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The hypothesis tested in these studies is that overexpression of USF in the mammary glands of transgenic mice will inhibit myc-dependent tumorigenesis. Expression of a FLAG-tagged form or USF-2 was targeted to the mammary gland under the control of the mouse mammary tumor virus (mmtv) long terminal repeat. Of eight lines of transgenic mice that were generated, one demonstrated expression of flag-tagged USF-2 in the lactating mammary gland at levels 12-fold over that of endogenous USF-2. Evaluation of tumorigenesis in these mice (n=17) out to 453 days of age suggests that USF-2 when overexpressed by itself is not oncogenic. In contrast, 72% of mmtv-myc mice (n=18) analyzed have developed mammary tumors with an average latency of 156±33 days. Tumor frequency and latency in bigenic mmtv-USF-2/mmtv-C-myc mice out to 200 days of age was similar to that in mmtv-C-myc mice. In contrast the growth of these myc-dependent tumors in bigenic mice was significantly lower ($P<0.01$) than that in mmtv-C-myc mice. This decreased growth, however was not associated with changes in cell proliferation or death. These data support the conclusion that while overexpression of USF-2 has only modest impact on mammary gland development and myc-dependent tumorigenesis

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Introduction

Upstream stimulatory factor (USF) consists of two helix-loop-helix/zipper (bHLH/zip) proteins, USF-1 and -2, which are highly conserved among species and related to c-*myc* (1) transcription factors. Previously published cell culture studies with cancer cell models show that USF is both antiproliferative and can antagonize c-*myc* (2). The research described in this proposal addresses the idea that expression/activity of USF is a determining factor in tumor initiation and/or growth. This idea was to be explored by testing the hypothesis that targeted overexpression of USF-2 in the mammary glands of MMTV-*myc* transgenic mice will cause withdrawal from the cell cycle and differentiation thereby preventing tumors. The overall approach was to make and characterize transgenic mice that overexpress USF-2 under the control of the mouse mammary tumor virus long terminal repeat. Once in hand this new line of transgenic mice would be crossed with a previously described line of transgenic mice that overexpress c-*myc* in the mammary gland. A decrease in tumor frequency and/or an increase in tumor latency among mice that carry both MMTV-*myc* and MMTV-USF-2 as compared with those which just carry MMTV-*myc* would confirm the hypothesis.

Body

The approved statement of work for this project described two specific tasks to be completed over a 36-month period. The first task was to determine the effect of mammary-specific USF-1 overexpression on mammary gland development and lactation. This was to be completed during months 1 through 24. The second task was to determine the ability of mammary-specific overexpression of USF-1 to prevent *myc*-induced mammary tumors. This task was to be completed during months 9 through 36.

the major focus of task 1 for the first 12 months of the funding period was to construct a transgene which overexpresses USF-1 in the mammary gland, use this transgene to produce transgenic mice, and then screen the resulting mice to identify 1 or 2 lines with biologically significant expression levels for use throughout the remainder of the project. During the first four months of the funding period, a transgene was constructed that was designed to target the expression of a tagged form of USF-2 to the virgin mammary gland under the control of the mouse mammary tumor virus long terminal repeat. Early in the period a decision was made to overexpress USF-2 as opposed to USF-1. This change was made because USF-2 was shown to display stronger antiproliferative as well as stronger myc antagonist activity than USF-1 (2). Consequently, USF-2 would be predicted show greater efficacy in the transgene model than USF-1. The overall design of the MMTV-USF-2 construct is presented in figure 1. The construct was produced from 2 starting plasmids, PN4 (2) and MMTV-SV40 (3) . Polymerase chain reaction was used to place a consensus Kozak (4) sequence and coding DNA for a FLAG peptide at the 5' end of the USF-2 cDNA. The primers used in this amplification also included unique restriction sites to facilitate subcloning. To produce the MMTV-USF-2 transgene, the resulting PCR product was then subloned as a HindIII/EcoRI fragment into the analogous sites of the MMTV-SV40 expression vector. The resulting plasmid was then sequenced to confirm the accuracy of the open reading frame for USF-2. Following the confirmation of the correct coding sequence the USF-2 transgene, the USF-2 coding sequence was subloned into a general-purpose expression vector, pSG5. This was used to evaluate that FLAG-tagged USF-2 in cultured cells. The ability of the FLAG-tagged transgene protein to be detected by immunohistochemistry was confirmed in transfected HeLa cells in culture (Fig 1 C). This result confirmed that the modified USF-2 cDNA was capable of producing a protein. To address the biological properties of the FLAG-tagged USF-2 protein, colony formation

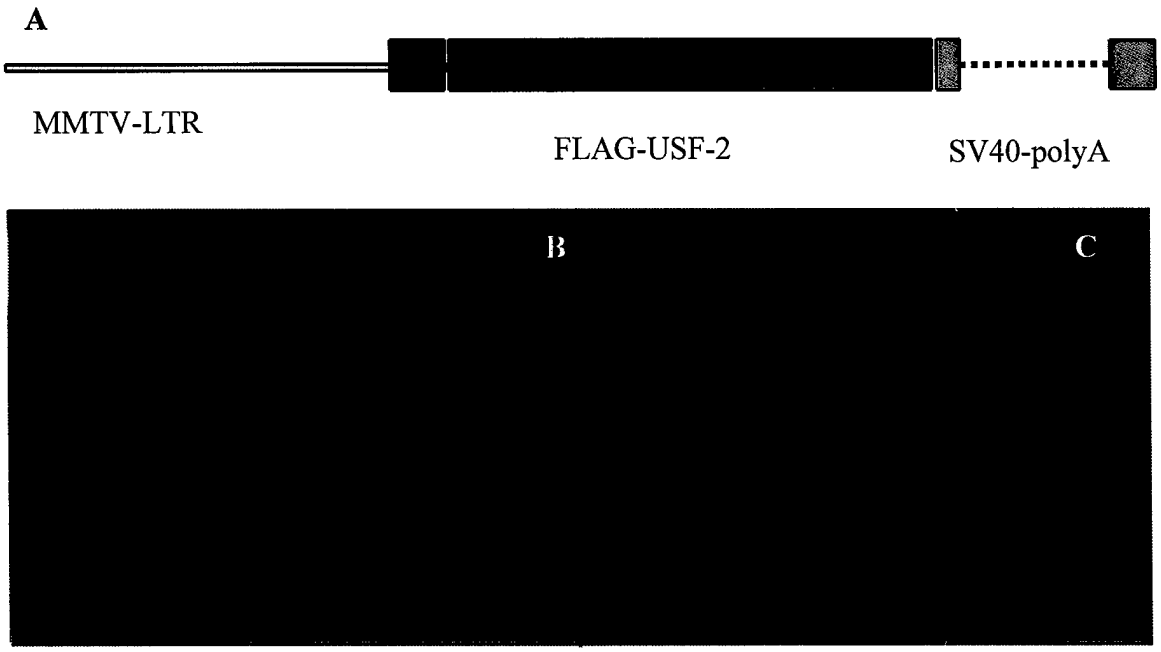


Figure 1. Design of the MMTV-USF-2 transgene (A) and immunofluorescent detection of FLAG-tagged USF-2 in transiently transfected HeLa cells (C). DAPI staining (blue) was used to detect both nontransfected and transfected cells (B). The transgene construct is driven by the MMTV-LTR (□) and contains coding sequence for the eight-residue FLAG peptide (■) fused in-frame to the N-terminal coding sequence for USF-2 (■). Sequence from SV40 T/t antigen provide an intron and poly A signal (▒). FLAG-tagged USF-2 expression (red) is visible in 4 of the 8 cells present in the field.

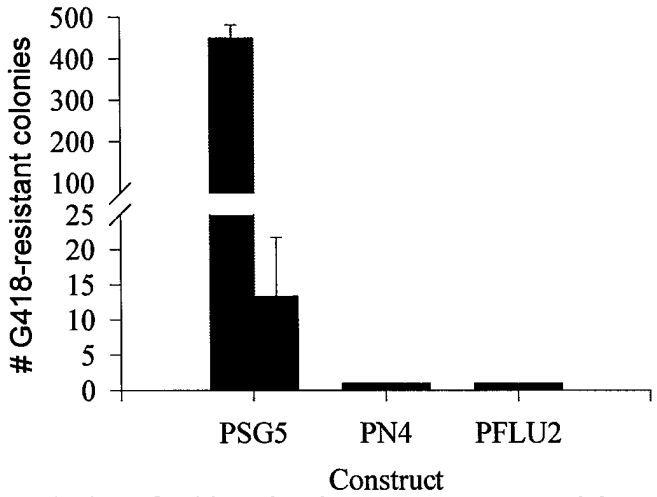


Figure 2. Inhibition of colony formation by USF-2 or a FLAG-tagged USF-2. Either HeLa cells (▬) or HC11 mammary cells (▬) were transfected with a neomycin resistance plasmid and either the empty expression vector (pSG5), the parental USF-2 expression vector (PN4), or the FLAG-tagged USF-2 expression vector (PFLU2). Colonies were then counted after a 4 weeks selection period in G418. Each bar represents the mean±SD. of three plates. Cells transfected with either PN4 or PFLU2 had zero colonies after 4 weeks of selection in G418.

during G418 selection was measured in cells transfected with either the parental USF-2 expression vector, the FLAG-tagged USF-2 vector, or an empty expression vector, pSG5 (Figure 2). This type of assay was originally used to demonstrate the antiproliferative effects of USF-2 (2) in tumor cells. Mammary epithelial cells or HeLa cells were transfected with one of the three vectors and a neomycin resistance vector. Following transfection the cells were selected in G418 for 1 month. After the selection period colony number was measured. Both the parental vector and the FLAG-tagged USF-2 inhibited colony formation in both HeLa cells and HC11 mammary epithelial cells. This supported the

conclusion that the modified USF-2 was in fact capable of eliciting a biological response similar to the parental, unmodified USF-2. Following this evaluation the MMTV-USF-2 transgene was purified away from bacterial plasmid DNA by digestion with BssHII and preparative agarose gel electrophoresis. The resulting transgene fragment was then injected in FVB mouse embryos for the production of founder transgenic mice. The injection data is summarized in table 1. From four independent rounds of microinjection a total of 12 founders were obtained. Of these, 8 transmitted the transgene to F1 offspring, 2 were fertile, but did not transmit the transgene, and 2 were incapable of reproducing. Expression of the transgene in the mammary gland was first evaluated in virgin founder females by western blot analysis of mammary tissue lysates using the M2-2 monoclonal antibody to the FLAG epitope (Fig. 3). Using this analysis, the transgene protein was easily detected in transiently transfected into HeLa cells (Fig 3, lane 1). In addition, a band of the correct size was detected in a single extract prepared from lactating mammary tissue of a single founder female (Fig 3, lane 2). This band was not present in an extract prepared from a lactating nontransgenic sibling (lane 3). This supports the conclusion that the transgene protein was in fact expressed at a detectable level in a single founder female during lactation. Expression of the transgene was undetectable in the virgin mammary gland for any of the founders (Fig 3, lanes 5, 6, 7, 9, 10 and 13). This result supported the conclusion that studies on development of the mammary ductal system in virgin USF-2 transgenic mice would not be possible.

Table 1. Production of transgenic mice with the MMTV-USF-2 transgene by microinjection of fertilized one-cell FVB embryos.

Injection	Embryos injected	Offspring obtained	Positive offspring	F1 lines
1	118	34	0	0
2	99	25	1	0
3	94	9	4	3/4
4	59	16	7	5/7

Because transcriptional activity of the MMTV-LTR is known to be dramatically induced with lactation, the major focus of task 1 for months 12 through 24 of the funding period was to complete a screen for transgene expression in lactating mammary tissue from mmtv-USF-2 lines and to determine the impact of such expression on mammary gland development and lactation. To complete the screen, females from each of six of the eight MMTV-USF-2 lines were mated and allowed to complete a normal pregnancy. At sixteen days postpartum, mammary gland biopsies were collected from these lactating females and used for the preparation of protein extracts for western blotting. Western blotting of these extracts using an anti-flag (Fig 4A) antibody to detect the epitope tag revealed that two of the transgenic females analyzed expressed detectable protein. Analysis of these extracts by western blotting with an

antibody to USF-2 (Fig 4B) demonstrated a dramatic increase in the abundance of USF-2 in an F1 female from line 2904. Comparison of the abundance of this line with nontransgenic females demonstrated an eight-fold elevation in the abundance of USF-2. This result suggested that females from line 2904 would be useful for studies on the effects of USF-2 overexpression on mammary gland development and tumorigenesis thus allowing for the completion of tasks 1 and 2 of the originally proposed studies.

Because the transgene was only expressed during lactation, studies on virgin development in the MMTV-USF-2 transgenic mice were not conducted. To determine the effects of USF-2 overexpression on lactation, nontransgenic (n=4) and MMTV-USF-2 (n=4) mice were studied during the first eight days of lactation. The study was initiated by crossfostering the dams to litters of 10 pups each on day 2 postpartum. Comparison of average pup weight over the remaining 7 days of the study demonstrated a only a slight negative effect of USF-2 overexpression of pup growth (Fig 5). This effect was not statistically significant, however (P=0.14).

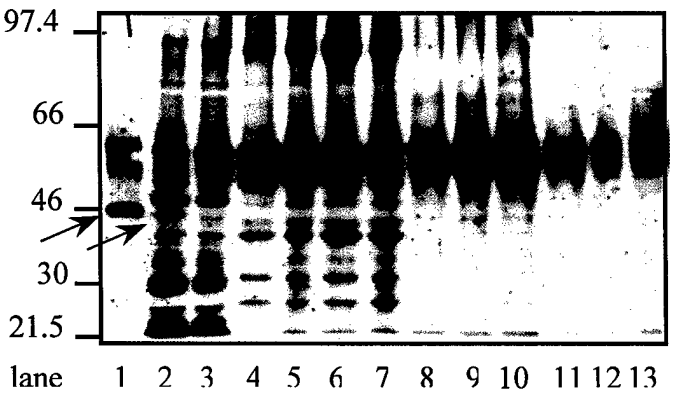


Fig 3. MMTV-USF-2 Transgene is not expressed in the virgin mammary gland. Western blot analysis for FLAG-tagged USF-2 in mammary tissue from transgenic and nontransgenic F1 females. Transiently transfected HeLa cells serve as a positive control (lane 1). The expected size of the protein is 44 kDa. Lanes 2 and 3 contain mammary tissue extracts from a lactating transgenic founder and her nontransgenic sibling respectively. Lanes 4, 8, 11 and 12 contain mammary tissue extracts from nonlactating F1 nontransgenic sibling females. Lanes 5,6,7 9,10, and 13 contain mammary tissue extracts from nonlactating F1 transgenic females. The arrows highlight FLAG-tagged USF in both the positive control sample and a single lactating founder female. molecular weight in kiloDaltons is indicated on the left.

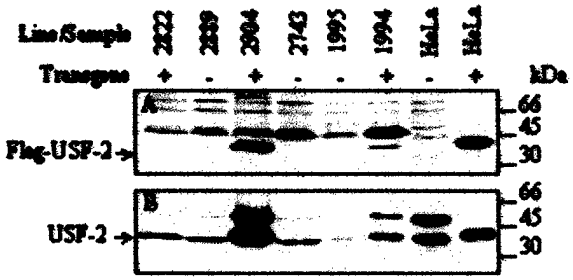


Fig 4. MMTV-USF-2 is expressed in the lactating mammary gland. Screening of lactating mice for USF-2 transgene expression. Western blotting was used to detect the flag-tagged USF-2 transgene protein (A) or total USF-2 (B). Extracts were prepared from mammary tissue of nontrangenic (-) of transgenic (+) females at 16 days of lactation. Extracts from transfected HeLa cells were used as controls.

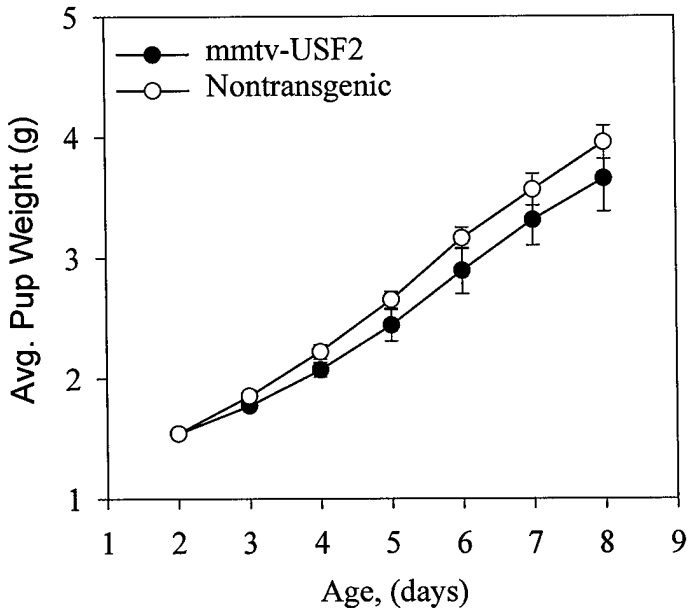


Fig 5. Lactation is not compromised in MMTV-USF-2 transgenic mice. Growth of pups reared by nontransgenic or MMTV-USF-2 mice. On day 2 postpartum, nontransgenic (○) or mmtv-USF2 (●) dams were placed with crossfostered litters consisting of 10 pups each. Litterweights were collected daily for 8 days. On day 8, a milk sample was collected. On day 9 the dams were euthanized and mammary tissue was collected for biochemical analysis. Each symbol represents the mean±S.D. of repeated measures on four

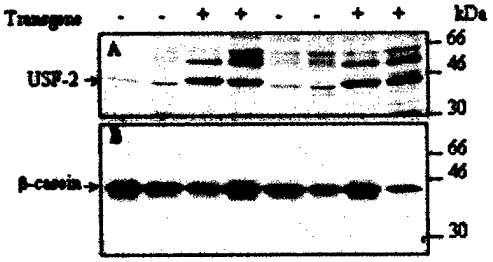


Fig 6. Milk protein gene expression is uninfluenced by overexpression of USF-2. Mammary tissue extracts were prepared for western blotting from nontransgenic (-) or MMTV-USF-2 (+) transgenic mice on day 9 postpartum. For the USF-2 blot (A), 10 mg of protein were loaded per lane. For the milk protein blot (B), 10 ng of protein were loaded per lane. Equivalency of protein loading was confirmed in parallel coumassie-stained gels (not shown).

Table 2 Analysis of mammary gland weigh, DNA and protein in mice that overexpress mmtv-USF-2 during lactation.

Gentoype	*Wet weight*	*DNA, (mg/g)*	*Protein, (mg/g)*	*Protein:DNA*
Nontransgenic	0.51±0.09[a]	4.6±1.6[a]	108.8±28.3[a]	25.0±10.9[a]
mmtv-USF-2	0.38±0.02[b]	4.3±0.6[a]	102.5±9.2[a]	25.6±7.4[a]

[ab]Means within the same column with different superscripts differ ($P<0.05$). Each value represents the mean±S.D. for four animals.

The effect of USF-2 overexpression on mammary gland development during lactation was determined by comparison of morphology, wet-weight, and the content of DNA and protein within the mammary glands on day 9 postpartum. Comparison of hematoxyolin-eosin-stained sections demonstrated little difference between the morphology of nontransgenic and mmtv-USF-2 mice (data not shown). However, mammary gland wet weight was significantly reduced in the mmtv-USF-2 mice as compared to nontransgenic mice (Table 2). This reduction was not however accompanied by significant changes in the DNA or protein content of the mammary tissue.

Overexpression of USF-2 in the mammary gland was confirmed by both western blotting and immunofluorescence. Western blotting for USF-2 abundance in mammary tissue extracts prepared at 9 days postpartum demonstrated an average elevation of 12-fold in the mmtv-USF-2 mice compared to nontransgenic mice (Fig 6A). To determine if overexpression of USF-2 affected mammary cell differentiation, milk protein abundance in mammary tissue extracts from these mice was analyzed by western blotting with an antibody specific for mouse milk proteins (Fig 6B). This antibody detects 7 different milk proteins, which can be distinguished by molecular weight. The most abundant of these is α-casein (fig 6B), which can be detected in as little as 10 ng of total lysate protein per lane. Little difference was observed for α-casein abundance among nontransgenic and mmtv-USF-2 mice supporting the suggestion that overexpression of USF-2 has minimal impact on mammary cell differentiation. Through immunofluorescent staining for USF-2 we demonstrated the dramatic elevation of USF-2 within the mammary cell nuclei of transgenic mice (Fig 7A) as compared to that of nontransgenic mice (Fig 7B). These results support the conclusion that overexpression of USF-2 has little impact on mammary gland development and function during lactation. This concluded the work on task 1.

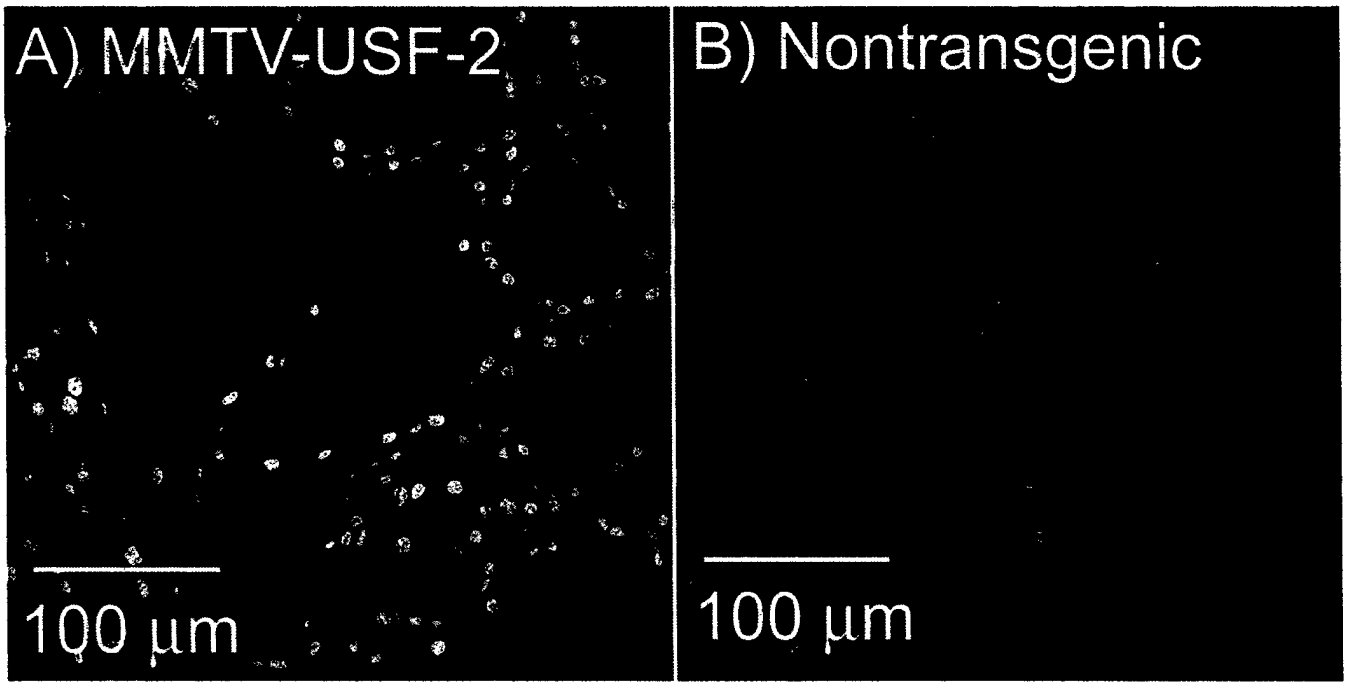


Fig 7. Immunofluorescent staining demonstrates a tremendous increase in nuclear USF-2 in mammary tissue from lactating MMTV-USF-2 mice. Frozen sections were prepared from mammary tissue collected at nine days of lactation from MMTV-USF-2 (A) or nontransgenic (B) mice. These sections were stained with antibodies against USF-2 (green/yellow), and α-smooth muscle actin (blue) and with the nuclear counterstain propidium iodide (red). Magnification is 400x.

Work on a modified version of task 2 commenced during month 15 and consisted of making genetic crosses between the mmtv-USF-2 mice and two other strains of transgenic mice; mmtv-myc (5) and mmtv-v-Ha-ras (6). The goal of these studies was to generate 20 female mice for each of five different genotypes (Table 3). At the termination of the sample collection period for this study, we were able to obtain a sample number that was 65 to 90 % of our original goal for all but one of the strains. In addition, the comparison of tumorigenesis among the Myc/Ras and USF-2/Myc/Ras mice was limited by the fact that surprisingly few tumors were observed in the 15 Myc/Ras females studied and even fewer trigenic USF-2/Myc/Ras females were obtained for study. Despite these limitations, the analysis of the tumor frequency data (Table 3) along with the comparison of tumor-free survival curves (Fig 8) among the Myc and Myc/USF-2 mice supports the conclusion that sample size was adequate to test our original hypothesis concerning the ability of USF-2 to inhibit myc-dependent mammary tumorigensis. This analysis demonstrated that tumor frequency, and latency were similar among Myc and Myc/USF-2 mice. Based on this analysis, we reject our initial hypothesis in favor of the alternative that overexpressed USF-2 is incapable of inhibiting myc-dependent mammary tumorigenesis.

Table 3. Summary of tumorigenesis study of transgenic mice that overexpress different combinations of USF-2, c-myc and v-Ha-ras.

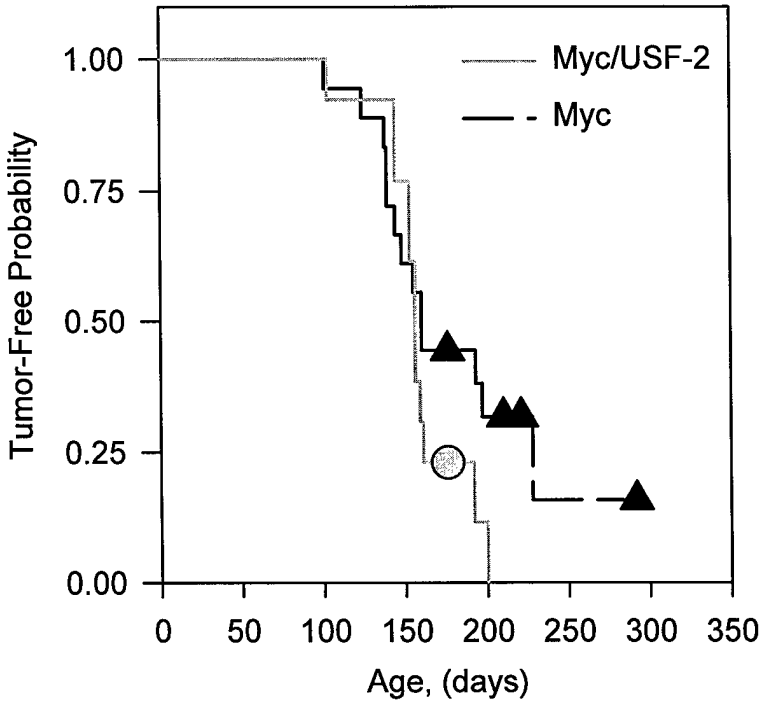
Genotype	*Mice obtained*	*Age[1], (days)*	*Pregnancies*	*Tumors*	*Latency, (days)*
mmtv-USF-2	17/20	453	4±3	0/17	--
mmtv-myc	18/20	292	2±2	13/18	156±33
USF-2/myc	13/20	200	2±1	12/13	156±24
myc/ras	15/20	399	<1	3/15	195±43
USF-2/myc/ras	6/20	362	1±1	2/6	230±17

[1]Age of oldest animal studied

In addition to collecting tumor frequency and latency data, tumor growth was measured and samples of both tumor tissue and adjacent noninvolved mammary glands were collected for further analysis. Comparison of Hematoxylin & Eosin-stained (H&E) tissue section demonstrated similar morphological characteristics among the Myc and Myc/USF-2 tumors. In general, tumor morphology was similar to the previously described cribiform morphology attributed to tumors in MMTV-Myc mice. Cells within the tumors contain two patterns of nuclei. Those with vesicular pale nuclei were often organized as solid pseudotubules with expansive and intraductal growth. Cells with small, uniform Hematoxylin-stained nuclei were frequently found as solid sheets. All tumors had frequently observed mitotic figures along with tremendous amounts of cell shedding. In most of the tumors, massive areas of secretion/necrosis were present (Fig 9A). In areas where this massive secretion/necrosis were not present, there were frequent small lacunae (Fig 9B) of apoptotic cells. The area occupied by necrosis within the tumors was estimated from TIFF images collected for each of the tumors and found to be similar among Myc and Myc/USF-2 tumors. In addition, the number of apoptotic lacunae per average field within these H&E-stained sections was similar among Myc and Myc/USF-2 tumors.

To determine if overexpressed USF-2 was capable of influencing tumor growth, weekly caliper measurements were made on the tumors for 4 weeks following their detection (Fig 10). The average tumor radius was estimated from two perpendicular measurements made on each tumor. This radius was then used to calculate tumor volume by the formula$V=4/3\pi r^3$. The results of this analysis demonstrated that the tumors in both populations grew slowly during the first two weeks and then began to grow exponentially during the last two weeks of the study period. The results also demonstrated that Myc tumors were significantly larger than Myc/USF-2 tumors by week 3 of the study. After 4 weeks of growth, the myc tumors were 49% larger ($P<0.01$) Myc/USF-2 tumors (Fig 10). This result supports the conclusion that overexpression of USF-2 slowed the growth of tumors in the MMTV-Myc mice.

Fig 8. Tumor—free survival is similar among Myc and Myc/USF-2 mice. Kaplan-Meier plots were constructed from tumor-free survival data collected from female MMTV-Myc (black triangles) or MMTV-Myc/MMTV-USF-2 (grey circles) mice housed continuously with a male. Censored data is illustrated by the symbols. Censored animals are those that remained tumor-free at the conclusion of the study.



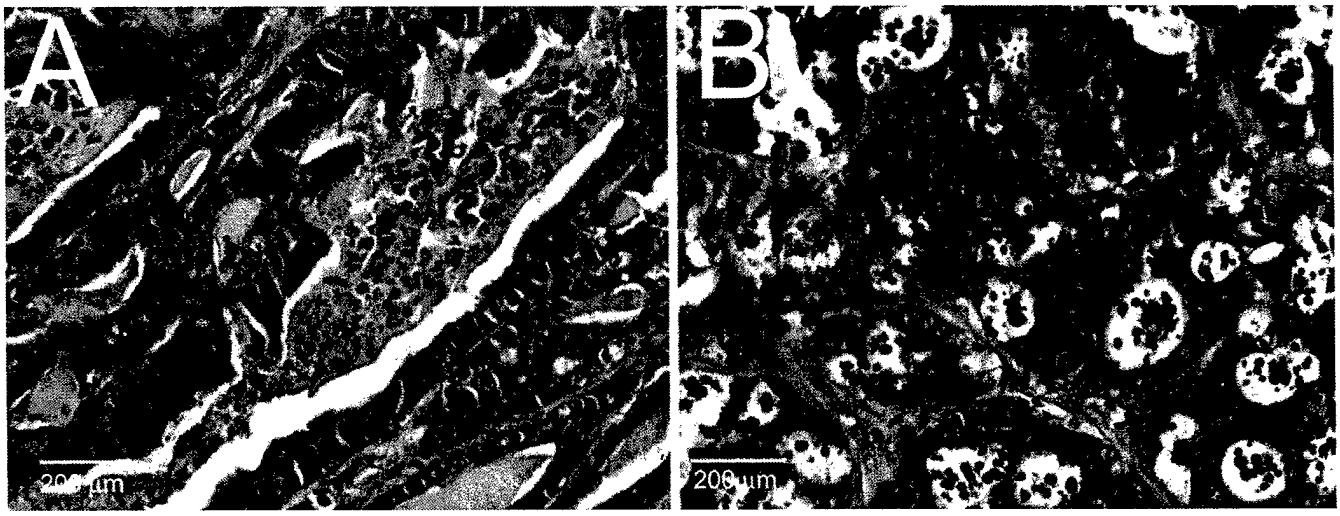
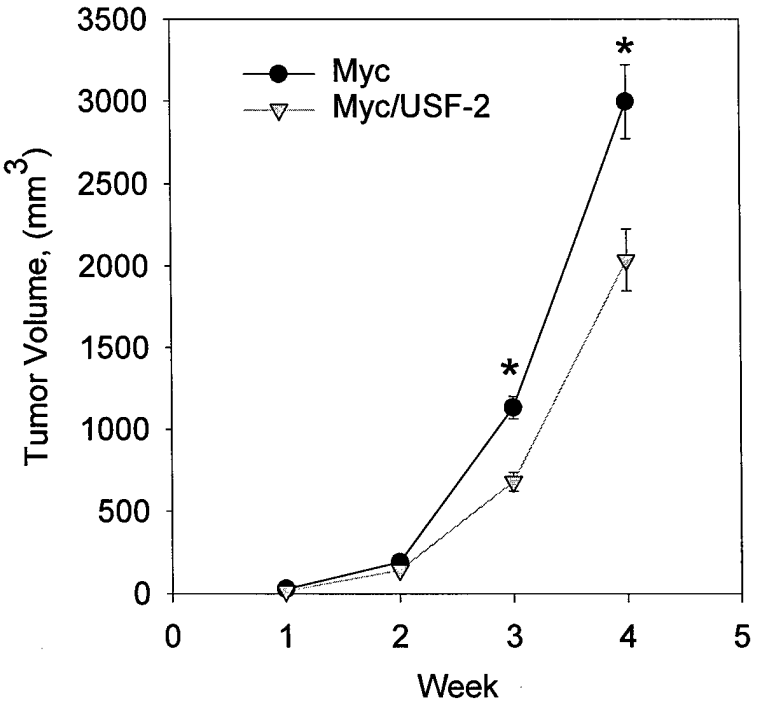


Fig 9. Morphology is similar among Myc and Myc/USF-2 tumors. Tumor samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin, sectioned and then stained with H& E. the Pictures in A and B are representative of 14 myc and 14 Myc/USF-2 tumors. Magnification is 400X.

To establish the potential mechanisms underlying the reduced growth observed in tumors from the Myc/USF-2 mice, cell proliferation and apoptosis were compared by immunofluorescent staining of frozen sections prepared from the Myc and Myc/USF-2 tumors. Each tumor was stained either by fluorescent TUNEL labeling or with an antibody specific for the G2M-phase marker, phospho-Histone H3. For both analyses, the double-stranded DNA-binding dye TOPRO 3 was used as a nuclear counterstain. Estimates of the percent positive cells were obtained by automated counting of 15 TIFF images collected at random from each specimen. Analysis for both of these markers demonstrated that both cell proliferation and cell death were similar among Myc and Myc/USF-2 tumors (Fig 11). These results support the conclusion that the diminished growth observed in Myc/USF-2 tumors is not due to changes in cell proliferation or cell death. An alternative explanation for the decrease in tumor growth could be related to the idea that myc and USF-2 might interact to regulate cell size independent of the rates of cell-cycle progression or cell death. A number of studies now link myc to the regulation of cell size (7). One key observation of relevance to this is that the myc antagonist MAD1 has been shown to reduce cell size in animals when overexpressed under the control of the actin promoter (8). This mechanism might also be at work in the Myc/USF-2 tumors.

Fig 10. Overexpressed USF-2 slows the growth of Myc-dependent tumors. Tumor size was measured once per week for 4 weeks in Myc (black circles) and Myc/USF-2 (grey triangles) mice. tumor volume was estimated as described in the text. Each point represents the mean±SEM for 14 tumors. Asterisks indicate significant differences (P<0.05).



Another possible explanation for the limited effects of the USF-2 transgene is that expression levels of the transgene were just not high enough to elicit the same types of biological responses in the tumors that were observed in previously described cell culture models of myc-USF-2 interaction (2). To address this issue the expression of the USF-2 transgene was measured by western blotting on tumor tissue extracts prepared from myc and myc/USF-2 transgenic mice (Fig 12). The results of this analysis demonstrated that detectable levels of the transgene were present in all, but one of the myc/USF-2 tumors. Densitometry analysis the USF-2 western blots revealed the the levels of USF-2 within the tumors of the Myc/USF-2 transgenic mice were elevated by about 2 – fold (P<0.05) (area under the curve, 24.9±6.6 vs 56.5±14.2 for Myc or Myc/USF-2 tumors, respectively). These results support the conclusion that the USF-2 transgene was expressed in the mammary tumors of the bigenic mice but at

much lower levels than that observed normal lactating mammary tissue from these mice. Hence the possibility remains at this point that the modest effects of the USF-2 transgene may have been due to the fact that expression levels were only modestly elevated.

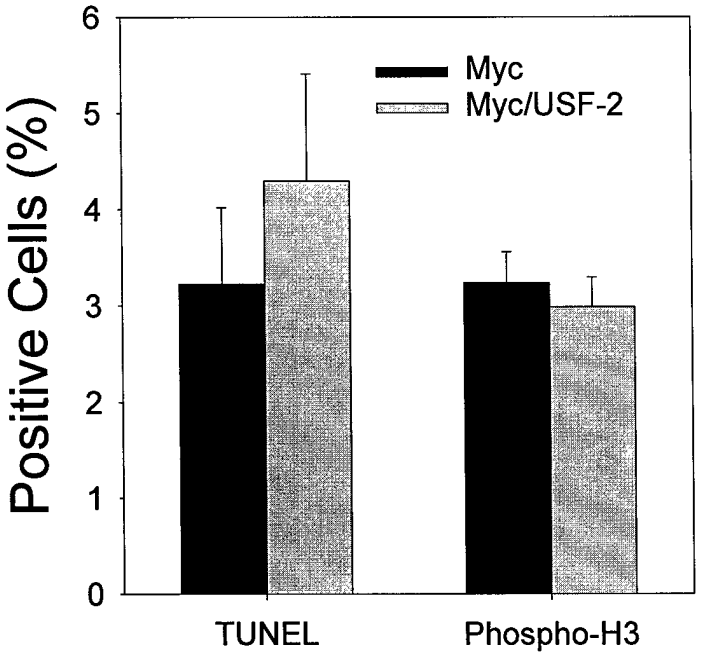


Fig 11. Overexpression of USF-2 has does not significantly influnce apoptosis or proliferation in Myc-dependent mammary tumors. Frozen sections were prepared from tumors arising in Myc (black) or Myc/USF-2 transgenic mice. Apoptosis was detected by fluorescent TUNEL. Proliferation was detected by immunofluorescent staining for phospho-histone H3 (phospho-H3). Percent positive cells was estimated by automated counting of tiff images collected from each tumor.

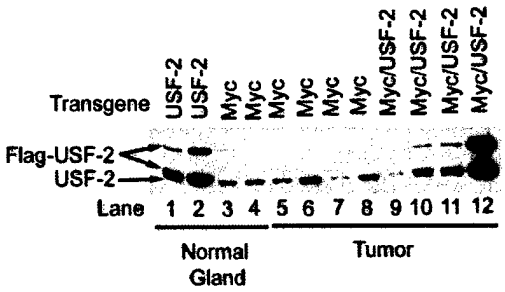


Fig 12. Increased USF-2 expression occurs in several of the tumors found in the Myc/USF-2 bigenic mice. Western blotting was used to detect USF-2 expression in mammary and tumor tissue. Tissue extracts were prepared from mammary tissue (lanes 1-4) or mammary tumors (lanes 5-12) of USF-2 (lanes 1 and 2), Myc (lanes 3-8) or Myc/USF-2 (lanes 9-12) transgenic mice. Note the presence of a doublet in extracts from the USF-2 transgenic tissue. This is indicative of the flag peptide on the N-terminus of the transgenic protein.

Key Research Accomplishments

- Constructed a transgene (MMTV-USF-2) designed to target USF-2 expression to the virgin mouse mammary gland.
- Validated the functionality of MMTV-USF-2 in cell culture
- Injected mouse embryos with MMTV-USF-2
- Obtained eight independent transgenic mouse lines that carry MMTV-USF-2.
- Screened six founder lines of transgenic mice for expression during lactation.
- Obtained one line of transgenic mice capable of 8-fold overexpression of USF-2 within the mammary gland during lactation.
- Determined the impact of overexpressing USF-2 on lactational capacity and the expression of milk proteins.
- Obtained transgenic (bigenic) mice carrying both mmtv-myc and mmtv-USF-2 transgenes.

- Determined that overexpression of USF-2 in the mammary glands of transgenic mice does not cause mammary tumors.
- Determined that overexpression of USF-2 in the mammary glands of MMTV-Myc mice has no effect on the incidence or latency of mammary tumors.
- Determined that overexpression of USF-2 in the mammary glands of MMTV-Myc mice causes a modest but statistically significant reduction in the growth of established tumors.
- Determined that decreased tumor growth rate in the Myc/USF-2 mice is not due to changes in cell cycle progression or cell death
- Determine that the MMTV-USF-2 transgene is expressed in the tumors obtained from Myc/USF-2 transgenic mice.

Reportable Outcomes

- Development and characterized a line of transgenic mice that express Flag-tagged USF-2 in the mammary gland during lactation at levels 12-fold above endogenous USF-2.
- Obtained bigenic mice with which to evaluate the ability of USF-2 to block myc-dependent tumorigenesis.
- Produced a bank of tumors and mammary tissue samples in transgenic mice that overexpress either Myc, or both Myc and USF-2.
- Presented an Abstract at the 2002 Era of Hope Meeting in Orlando, Fla.

Conclusions

In summary, the data obtained from these studies support the conclusion that overexpression of USF-2 in the mammary gland has little, if any effect, on the overall mammary gland development or lactation. The data obtained from the tumorigenesis studies, supports the conclusion that USF-2 has little impact on the ability of overexpressed Myc to cause mammary tumors and has only modest influence over the growth of these Myc-dependent tumors. The observation that neither proliferation nor apoptosis appear to account for the differences is perplexing, but may be accounted for by several possibilities. The first is that the difference difference in tumor growth is the result of sustained, but small differences in apoptosis or proliferation that are beyond the current sensitivity or our assays to detect. The second possibility is that earlier during the tumor growth period, larger differences were present in proliferation and/or apoptosis and that as the tumors expanded these differences disappeared as a result of other factors unrelated to the overexpression of USF-2. A third possiblility, is that the expression of the transgene, though capable of producing a 2-fold elevation in USF-2 level within the tumors, was not high enough to antagonize myc. A manuscript is currently in preparation to describe the obervations made in these studies.

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Appendices

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