30 µl kinase buffer and the

reaction was started by adding ATP (200 μM), γ^{32}P-ATP (5 μCi) and histone H1 (1 μg). Samples were incubated at 30 °C for 15 min before stopping the reaction with 2x loading buffer (62.5 mM Tris [pH 6.8], 10% sucrose, 2% SDS, 5% ß-mercaptoethanol, 1% bromphenol blue). Labeled proteins were run on a 10% polyacrylamide gel which was dried prior to visualization with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale CA).

RNA Isolation

A1N4 and A1N4-myc cells were plated sparsely (1.5 x10^6 cells) in culture flasks (225 cm^2; Costar) and growth arrested as described above. Following re-stimulation with EGF (10 ng/ml), total RNA was harvested at three hour intervals by the guanidine thiocyanate-acid phenol method (36).

Northern Analysis

Total RNA (12 μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were hybridized overnight with a 700 bp ^{32}P-labeled, random-primed human probe for cdc25A (nt 936-1637). Bands were detected with a PhosphorImager 445 SI.

RNase Protection Assay

Changes in cyclin RNA expression were examined using a nonradioactive RNase protection assay, as previously described (37). A pGEM-4z vector containing a 400 bp fragment of the human cyclin A cDNA was linearized with EcoRI prior to synthesis of a 440 bp riboprobe. A 1.3 kb NotI fragment of human cyclin D1 in a Bluescript KS- plasmid was linearized with EcoNI to synthesize a 360 bp probe.

Results

We began our studies by comparing the effect of constitiutive Myc expression on the growth rate of human and mouse mammary epithelial cell lines. In both mouse (HC14-myc) and human (A1N4-myc) MECs, c-*myc* overexpression decreased the doubling time by about 6 h compared to parental lines (Table 1). Our results are also in agreement with previously published data which indicated a similar decrease in doubling time by MMLV LTR-driven c-*myc* overexpression in MMEC cells, another cell line derived from normal mouse mammary tissue (27, Table 1). In order to determine whether the faster growth rate was simply due to increased sensitivity to growth factors, the two human cell lines were grown in the presence of various concentrations of EGF for three days. The two resulting dose-response curves were parallel, with the A1N4-myc cells growing faster than the parental cells at all concentrations tested (Figure 1).

The A1N4 and A1N4-myc cells were used to further investigate the observed change in growth rate. In the absence of EGF, neither the parental nor the c-*myc*-infected cell line showed significant growth (Figure 2A). That observation was due to the fact that both cell lines arrested in G_1 upon EGF deprivation (Figure 2B). In order to determine the kinetics of cell cycle progression in the two lines, cells were arrested in G_1 in the absence of EGF and were allowed to re-enter the cell cycle by replacing EGF. FACS analysis demonstrated that the A1N4-myc cells began to enter S phase 12 h after EGF addition and percent cells in S phase peaked at 18 h (Figure 3). In contrast, parental cells did not enter S phase until 18 h and peaked at 24 h. The results suggested that the difference in doubling time was due to a shortened G_1 phase of the cell cycle.

Since Rb is believed to play an important role in the G_1 phase of the cell cycle, we next examined Rb expression and phosphorylation and observed a significant difference between the two cell lines. In arrested A1N4 cells, Rb expression was relatively low and the protein was present only in the hypophosphorylated state (Figure 4). About 6 hours after EGF stimulation, approximately 50% of the protein was found in the hyperphosphorylated state. At all time points beyond 6 hours, Rb protein levels were greatly increased and most of the protein was

hyperphosphorylated. In contrast, Rb was highly expressed and phosphorylated at all time points tested in A1N4-myc cells.

We then examined the expression of several proteins which are known to be involved in the regulation of G_1 progression and have been implicated in the regulation of Rb function (Figure 5). Cyclin D1 protein expression was very low in arrested cells, was rapidly induced following EGF stimulation, and remained elevated throughout the remainder of the cell cycle. Cyclin D1 levels were maximal at 6 h after stimulation in A1N4 cells, and at 3 h in A1N4-myc cells. Cyclin E protein was detectable in arrested cells of both lines, but it was further stimulated by EGF addition and then down regulated later in the cell cycle. Peak levels of this cyclin were observed between 9 and 15 h in parental cells, and from 3 to 6 h in c-*myc*-expressing cells. Expression of two cyclin dependent kinases which interact with cyclins D1 and E were also examined. Cdk4 expression was constant throughout the cell cycle in both cell lines, but protein levels were higher in A1N4-myc cells. Western analysis of cdk2 demonstrated a dramatic mobility shift in the protein due to CAK phosphorylation. In A1N4 cells, the shift was observed about 12 h after EGF addition, while A1N4-myc cells already contained low levels of phosphorylated protein even when arrested, with a maximal shift at about 6-9 h post stimulation. As expected, those mobility shifts corresponded to the time of cyclin E induction. CAK is believed to be constitutively active, but can only phosphorylate cdks which are complexed with a cyclin (22). Finally, expression of a cdk inhibitor, p27, was analyzed. The function or expression of any G_1 cdk inhibitors could potentially be altered in cells with a shortened G_1 phase, but we chose to examine p27 first, since there was precedent for modified p27 levels in fibroblasts with deregulated c-*myc* expression (21). Protein levels were quite high in arrested A1N4 cells and decreased as the cells progressed through the cell cycle. In contrast, p27 was barely detectable in arrested A1N4-myc cells, and was rapidly eliminated following EGF addition.

The results presented in Figure 5 suggested that differences in cdk2 activity might play a role in the shortened G_1 phase in c-*myc*-overexpressing cells. We therefore directly examined activation of cdk2 in the cells with an *in vitro* kinase assay (Figure 6). As predicted, arrested

parental cells contained very little active cdk2, and a major increase in activity was observed 12 hours after EGF stimulation, the time at which cyclin E was maximally expressed, p27 levels were reduced, and cdk2 was phosphorylated by CAK. In contrast, cdk2 was active even in EGF-deprived A1N4-myc cells, with maximal activation at 6 h after EGF stimulation.

Since a recent study identified the cdk2 phosphatase Cdc25A as a direct transcriptional target of myc, we also wished to examine its expression in our MEC system. In unsynchronized cells, cdc25A RNA was elevated compared to parental cells (Figure 7A). That result was somewhat unexpected since a similar analysis of cyclins A and D1 RNA showed no significant differences between the two cells lines during asynchronous growth (not shown). However, despite the elevated RNA levels, the time of cdc25A induction in synchronized cells was similar in both cell lines, beginning at 12 h and reaching maximal levels between 15 and 21 h after EGF stimulation (Figure 7B).

The shortened G_1 phase did not appear to be a consequence of any gross changes in cyclin A or D1 RNA expression (not shown). Cyclin RNA was undetectable in arrested cells and induction was closely correlated with changes in cell cycle phase. In both cell lines, cyclin D1 expression was detectable by three hours after EGF treatment and levels remained relatively constant throughout the cell cycle, in agreement with the results by Western blot. In the A1N4-myc cells, cyclin A RNA expression began about 9 hours after EGF stimulation, with a peak at 18 hours (37).

Discussion

The results presented here show that constitutive, elevated expression of c-*myc* leads to altered cell cycle regulation in MECs, with accelerated passage through G_1. The faster growth rate of c-*myc* expressing MECs was correlated with constitutive phosphorylation of Rb and increased cdk2 activity. Furthermore, the elevated cdk2 activity in arrested and synchronized A1N4-myc cells compared to parental cells was associated with increased cyclin E expression and diminished expression of the cdk inhibitor p27.

Control of Cdk2 activity by p27 and cyclin E

In normal cells, p27 protein levels undergo cell cycle dependent oscillations, with highest levels in G_1 (38). The protein is also induced, through translational and posttranslational mechanisms, by several conditions which facilitate G_1 arrest, including high density, or exposure to TGFß or lovastatin (38-40). Interestingly, Myc overexpression can block TGFß-dependent growth arrest in keratinocytes (41). It is thought that p27 associates with cyclin E/cdk2 until cyclin D levels are high enough to sequester the inhibitor in cyclin D/cdk complexes. p27 may thereby determine the order of cdk activation by inhibiting cdk2 activity until the cyclin D level (and therefore cdk4 activity) is maximal (31, 40). Our observations therefore indicate that this level of regulation is reduced or eliminated in MECs which overexpress c-*myc*. In the parental A1N4 line, p27 was expressed in arrested cells and was down-regulated following EGF addition, in agreement with a recent report which demonstrated that growth arrest by anti-EGFR antibody involves p27 expression (42). The A1N4-myc cells, in contrast, had markedly reduced p27 protein levels, even in the absence of EGF stimulation.

Our findings are therefore, in part, similar to those observed in density arrested fibroblasts following induction of a regulatable c-*myc* expression construct (21). In that study, Myc activation led to a rapid increase in G_1 cdk activity and subsequent Rb phosphorylation. Since those density arrested cells already contained relatively high levels of cyclins D1 and E, an increase in cyclin expression was neither necessary nor observed prior to Myc-induced cdk activation. Rather, the authors suggested that the change in cdk kinase activity was specifically due to a Myc-dependent

decrease in p27 levels. However, our results and those of Steiner et al. (21) appear to contrast the findings of a more recent study in fibroblasts (43). It was reported that Rat1 cells infected with a p27 retrovirus had inactive cyclin E/cdk2 complexes and arrested in G_1. Co-expression of Myc with p27 promoted cdk2 activation and released the cells from the G_1 arrest without altering the p27 protein levels. The authors proposed that Myc indirectly promoted the sequestration and inactivation of p27.

Rb and the G_1 cyclin dependent kinases

In its hypophosphorylated state, the retinoblastoma protein prevents cells from exiting the G_1 phase of the cell cycle (reviewed in 44). Normally, as cells progress through G_1, Rb becomes increasingly phosphorylated, allowing the cells to proceed into S phase to complete the rest of the cycle.

The high levels of phosphorylated Rb in A1N4-myc cells may be due to elevated cdk2 activity, which was significant even in arrested cells. *In vitro*, several cyclin/cdk complexes can phosphorylate Rb, but *in vivo*, the mechanism of Rb phosphorylation is not fully understood. Both cyclin D- and cyclin E- associated kinases have been implicated in Rb phosphorylation (44-49), but timing of the major shift in Rb hyperphosphorylation in normal cells most closely corresponds with the activation of cyclin E/cdk2 (44, 50-53). Indeed, it has been proposed that cyclin D1-associated kinase activity may promote a low, basal level of Rb phosphorylation during the early portion of G1 in preparation for the sudden change in hyperphosphorylation late in G1 (54). A recent study in Rat1a cells also points to an important role for cdk2 activity in Myc-driven cell cycle progression (55). The authors found that cyclin A expression following Myc induction could be blocked by microinjected expression plasmids encoding cdk inhibitors or kinase negative cdk2, or by treatment with a cdk2-specific chemical inhibitor.

Alternatively, the constitutively hyperphosphorylated state of Rb could be due to an inability of the A1N4-myc cells to dephosphorylate Rb. Normally, Rb is dephophorylated by protein phosphatase types 1 and 2 during mitosis (56, 57). It is difficult to distinguish between the

two scenarios in our system, since c-*myc* expression is constitutive. An inducible c-*myc* expression construct may provide a better system for distinguishing between the two possibilities.

It is interesting to note that fibroblasts prepared from Rb knockout mouse embryos also exhibit a shortened G_1 phase compared to wild type fibroblasts, and like the A1N4-myc cells, the Rb deficient cells are still dependent on an external growth signal and can be arrested in G_1 by serum withdrawal (58). Furthermore, the Rb negative cells display premature and elevated expression of cyclin E, but comparatively insignificant changes (either quantitative or temporal) in the expression of several other cell cycle regulated genes, including cyclin D1. Those results reiterate the likely connection between Rb function and cyclin E expression (and thus cdk2 activity).

No changes in Cdk4 protein expression or phosphorylation were observed in either cell line under our experimental conditions, but Cdk4 levels appeared to be elevated in A1N4-myc cells compared to parental cells. Cyclin D1 expression (at both the RNA and protein level) was absent in arrested cells and was rapidly induced by EGF stimulation in both cell lines, with maximal levels achieved within 3 hours in A1N4-myc cells and 6 hours in parental cells. Thus, cyclin D1-cdk4 complexes may contribute to the accelerated growth rate of Myc-expressing cells, but clearly the high level of hyperphosphorylated Rb in arrested A1N4-myc cells cannot be attributed to cyclin D1-associated kinase activity.

Our finding that cyclin D1 expression was not up-regulated by Myc overexpression alone is in agreement with the hypothesis that Myc and cyclin D1 function in complimentary, rather than linear pathways (59). In the Rat1a fibroblast system, it was also determined that c-*myc* overexpression did not eliminate the requirement of cyclin D1 induction by serum for cell cycle progression (21). Furthermore, since Myc appeared to induce phosphorylation of Rb prior to induction of either cyclin D1 expression or cdk4 activity in both Myc-transfected Rat1a and parental cells, those results suggest that cyclin D1-associated activity is necessary for some other aspect of G_1 progression. Recently, a novel target of cdk4 and cdk6 was identified in a human breast cancer cell line (60), and certainly there could be other, as yet undefined, targets of cyclin

D1-associated kinases. Nonetheless, cyclin D1 is not required for cell cycle progression in some cells that are functionally deficient for Rb due to mutation or viral oncoprotein expression, (61), and cyclin D1 mRNA and protein expression is often low in breast cancer cell lines which lack Rb function (62). Perhaps the effect of constitutive phosphorylation of Rb is different from that of functional Rb inactivation by mutation, deletion, or association with viral oncoproteins, with regard to the cyclin D1 requirement in the cell cycle. It should also be noted that cyclin D1 binds to Rb through a domain similar to those found in viral oncoproteins which interact with and inactivate the tumor suppressor (54), suggesting that a physical interaction between the two proteins may lead to further inactivation of one or the other.

A role for Cdc25A?

The Cdc25 family of phosphatases have also been implicated in the regulation of cdk activity, since they remove inhibitory phosphate groups at serine 14 and tyrosine 15 on cdks (22). Although UV irradiation stimulates tyrosine phosphorylation of cdk4 with subsequent G_1 arrest (63), a clear function for such a cdk4 species in normal cell cycle progression has not been demonstrated. Thus Cdc25 expression may not be as important for cyclin D1/cdk4 activity as it is for cdk2 activation.

The A and B forms of Cdc25 can function as transforming oncogenes in cooperation with activated Ha-ras or loss of Rb (64). The synergism between Ras and Cdc25 may be explained by the observation that Raf1, a component of the Ras pathway, can phosphorylate and activate Cdc25 proteins (65). That assertion could also explain the results of Steiner et al., (21). They found that full induction of cdk2 kinase activity by Myc also required Cdc25A activity, which could apparently be stimulated by serum growth factors, perhaps through the Ras-Raf pathway. A more recent study indicates that cdc25A expression can be directly induced by Myc in fibrobasts (23). However, another study using a similar rat fibroblast cell line showed no increase in Cdc25A steady state levels when Myc was overexpressed (43). In our MEC system, cdc25A RNA levels were elevated by Myc overexpression, but the timing of cdc25A expression induction following exposure to EGF was quite similar in the two MEC lines. The RNA was first detected 12 hours

after EGF addition, suggesting that other factors in addition to Myc are required for cdc25A expression.

Distinguishing the roles of Myc in cell cycle, apoptosis, and malignant progression

In contrast to fibroblast models in which c-*myc* expression was sufficient to force quiescent cells to re-enter the cell cycle (13, 14), c-*myc* overexpression was not sufficient to drive the mammary epithelial cells through the cell cycle in the absence of a growth stimulus (EGF). That difference may simply be due to cell type specificity or experimental conditions, but it should also be pointed out that although the fibroblasts re-entered the cell cycle, they were executing an apoptotic pathway rather than an actual proliferative response. The A1N4-myc cells, like the parental A1N4 cell line, reversibly arrest in G_1 in the absence of EGF, rather than undergoing apoptosis. Deregulated c-*myc* expression can induce apoptosis in primary mouse tumor MECs in the absence of growth/survival factors (29, 30). Thus, the results suggest that A1N4 cells, presumably during the process of immortalization, have undergone some change which makes them incapable of executing the apoptotic pathway in response to Myc. Although mutation of p53 is an attractive postulation to account for the difference, no mutations were found in the highly conserved exons 4-9 in the immortalized cell line (66). However, it was noted in that study that p53 proteins levels were unusually high, suggesting that the protein may be posttranscriptionally modified. Whatever the cause, the end result is that these cell lines provide an excellent model for studying alterations in cell cycle control due to c-*myc* overexpression in the absence of the confounding effects of apoptosis induction. That is an important distinction to make since a recent study found that the effects of Myc on cell cycle progression and apoptosis are indeed distinct (55).

In summary, our results may provide at least a partial explanation as to why Myc and EGF can cooperate to transform MECs and similarly, why there is such a strong synergism between Myc and TGFα in mammary tumorigenesis, as demonstrated by transgenic mouse models. We have previously shown that EGF can act as a survival factor for mammary tumor cells which overexpress Myc (29-30). The current results reported here indicate that Myc overexpression, in

conjunction with EGF receptor stimulation, can also force MECs through G_1 at a faster rate, resulting in accelerated growth. Taken together, these two characteristics may allow epithelial cells within the mammary gland to survive and proliferate under some conditions which would normally prevent DNA replication through the induction of apoptosis or a G_1 arrest. Thus, increased genetic instability may also be a logical endpoint of such a phenotype, analogous to the phenomenon which was demonstrated for p53 mutations (67, 68). Indeed, it has already been demonstrated that prolonged Myc overexpression in Rat1a cells can promote a variety of genetic aberrations, including numerical changes, chromosome breakage, and the formation of circular chromosomal structures, chromosome fusions, and extrachromosomal elements (69). In further support of that hypothesis, a recent study demonstrated that ectopic expression of p27 (which was down-regulated in the A1N4-myc cells) suppressed tumor growth and the accumulation of aneuploid cells in a brain tumor model (70).

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Cell Line	Doubling Time	Difference
A1N4	27.4 +/-0.6 h	5.9 h
A1N4-myc	21.5 +/-0.3 h	
HC14	25.1 +/- 0.5 h	6.3 h
HC14-myc	18.8 +/-0.3 h	
MMEC[1]	24.0 h	5.8 h
MMEC-myc[1]	18.2 h	

Table 1: Doubling times for 1 human pair and 2 mouse pairs of cell lines, +/- S.E. (pair=c-*myc*-overexpressing line and its parental line). The last column indicates the decrease in doubling time of the Myc line compared to its parental line.

[1]Reference 27.

Figure Legends

Figure 1: Growth of A1N4 and A1N4-myc cells in response to EGF is concentration dependent. Cells were plated in 96 well plates with increasing concentrations of EGF and incubated for three days before being stained with crystal violet. Note that the two curves are parallel. n=8, +/- S.E.

Figure 2: A1N4 and A1N4-myc cells arrest in G_1 in the absence of EGF.

A: Growth of both A1N4 and A1N4-myc cells is dependent on EGF. Cells were plated in 96 well plates (10^3 cells/well) in the presence or absence of EGF and were stained with crystal violet at the indicated times. Relative cell number was then measured as absorbance at 540 nm. n=8. **B:** Cell cycle histograms for unsynchronized cells grown in normal media with EGF and arrested A1N4 and A1N4-myc cells which had been deprived of EGF for 48 h.

Figure 3: Cell cycle analysis of A1N4 and A1N4-myc cells re-stimulated with EGF following growth arrest for 48 hours. Arrested cells were treated with 10 ng/ml EGF and harvested at 3 hour intervals. Propidium iodide staining and FACS analysis was performed with isolated nuclei.

Figure 4: Expression and phosphorylation of Rb in synchronized cells (A1N4 and A1N4-myc). Arrested cells were re-stimulated with EGF and whole cell lysates were prepared at the times indicated. 20 μg of protein were separated on a 6% gel before transfer to nitrocellulose for Western analysis. The faster moving band is due to hypophosphorylated (inhibitory) Rb and the upper band contains hyperphosphorylated Rb. A0=A1N4 at time 0. M0=A1N4-myc at time 0, +=unsynchronized cells.

Figure 5: Expression of the G_1 cyclins D1 and E, their associated kinases cdk4 and cdk2, and the cdk inhibitor p27 in synchronized A1N4 and A1N4-myc cells. Lysates were harvested as in Figure 4 and were separated on 10% acrylamide gels prior to transfer to nitrocellulose for western analysis. In the case of cdk2, phosphorylation by CAK leads to a downward shift in mobility, producing the observed doublet. +, unsynchronized cells.

Figure 6: Kinase activity of cdk2 in synchronized A1N4 and A1N4-myc cells. Cdk2 was immunoprecipitated from whole cell lysates at the indicated times following EGF re-stimulation. The precipitates were then incubated for 15 min at 37 °C in the presence of histone H1 and γ^{32}P-ATP. Labeled substrate was detected by phosphorimager analysis following fractionation on a 10% PAGE gel.

Figure 7: Northern analysis of cdc25A RNA in A1N4 and A1N4-myc cells. **A**: Expression in unsynchronized cells. Cells were grown in the presence of EGF and were harvested at approximately 75% confluence. **B**: Cell cycle dependent expression. Cells were arrested and re-stimulated by addition of EGF as in Figure 3. At the times indicated, total RNA was harvested.

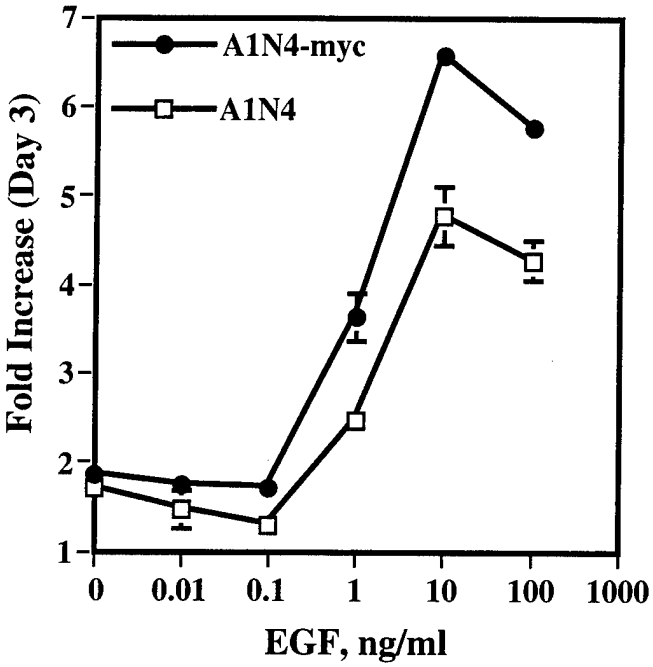

A1N4-myc
A1N4
Fold Increase (Day 3)
7
6
5
4
3
2
1
0
0.01
0.1
1
10
100
1000
EGF, ng/ml

Figure 1

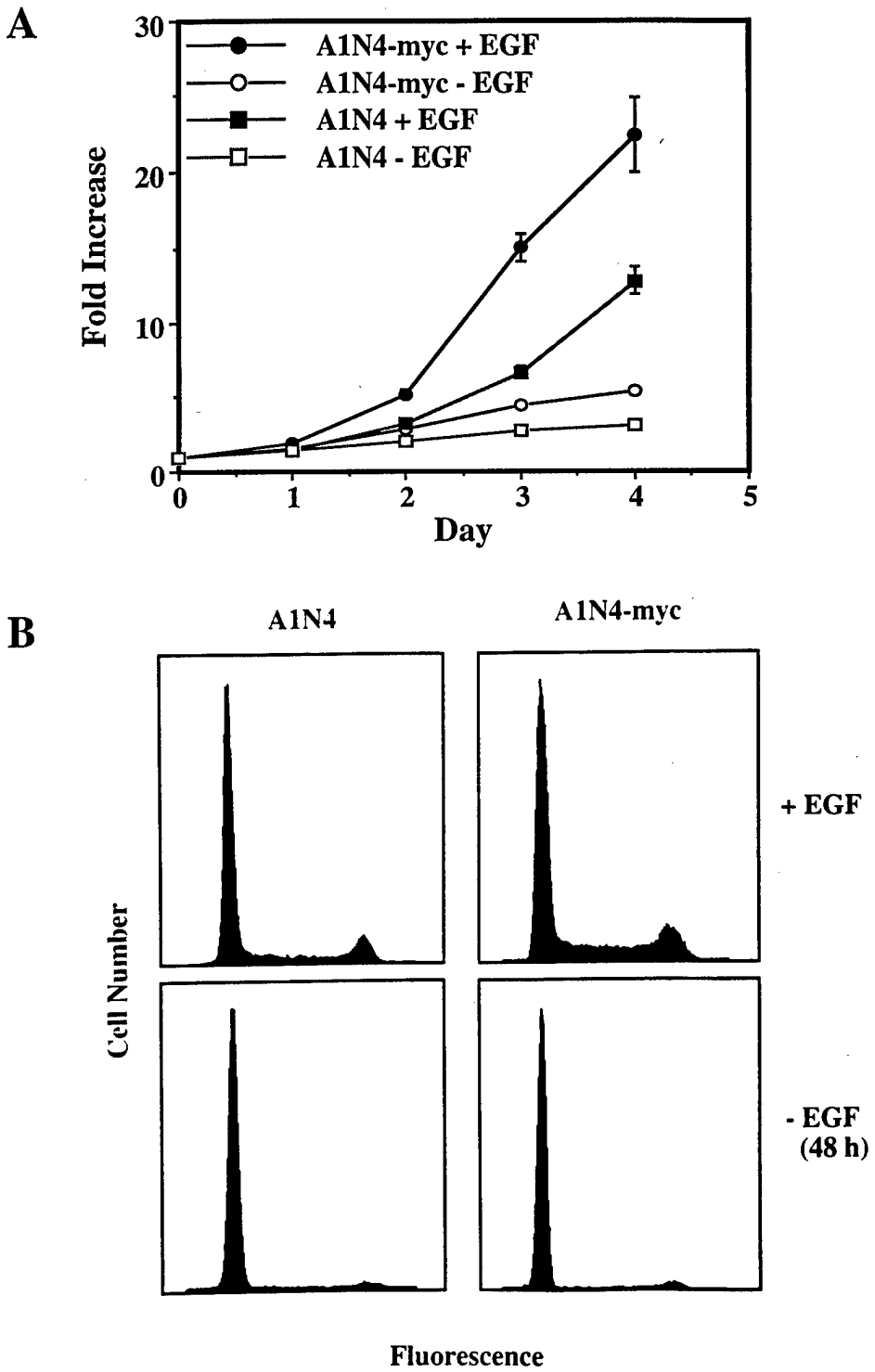

A
30
20
10
0
Fold Increase
A1N4-myc + EGF
A1N4-myc - EGF
A1N4 + EGF
A1N4 - EGF
0
1
2
3
4
5
Day
B
A1N4
A1N4-myc
+ EGF
- EGF
(48 h)
Cell Number
Fluorescence

Figure 2

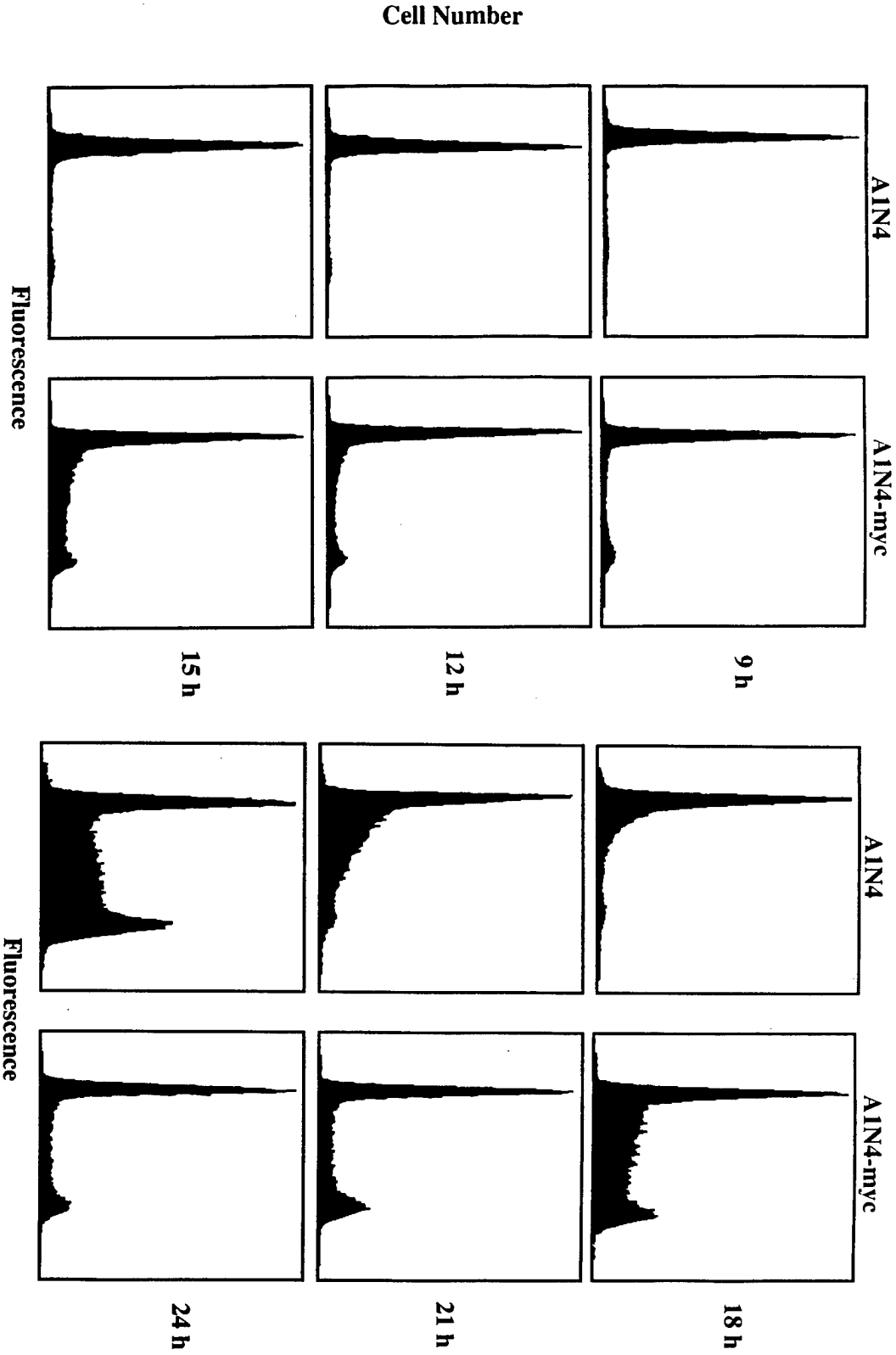

Cell Number
A1N4
A1N4-myc
9 h
12 h
15 h
18 h
21 h
24 h
Fluorescence
Fluorescence

Figure 3

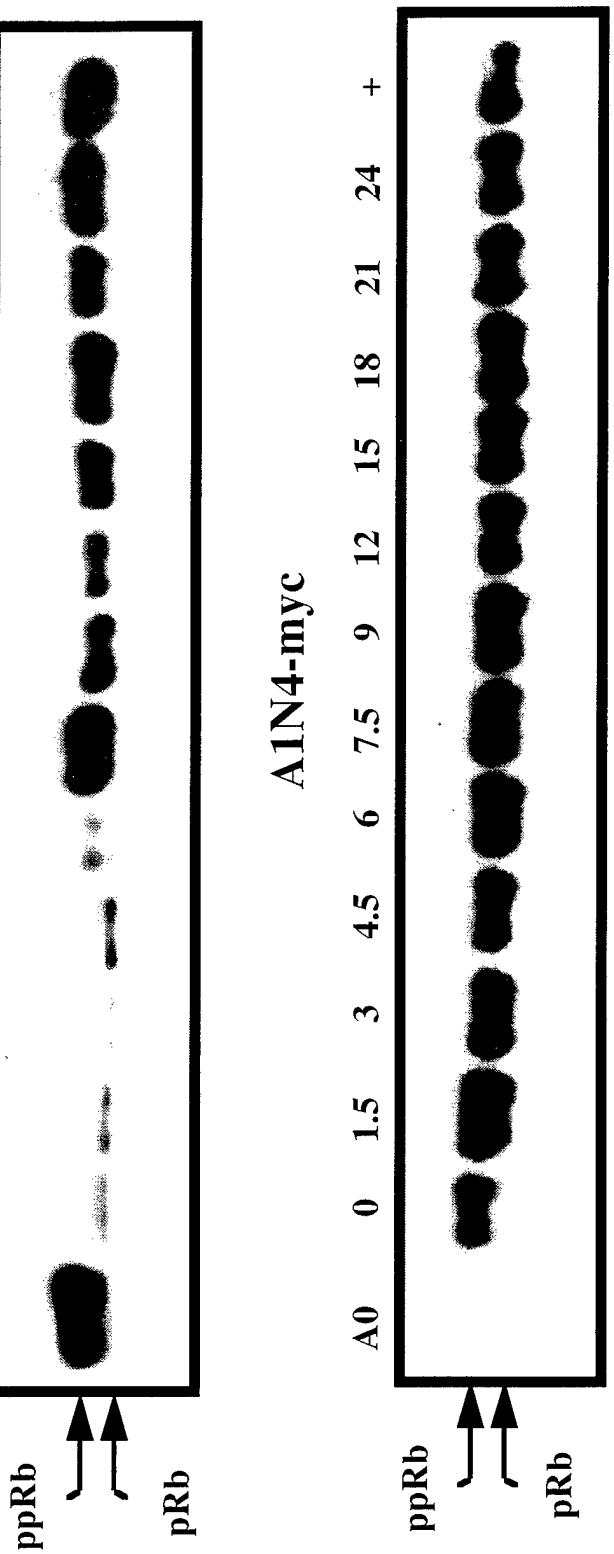

A1N4
M0
0
1.5
3
4.5
6
7.5
9
12
15
18
21
24
+
ppRb
pRb
A1N4-myc
A0
0
1.5
3
4.5
6
7.5
9
12
15
18
21
24
+
ppRb
pRb

Figure 4

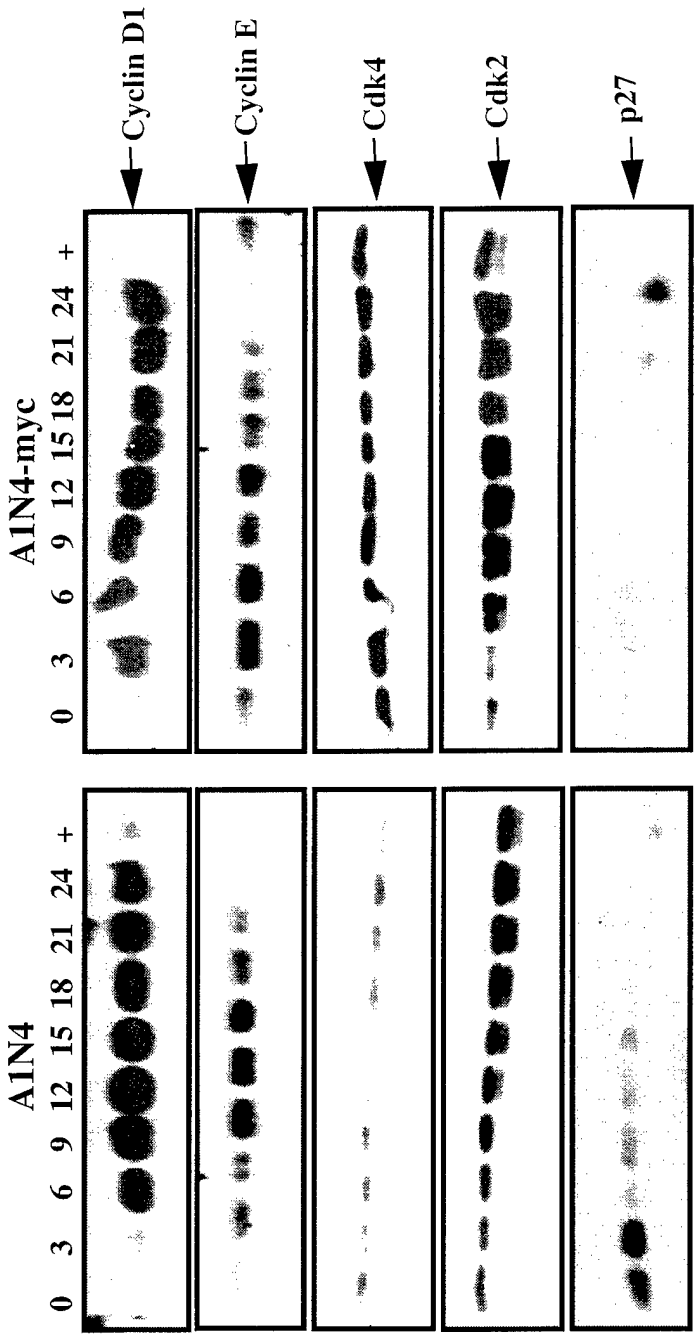

A1N4
0 3 6 9 12 15 18 21 24 +
A1N4-myc
0 3 6 9 12 15 18 21 24 +
Cyclin D1
Cyclin E
Cdk4
Cdk2
p27

Figure 5

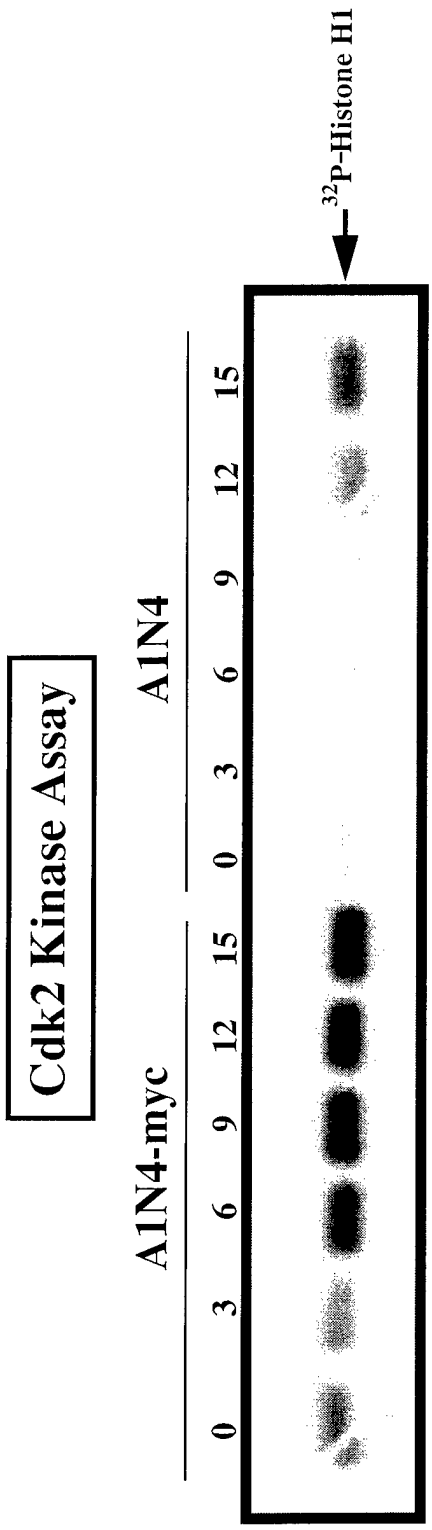
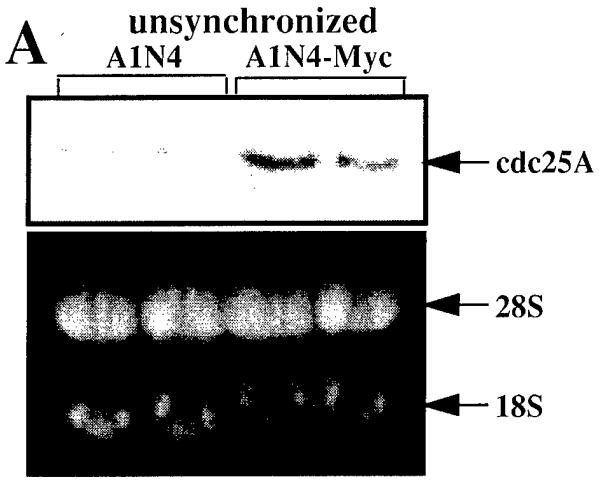

Cdk2 Kinase Assay
A1N4-myc
0 3 6 9 12 15
A1N4
0 3 6 9 12 15
32P-Histone H1

Figure 6


A
unsynchronized
A1N4
A1N4-Myc
cdc25A
28S
18S

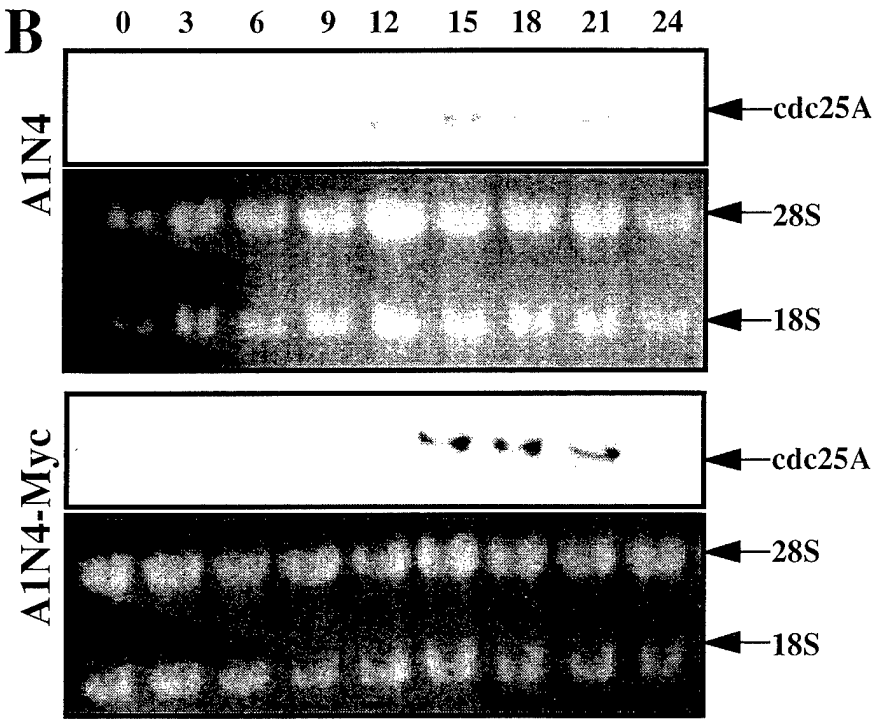

B
0 3 6 9 12 15 18 21 24
A1N4
cdc25A
28S
18S
A1N4-Myc
cdc25A
28S
18S

Figure 7