An Activated Allele of the c-*erbB-2* Oncogene Impairs Kidney and Lung Function and Causes Early Death of Transgenic Mice

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Abstract. The pathogenicity of the human c-*erbB-2* oncogene was evaluated in transgenic mice. A DNA sequence comprising the promoter–enhancer region of the MMTV LTR and a constitutively activated allele of the human c-*erbB-2* growth factor receptor gene was introduced into the germ line of mice. Expression of the transgene was observed in kidney, lung, mammary gland, salivary gland, Harderian gland, and in epithelial cells of the male reproductive tract. All transgenic mice expressing the c-*erbB-2* receptor died within four months of birth. Histopathological analysis suggests that preneoplastic lesions in kidney and lung most likely caused organ failure and the early death of the transgenic mice. Focal dilatation and atypical proliferation of the tubular epithelial cells was found in the kidney. These hyperplastic lesions were found adjacent to normal tubules.

Immunohistochemistry showed that normal renal structures were completely negative for c-*erbB-2* protein expression. Atypical pseudopapillary proliferation of bronchial and bronchiolar epithelial cells narrowed the bronchial lumen in lung. Alveoli appeared normal. The expression of c-erbB-2 protein was strictly limited to the proliferating epithelial cells and not detected in normal tissue. The mammary glands of two parous mice were underdeveloped, lacking lobular–alveolar structures and were lactation deficient. Only a few ducts were interspersed in the fat pad. A virgin mouse developed a focal adenocarcinoma infiltrating the mammary fat pad. Expression of the c-erbB-2 protein was enhanced in the proliferating epithelial cells. Transgenic males were sterile. Epithelial hyperplasia and hypertrophy in the epididymis, vas deferens and seminal vesicles was found. The transgene is not uniformly expressed in the tissues where the MMTV LTR is transcriptionally active. The scattered transgene expression invariably coincides with epithelial hyperplasia.

TRANSMEMBRANE glycoproteins with tyrosine specific protein kinase activity confer signals from extracellular ligands, such as polypeptide hormones and growth factors, to the intracellular signal transduction and transcription machinery. The ligand activated receptor molecules regulate central cellular functions such as growth and differentiation. Mutated forms of these receptors as well as their deregulated expression can contribute to cellular transformation (Ullrich and Schlessinger, 1990; Aaronson, 1991; Hanks, 1991). The c-*erbB-2* gene, the homologue of the rat c-*neu* gene, encodes a typical representative of this receptor gene family. Investigated in different cell types, and probed under different circumstances it was found to contribute to cell proliferation, cell differentiation, and to exert transforming potential.

The human c-*erbB-2* gene was identified due to its similarity with the EGF receptor gene; its homologue is the rat c-*neu* gene (Schechter et al., 1984, 1985; Semba et al., 1985). The two receptors are structurally related and have overlapping as well as distinct biological effects (di Fiore et al., 1990; Segatto et al., 1991). The c-*erbB-2* gene encodes a 185-kD transmembrane glycoprotein, gp185 c-*erbB-2* (Coussens et al., 1985; Yamamoto et al., 1986). It is commonly expressed in fetal epithelial cells of kidney, lung, gastrointestinal tract, placenta at term, and to a lesser degree in normal adult tissues (Gullick et al., 1987; Kokai et al., 1987; Mori et al., 1989; Press et al., 1990).

The c-*erbB-2* receptor can be activated by binding to its ligand heregulin (Holmes et al., 1992) or *neu* differentiation factor (Wen et al., 1992). Activation causes an increase in the tyrosine phosphorylation of the receptor and a mitogenic response, e.g., in the human breast carcinoma cell line SK-BR-3 (Holmes et al., 1992). Differentiation-specific effects were observed in the human breast carcinoma cell line AU-565 upon ligand activation of the receptor. These include alterations in cell morphology, the synthesis of milk components, an increase in the nuclear size, inhibition of cell growth, and induction of DNA polyploidy (Peles et al., 1992). Differentiation promoting effects of c-*erbB-2* receptor activation, e.g., conferal of sensitivity towards the action of lactogenic hormones, have been observed in the mouse mammary epithelial cell line HC11 (Hynes et al., 1990; Taverna et al., 1991).

Both the human c-*erbB-2* gene and the rat c-*neu* gene have

been implicated in transformation of cells in culture and in tumor formation in vivo. Amplification of the c-*erbB-2* gene and over-expression of its gp185 product has been shown to occur in human adenocarcinomas of the breast and ovary (Slamon et al., 1989), as well as in gastric (Yonemura et al., 1991) and nonsmall cell lung adenocarcinoma (Kern et al., 1990). c-*erbB-2* over-expression is predictive of clinical outcome and associated with an unfavorable prognosis (Slamon et al., 1987, 1989; Varley et al., 1987; Berger et al., 1988; Borg et al., 1990). The rat c-*neu* gene was isolated as a dominantly transforming oncogene from a chemically induced rat neuroblastoma (Shih et al., 1981). The activation of the c-*neu* gene was shown to be due to the mutation of a valine residue to glutamic acid in the transmembrane domain (Schechter et al., 1984; Bargmann et al., 1986*a*,*b*). This mutation results in an oncogenic variant which displays constitutive high phosphorylation on tyrosine and is functionally equivalent to the ligand stimulated receptor (Yarden, 1990). The c-*erbB-2* gene is not mutated in human carcinomas and its transforming activity is due to gene amplification and the resulting overexpression of the gene product. It is also highly phosphorylated on tyrosine in primary breast tumor cells which overexpress the gene (Wildenhain et al., 1990).

Transgenic mice have become useful models for the study of oncogenes. The tissue-specific action of oncogenes in vivo and their causality in carcinogenesis have been demonstrated. Consistent patterns of widespread cell proliferation and dysplasia that precede tumor development have been described (Adams and Cory, 1991). To study the influence of the activated c-*erbB-2* oncogene on cell proliferation, differentiation and transformation, we introduced a constitutively activated, transforming allele of the human c-*erbB-2* oncogene under the control of the long terminal repeat of the mouse mammary tumor virus, MMTV LTR, into transgenic mice. Experiments have been described previously in which the activated rat c-*neu* gene and the normal c-*erbB-2* allele under the control of the MMTV LTR have been used (Muller et al., 1988; Bouchard et al., 1989; Suda et al., 1990). The transgenes introduced in these earlier studies are similar, but not identical to the one described here. Our experiments confirm some of the earlier conclusions, clarify conflicting claims and extend the observations. We show that transgene expression is not uniform in tissues in which the MMTV LTR is transcriptionally active and invariably correlates with epithelial hyperplasia. Non-expressing normal cells in lung, kidney, mammary, and salivary glands reside side-by-side with expressing hyperplastic cells. Mammary cells are most prone to transformation and focal adenocarcinomas occur very early. The life span of the transgenic mice is limited by the severe preneoplastic lesions in kidney and lung which result in organ failure.

Materials and Methods

Construction of the MMTV LTR c-erbB-2 Gene

The MMTV LTR contained within a 1.7-kb SalI-HindIII fragment (Jaggi et al., 1986) was converted to a SalI-EcoRI fragment by linker ligation. The HindIII sites of the activated c-*erbB-2* (VE) fragment (Suda et al., 1990) were converted into EcoRI sites and linked to the EcoRI sites of MMTV LTR and to the splicing and polyadenylation signal from SV-40 and cloned into the pSP72 plasmid as depicted in Fig. 1. Standard techniques were used in the cloning experiments (Sambrook et al., 1989).

Generation of Transgenic Mice

Transgenic mice were generated and analyzed as described (Botteri et al., 1987). Fertilized eggs used for microinjection were derived from C57BL/6×CB6F1 mice.

Analysis of the Transgene RNA Transcript

Mouse tissues were removed at necropsy and immediately frozen in liquid nitrogen. RNA was prepared by the single step method of Chomczynski and Sacchi (1987). 15 μg of total RNA per lane were electrophoresed on 1% agarose-formaldehyde gels, transferred to nylon membranes (Gene screen plus) and hybridized either with the 1.6-kb SV-40 fragment or the 4.4-kb c-*erbB-2* fragment labeled with α-[^{32}P]dCTP by the random priming procedure (Boehringer Mannheim Corp., Indianapolis, IN). Prehybridization, hybridization, and washes were carried out as described by Sambrook et al. (1989).

Histology

Complete autopsies were performed and both gross and microscopic examination were done. Tissues for histology and immunohistology were fixed in 10% buffered formalin and embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined for pathological findings.

Immunohistology

For the detection of the *c-erbB-2* protein, paraffin embedded tissues were sectioned at 4 μm, deparaffinized, and immunostaining was performed with the biotin-streptavidin amplified detection system according to StrAviGen™ super sensitive universal immunostaining kit (Bio Genex Laboratories, San Ramon, CA). The specific antibody used to detect *c-erbB-2* protein was the polyclonal antiserum 21N, raised against the COOH-terminus of the *c-erbB-2* protein (Gullick et al., 1987). The enzyme used was alkaline phosphatase and to detect the bound phosphatase complex the chromogen Fast red was applied. All sections were counterstained with Mayer's hematoxylin.

Results

Generation of MMTV LTR c-erbB-2 Transgenic Mice

We derived transgenic mouse lines expressing the activated human c-erbB-2 gene. The transforming gene product of the c-*erbB-2* gene differs from its normal counterpart by a single amino acid substitution (valine 659 to glutamic acid) within the transmembrane domain (Suda et al., 1990) and is homologous to the mutant rat c-*neu* oncogene. A cDNA encoding the activated human c-*erbB-2* (VE) protein was linked to the MMTV LTR promoter/enhancer (Jaggi et al., 1986) and the splicing and polyadenylation sequences from SV-40 (Fig. 1). The biological activity and transformation ability of this MMTV LTR c-*erbB-2* (VE) construct was confirmed in transfected NIH-3T3 cells. Glucocorticoid hormone dependent cell transformation in vitro and tumor formation in nude mice were observed (data not shown).

Transgenic mice were generated. A 7.7-kb SalI fragment comprising the MMTV LTR c-*erbB-2* hybrid gene was microinjected into the male pronucleus of fertilized mouse eggs. 14 positive animals carrying between 2 and 50 copies of the transgene per cell, were identified by Southern blot analysis of DNA from tail biopsies (data not shown). One of the positive animals, TM1631, contained two separate unlinked insertions of the transgene. Only one positive female mouse, TM1165, was able to establish a transgenic line and transmit the transgene to her progeny in a typical Mendelian fashion. These mice, however, did not express the transgene (see below). Of the six positive male animals mated, only one sired a litter. However, this animal was genetically mo-

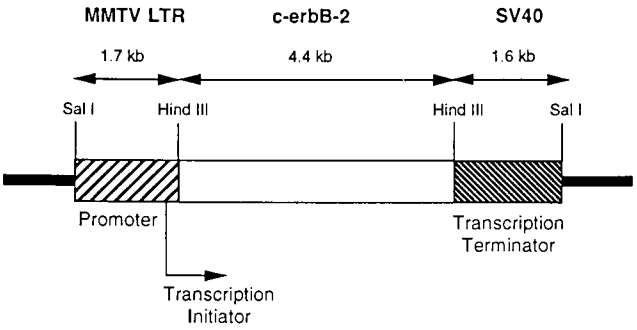

Figure 1. Schematic representation of the MMTV LTR c-*erbB-2* transgene. (□) The c-*erbB-2* cDNA encoding the activated human c-*erbB-2* protein; (▨) The MMTV LTR promoter with the transcription initiation site; (▧) The termination signal from the SV-40 early gene. Relevant restrictions sites are identified.

saic and the transgene was not inherited in its progeny. The other five positive males failed to sire offspring. This is most likely explained by the finding that these animals developed severe hyperplasia and hypertrophy of the epididymis and seminal vesicles (discussed below). Four positive females, TM1630, TM1162, TM1624, and TM1723 were mated successfully and carried pups to term (Table I). All pups died one day after birth, most likely because the transgenic mothers were unable to nurse their young (discussed below). Eight of the positive transgenic mice died unexpectedly of unknown causes between one and four months of age and were not available for necropsy. Two males, TM1623 and TM1696, showed symptoms of respiratory disease and were sacrificed at the age of 4 and 8 wk, respectively. We also examined three apparently healthy females: two 11-wk-old and a 9-wk-old positive females, TM1631, TM1624, and TM1723. TM1631 was a virgin mouse. TM1624 and TM1723 became pregnant and delivered a litter at term, but all pups died within 1 d. These two mothers were sacrificed 2 d later and analyzed together with the other five positive animals. We investigated transgene expression in different tissues and carried out histological and immunohistological evaluations.

Table I. Transgenic MMTV LTR c-erbB-2 Mice

Mouse-Nr	Sex	Life span (weeks)		Pregnant	Pups survival
		Died	Sacrificed		
TM1724	f	4			
TM1755	f	7			
TM1613	m	9			
TM1621	m	10			
TM1630	f	10		+	−
TM1166	m	12			
TM1609	m	13			
TM1162	f	16		+	−
TM1623	m		4		
TM1696	m		8		
TM1723	f		9	+	−
TM1631	f		11	−	
TM1624	f		11	+	−
TM1165*	f		20	+	+
TM11F1‡	f		14	+	+

* Only established line, but not expressing.
‡ Offspring from TM1165.

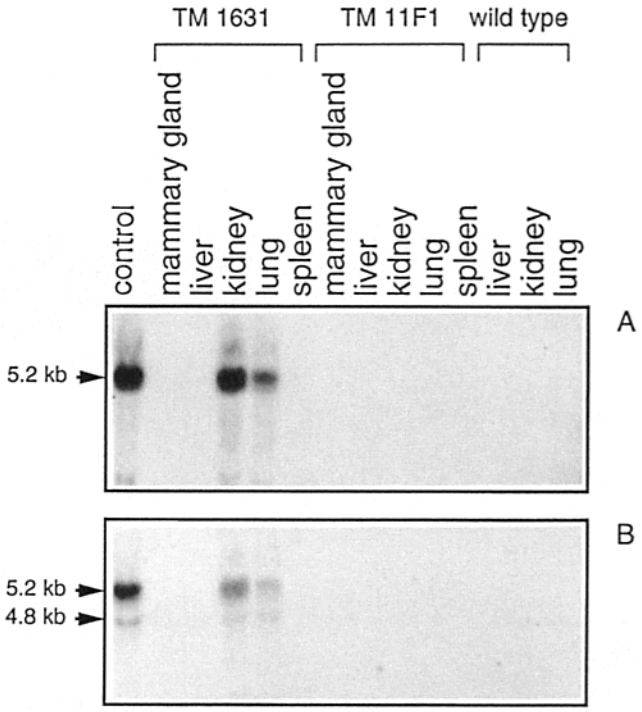

Figure 2. Detection of MMTV LTR c-*erbB-2* RNA in transgenic mice. 15 μg of total RNA from various tissues was analyzed on Northern blots for the presence of c-*erbB-2* transgene specific RNA with the 1.6-kb SV-40 fragment (*A*) or the 4.4-kb c-*erbB-2* fragment (*B*). The equal loading of RNA was verified by methylene blue staining.

Tissue Specific Expression of the MMTV LTR c-erbB-2 Transgene

Transcription from the MMTV LTR promoter is known to be regulated by different classes of steroid hormones in transfected cultured cells (Cato et al., 1987). Its potential to direct the expression of transgenes has been evaluated. Despite the implication in its name, the MMTV LTR is not exclusively active in mammary gland cells. High levels of expression have been detected in mammary epithelium, but expression was also found in the epithelial cells of salivary glands, lung, kidney, seminal vesicles, testes, and also in lymphoid cells in spleen and thymus (Choi et al., 1987, 1988; Stewart et al., 1988; Henrard et al., 1988; Ross et al., 1990).

To determine the MMTV LTR c-*erbB-2* transgene expression in mouse organs, RNA was analyzed by Northern blotting. We used two hybridization probes to detect transgene expression, the 1.6-kb SV-40–specific DNA fragment and the 4.4-kb c-*erbB-2* specific DNA fragment (shown in Fig. 1). The 1.6-kb SV-40 fragment detects exclusively the transgene transcript (predicted length 5.2 kb). The 4.4-kb c-*erbB-2* fragment recognizes the transgene as well as the endogenous mouse c-*erbB-2* gene transcript (predicted length 4.8 kb). The Northern blot analysis of the RNA from tissues of TM1631, a virgin female, and TM11F1 an offspring of founder TM1165, is shown in Fig. 2 *A*. The hybridization was carried out with the 1.6-kb SV-40 DNA probe. A high level of expression of the 5.2-kb transgene transcript was found in kidney and lung of TM1631, but not in TM11F1 and wild-type control mice. TM11F1 is an offspring of TM1165,

Table II. Transgene Expression in MMTV LTR c-erbB-2 Mice

Mouse No/ gender	Tissue						
	Kidney	Lung	Mammary gland	Muscle	Spleen	Brain	Liver
TM1623 m	+++	++	ND	−	−	−	−
TM1624 f	++	+++	−*	−	++	−	−
TM1631 f	+++	++	−*	−	−	−	−
TM1696 m	+	+	ND	ND	++	ND	−
TM1723 f	−‡	−‡	+++	ND	−	ND	−
TM1165 f	−	−	−	−	−	−	−
TM11F1 f§	−	−	−	−	−	−	−
wild-type f	−	−	−	ND	−	−	−

Northern blot analysis was performed on 15 μg of total RNA extracted from a variety of organs from MMTV-c-*erbB-2* mice. Relative levels of transgene expression are indicated by: +, low; ++, intermediate; or +++, high; ND, not done.
* Positive expression in immunohistology.
‡ No significant lesions in histology.
§ Offspring from TM1165.

the only founder mouse capable of producing transgenic progeny.

The pattern of tissue-specific transgene expression in TM1631 was confirmed using the c-*erbB-2* probe (Fig. 2 *B*). The endogenous mouse c-*erbB-2* gene transcript was detected only in lung and kidney of all transgenic mice which also expressed the transgene. The endogenous mouse c-*erbB-2* transcript was not found in lung and kidney RNA of wild-type controls.

The results of the Northern blot analysis of all transgenic MMTV LTR c-*erbB-2* mice investigated are summarized in Table II. The positive animals TM1623, TM1624, and TM1631 showed high levels of transgene expression in lung and kidney while TM1696 showed low expression in these organs. TM1624 and TM1696 displayed in addition high transgene expression in the spleen. A high level of transgene expression in the mammary gland was only observed in TM1723. Transgene-specific RNA expression in the other investigated mammary glands was barely detectable by Northern blot analysis. The immunohistochemical analysis of the tissues was more informative (described below). The deregulated c-*erbB-2* gene expression was consistently high in lung and kidney of four independent transgenic animals. We suspected that the early death of the transgenic animals might be causally related to these observations. For this reason we carried out histological and immunohistochemical analyzes of these organs.

Histological and Immunohistochemical Analysis of Lung and Kidney Tissue

Consistent phenotypic abnormalities were detected only in tissues in which the Northern blot analysis had indicated transgene expression (Tables II and III). Histological examination revealed that the lungs were dominated by mild to severe hyperplasia of bronchial and bronchiolar epithelium with marked basophilia. Pronounced, atypical, pseudopapillary proliferation of epithelial cells caused partial or complete obstruction of the bronchial lumen. Alveoli did not show significant lesions (compare Fig. 3, *A* and *B*). To determine the cellular distribution pattern of the *c-erbB-2* protein, we used the polyclonal antiserum 21N, raised against the COOH-terminus of the *c-erbB-2* protein (Gullick et al., 1987). Immunostaining showed that expression was limited to the proliferating epithelial cells (Fig. 3 *C*). Cells in adjacent normal tissue could not be stained or stained only very weakly.

In kidney the predominant phenotypic alterations were found in the cortex (Fig. 3 *E*). Multifocal, severe hyperplasia of renal tubules resulting in dilatation and microcystic changes were observed. The tubules revealed atypical proliferation of tubular epithelial cells and some even showed pseudopapillary intraluminal proliferation. It can be seen at

Table III. Summary of Phenotypic Abnormalities in MMTV LTR c-erbB-2 Transgenic Mice

Mouse No/ gender	Kidney	Lung	Mammary gland	Salivary gland	Spleen	Epididymis/ Seminal vesicles	Lacrimal/ Harderian gland
TM1623 m	dilated tubules hyperplasia	hyperplasia	NA	ND	NSL	hyperplasia hypertrophy	ND
TM1624 f	dilated tubules hyperplasia	hyperplasia	development incomplete/ nonlactating hyperplastic nodule	hyperplasia	diffuse malignant mastocytosis	NA	ND
TM1631 f	dilated tubules hyperplasia	hyperplasia	development incomplete adenocarcinoma	NSL	NSL	NA	ND
TM1696 m	dilated tubules hyperplasia	hyperplasia	NA	hyperplasia	diffuse malignant mastocytosis	hyperplasia hypertrophy	ND
TM1723 f	NSL	NSL	development incomplete/ nonlactating adenocarcinoma	hyperplasia	focal malignant mastocytosis	NA	hyperplasia
TM11F1 f	NSL	NSL	fully developed lactating	NSL	NSL	NA	ND
wild-type f	NSL	NSL	fully developed	ND	NSL	NA	ND

* Offspring from TM1165
NA, not applicable; ND, not done; NSL, no significant lesions.

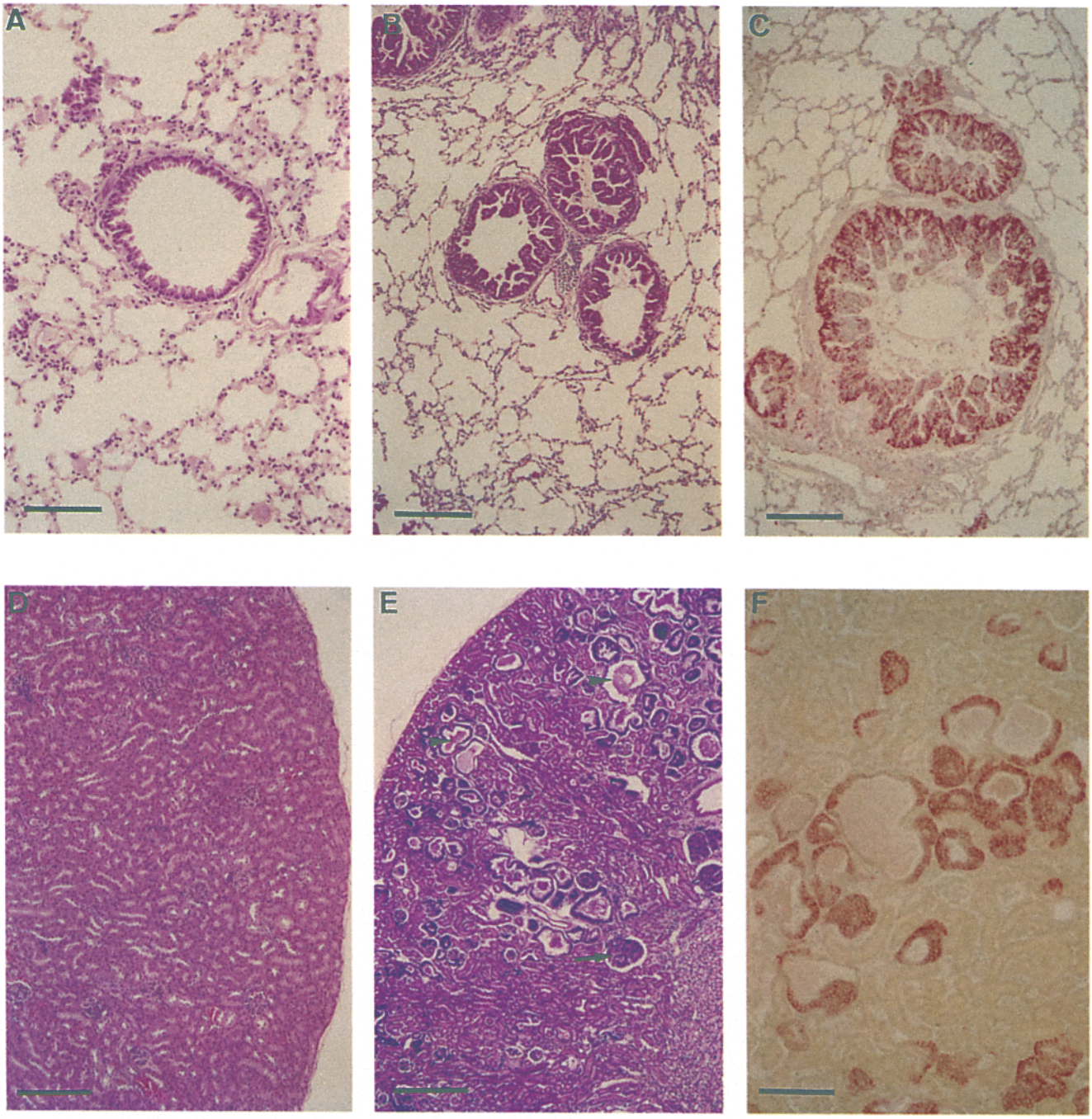

Figure 3. Histology and immunohistology of lung and kidney from MMTV LTR c-*erbB-2* transgenic and wild-type mice. (*A*) Lung from a wild-type mouse. (*B*) Lung from a transgenic mouse. Note the severe pseudopapillary proliferation of bronchial epithelial cells obstructing the bronchial lumen. Alveoli are normal. (*C*) Immunostaining of the same lung as in *B*. Note the characteristic membrane staining for the *c-erbB-2* protein in the hyperplastic bronchial epithelial cells. (*D*) Kidney from a wild-type mouse. (*E*) Kidney from a transgenic mouse. Note the multifocal dilatation of the tubules (*arrowhead*). Atypical renal tubular epithelial proliferation with marked basophilia. Some glomeruli at the cortico–medullary junction are enlarged and hypercellular (*arrow*). (*F*) Immunostaining of the same kidney as in *E*. Note that the membrane staining is strictly localized to the hyperplastic epithelial cells. The adjacent normal tubules are not stained. Bars: (*A*, *C*, and *F*) 100 μm; (*B*, *D*, and *E*) 200 μm.

higher magnification that the tubular epithelial cells were markedly basophilic and atypical with increased cell volume and enlarged nuclei (data not shown). Normal tubules were seen adjacent to these lesions. Glomeruli also showed abnormalities which were found predominantly at the cortico–medullary junction. Here, the glomeruli were enlarged in size and hypercellular, with loss of glomerular capillary loops and mesangium. Towards the renal capsule the glomeruli appeared normal (data not shown). Immunohistochemical analysis showed very strong c-*erbB-2* expression throughout these hyperplastic lesions. There was no staining of histologically normal renal structures detectable (Fig. 3 *F*).

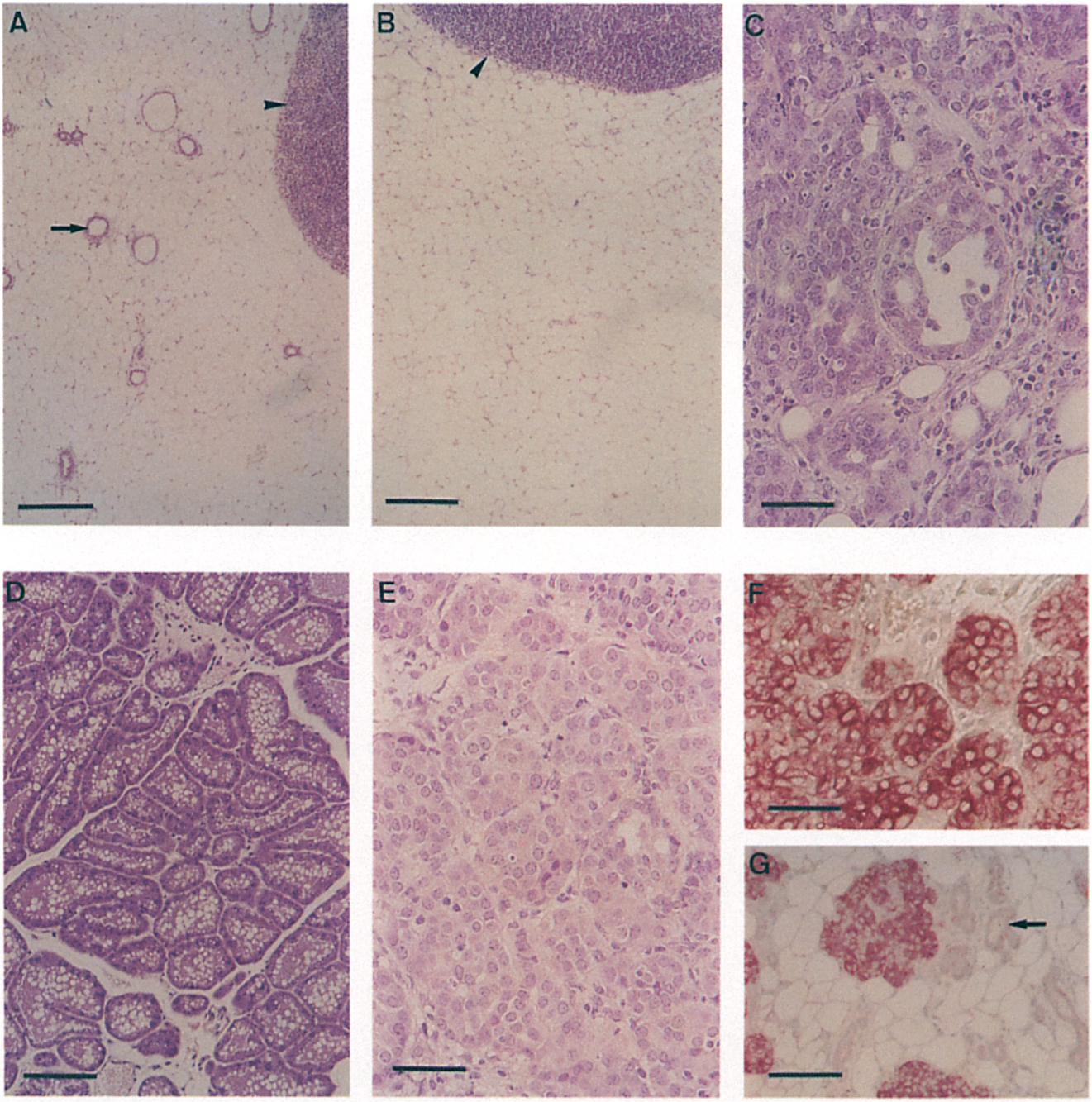

Figure 4. Histology and immunohistology of mammary glands and epididymis of MMTV LTR *c-erbB-2* transgenic and wild-type mice. (*A*) Mammary tissue from a wild-type virgin mouse. Note the presence of mammary ducts (*arrow*) interspersed through the mammary fat pad. Lymph node (*arrowhead*). (*B*) Mammary tissue from a virgin transgenic mouse. Mammary glandular ducts are largely missing from the fat tissue. Lymph node (*arrowhead*). (*C*) Adenocarcinoma from a virgin transgenic mouse. Note the presence of neoplastic pleomorphic epithelial cells narrowing the glandular lumen. (*D*) Mammary tissue from a parous wild-type mouse. Note the regular pattern of a fully developed alveolar–lobular structure with secreted milk in the alveoli. (*E*) Hyperplasia of the mammary gland from a parous transgenic mouse. Note the atypical, severe proliferation of epithelial cells completely filling the glandular lumen. (*F*) Immunostaining of the same mammary gland as in *E*. Note the characteristic membrane staining for c-*erbB-2* in the hyperplastic epithelium. (*G*) Immunohistology of a mammary gland from a parous transgenic mouse. Note the nonuniform expression pattern of the c-erbB-2 protein. The hyperplastic nodule shows a strong surface staining, whereas the adjacent morphologically normal ducts appear negative (*arrow*). (*H*) Epididymis from a wild-type mouse (8 wk). Note the lumina of the tubules contain many spermatozoa. (*J*) Epididymis from a transgenic mouse (8 wk). Tubular lumens are extremely dilated. Note the focal pseudopapillary epithelial hyperplasia. Spermatozoa are largely missing in the tubular lumen. (*K*) Immunohistology of the same epididymis as in *J*. The staining pattern for c-*erbB-2* reveals partially intense, and partially faint staining throughout the hyperplastic epithelium. Bars: (*A*, *B*, *H*, and *J*) 200 μm; (*C*, *E*, and *F*) 50 μm; (*D*, *G*, and *K*) 100 μm.

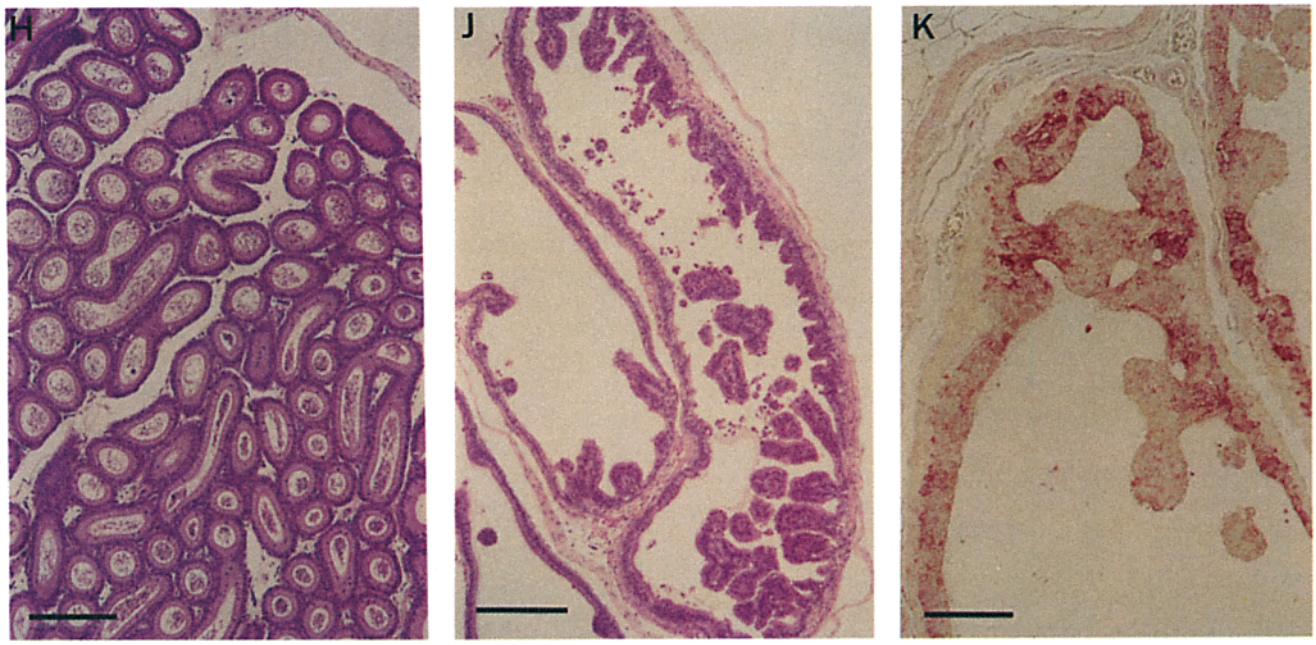

Figure 4.

Predominant membrane staining, indicative for cells with high c-erbB-2 protein expression at the cell surface was seen at higher magnification (data not shown). These results differ from the ones obtained with the activated MMTV LTR c-*neu* transgene (Muller et al., 1988; Bouchard et al., 1989, see discussion).

Histopathological Evaluation of the Mammary Glands

The analysis of the mammary tissue of the transgenic mice was particularly important in light of the frequent involvement of the c-*erbB-2* gene in human breast cancer, and the conflicting observations made in studies with MMTV LTR c-*neu* transgenic mice. The most striking finding of the histological evaluation of mammary glands of several transgenic female mice was the abnormal development and structure of the mammary tissue and its infiltration by hyperplastic or neoplastic nodules. Histopathology on the virgin mammary glands from TM1631 showed that the gland was dominated by the presence of a typical adenocarcinoma infiltrating the mammary fat pad (Fig. 4 *C*). There was a marked atypical proliferation of the alveolar ductal epithelium with pleomorphic changes and numerous mitotic figures. In some areas of the tumor pseudopapillary proliferation narrowed the ductal lumen. The adjacent mammary fat tissue was only sparsely scattered with ductal structures, this is in contrast to their frequent appearance in wild-type virgin mammary glands (Fig. 4, *A* and *B*).

Two parous transgenic mice, TM1624 and TM1723, lost their pups shortly after birth, suggesting lactation deficiency. Histological examination of their mammary glands revealed completely underdeveloped mammary glands composed mainly of fat tissue interspersed by only a few ducts and multiple infiltrating hyperplastic nodular lesions. The mammary glands lacked the typical lobular-alveolar structure of the lactation state, in particular the secretory, lactating alveoli were absent. Similar underdeveloped mammary glands were seen in MMTV LTR int-3 transgenic mice (Jhappan et al., 1992).

At higher magnification the hyperplastic nodular lesions showed severe, atypical proliferation of the alveolar–ductal mammary epithelium partially, or completely filling the ductal lumen, and in some areas even forming solid cords of cells (Fig. 4 *E*). In TM1624 the hyperplastic nodules seemed not yet fully transformed. In contrast, in TM1723 one out of three histologically evaluated mammary glands revealed the characteristics of an adenocarcinoma. The pleomorphic epithelial cells infiltrate the skeletal muscle. The adenocarcinoma was adjacent to atypical hyperplasia. These growth disturbances were always localized and never involved the epithelial cells of the entire mammary gland. Interspersed in these hyperplastic lesions were a few normal ducts. These results indicate the stochastic appearance of mammary tumors in the MMTV LTR c-*erbB-2* mice.

Immunohistochemical analysis of these mammary glands showed strong c-*erbB-2* staining in the hyperplastic lesions (Fig. 4 *F*) and the adenocarcinomas. The staining was limited to the proliferating epithelial cells. No or only very weak immunostaining was detected in the remaining normal mammary ducts (Fig. 4 *G*). These results are in contrast to reports in which the transgene was found to be uniformly expressed at high levels in both malignant and normal tissue of transgenic mice (Stewart et al., 1984; Leder et al., 1986; Sinn et al., 1987; Tsukamoto et al., 1988; Bouchard et al., 1989).

Consequences of c-erbB-2 Expression in the Male Reproductive Tract

Dramatic changes of the male reproductive tract were already observed macroscopically. Abnormal, bilateral enlargement of the epididymis, vas deferens, and seminal vesicles was noted in two transgenic males, TM1623 and TM1696. Subsequent histopathology of these lesions revealed severe

dilatation and hypertrophy of the tubules with focal areas of atypical pseudopapillary epithelial hyperplasia causing an abnormal architecture. Immunohistochemical analysis showed c-*erbB-2* expression throughout these extensive epithelial proliferation. The staining does not seem homogeneous. The pseudopapillary proliferations show weaker staining than the less proliferating areas (Fig. 4 *K*). No staining was observed in epididymis of wild-type control animals (data not shown). The impediment of spermatogenesis probably explain the sterility of these animals. None of the male transgenic mice in our study sired a litter. Similar results have been reported in transgenic mice expressing the c-*neu* (Müller et al., 1988; Bouchard et al., 1989), N-*ras* (Mangues et al., 1990), *int-2* (Müller et al., 1990) and *int-3* (Jhappan et al., 1992) genes under the control of the MMTV LTR.

Phenotypic Abnormalities in the Salivary, Harderian, and Lacrimal Glands, the Spleen and the Mast Cells of the Transgenic Mice

In addition to mammary tumors and hyperplasia of the epithelium in kidney, lung, and male reproductive tracts, the MMTV LTR c-*erbB-2* mice exhibited additional growth abnormalities, primarily in organs and cells known to use the MMTV LTR promoter/enhancer (Choi et al., 1987; Stewart et al., 1988; Henrard et al., 1988; Ross et al., 1990). MMTV LTR c-*erbB-2* mice developed malignant mast cell neoplasia and hypertrophy or hyperplasia of the epithelium in the salivary glands (Table III). The Harderian gland of animal TM 1723 revealed severe bilateral hyperplasia of the epithelium that arose adjacent to morphologically normal tissue. The c-*erbB-2* protein showed a nonuniform expression pattern, and distinct staining was only detected in the hyperplastic nodule. Adjacent normal glandular cells were not stained (data not shown). Dissection of the lacrimal gland of the same animal showed abnormalities of the glandular architecture. Severe hyperplasia and dysplasia were seen almost throughout the entire gland. A solid-like pattern occurred showing cytological atypia and suggesting a possible malignant transformation (data not shown).

Splenomegaly was another abnormality found in two transgenic mice, TM 1624 and TM 1696. Histologically, the red pulp of the spleen was markedly hypercellular and extensively infiltrated with mastocytes. In both animals this observation correlated with high levels of MMTV LTR c-*erbB-2* transgene expression (Table II). Several other organs were infiltrated by mastocytes, but not as dramatically as the spleen. In one animal, TM 1723, only small foci of mastocytes were noted, sparsely scattered in the red pulp of the spleen.

Abnormalities were found on histological examination of the salivary gland. Multiple small foci of epithelial hypertrophy or hyperplasia were scattered throughout the salivary gland in three transgenic mice examined. Immunochemistry revealed that only the hyperplastic or hypertrophic lesions expressed the transgene at a moderate level (data not shown). No expression was seen in the surrounding normal tissue.

Discussion

Amplification and overexpression of the c-*erbB-2* gene occurs frequently in human adenocarcinomas of the breast, ovary, lung, stomach, and salivary gland (Hynes, 1992). These findings have demarcated this growth factor receptor as a potent oncogene and attracted considerable attention to the study of its function. Biochemical experiments have defined the enzymatic properties of the intrinsic tyrosine specific protein kinase and its substrates. Transfection experiments have shown its oncogenic potential in cultured cells and transgenic mouse models have been developed to assess in vivo the consequences of ectopic expression.

Two publications have described the introduction of the activated rat c-*neu* oncogene under the control of the MMTV LTR into transgenic mice (Muller et al., 1988; Bouchard et al., 1989). The most striking difference between these studies and the one reported here is the viability of the transgenic animals. Our transgenic mice died early. In the kidney, focal dilatation and atypical proliferation of the renal tubular epithelial cells and lesions in the glomeruli were observed. In the lung, bronchial and bronchiolar epithelial proliferation caused narrowing of the bronchial lumen and possibly suffocation. These preneoplastic lesions in kidney and lung most probably caused organ failure. For these reasons it was not possible to establish lines. This has not been reported for the MMTV LTR c-*neu* mice described earlier. Differences in the biological activities of the activated rat c-*neu* and the human c-*erbB-2* gene could be responsible. The drastic aberrations of kidney and lung and the early death of these transgenic animals limit their usefulness for experimental therapeutic anti-tumor studies. The severe premalignant lesions observed here, however, might indicate that c-*erbB-2* overexpression is not only correlated with the development of cancer, but might also underlie other conditions.

Our observations concerning the effects of transgene expression in the mammary gland are important complements to the observations reported earlier by Muller et al. (1988) and Bouchard et al. (1989). These authors obtained concurring, as well as contradictory results. Both studies reported hypertrophy and hyperplasia in the salivary glands and epididymis, but differed significantly in their findings and interpretations of the c-*neu* effects on the mammary gland. Muller et al. (1988) reported that the transgenic mice develop polyclonal, synchronously arising adenocarcinomas that involve the entire epithelium in each gland and concluded that the expression of the activated c-*neu* oncogene is sufficient to induce malignant transformation in a single step. Since independently and asynchronously arising tumors appeared late after birth at multiple sites Bouchard et al. (1989) concluded that c-*neu* expression was important for initiation and maintenance, but not sufficient for tumor formation. They claimed that high levels of transgene RNA was present in morphologically normal tissue and preceeded the appearance of stochastically arising mammary tumors. Our immunohistochemical analysis shows heterogeneity of transgene expression. The *c-erbB-2* protein can barely be detected in normal mammary tissue. High expression of the c-*erbB-2* protein and hyperplasia are concomitant. The nonuniform expression of the transgene is not restricted to the mammary tissue. We made equivalent observations in all organs examined. Ectopic c-*erbB-2* expression cannot be tolerated and is invariably associated with severe cellular hyperplasia in a variety of epithelial cell types. The powerful dominant phenotype of c-*erbB-2* expression in the transgenic mice might be a reflection of the potent oncogenic activity observed in so many human tissues.

The c-*erbB-2*-induced hyperplasia are clearly distinguishable from malignant tumors. Tumors found, e.g., in the mammary gland, still develop stochastically. Our results do not support a model which suggests that the expression of the activated c-*erbB-2* gene is sufficient for malignant transformation. Oncogenes regulated by the MMTV LTR promoter have been introduced and expressed in transgenic mice. The predominant phenotype observed was the development of mammary carcinoma. A high incidence was found in transgenic mice expressing c-*myc* (Stewart et al., 1984; Leder et al., 1986), v-Ha-*ras* (Sinn et al., 1987; Tremblay et al., 1989), *int-1* (Tsukamoto et al., 1988), activated c-*neu* (Müller et al., 1988; Bouchard et al., 1989), *ret* (Iwamoto et al., 1990), TGF-α (Matsui et al., 1990), N-*ras* (Mangues et al., 1990), and *int-3* (Jhappan et al., 1992). In all cases the tumor formation appeared to be a stochastic event and tumors were clonal in origin.

The tumors which occur in the transgenic mice described here, however, were found at an earlier age than in most other studies cited above. This early onset indicates that the c-erb-2 oncogene very strongly predisposes cells for neoplastic transformation and that mammary epithelial cells seem to be most sensitive. This sensitivity might in turn be related to the extensive inhibition of mammary gland differentiation. Strongly proliferating cells were observed in the mammary glands of the transgenic animals at a few weeks of age, but the glandular tree never developed properly. It is reasonable to assume that cells responsible for the early stages of mammary development are stimulated in their proliferation, but inhibited in their differentiation potential. These cells seem particularly prone for transformation.

In all tissues analyzed, we consistently observed hyperplastic, c-*erbB-2* expressing cells coexisting side-by-side with phenotypically normal, non-expressing cells. The question which arises from these observations concerns the mechanism by which a transgene, present in an integrated state in all somatic cells, is subject to differential regulation in a particular tissue. It is possible that nuclear factors necessary for the transcription of the MMTV LTR are not uniformly distributed in the cells of individual tissues. Since the transcription of the MMTV LTR is regulated by steroid hormone receptors, it is conceivable that these receptors are not homogeneously expressed or activated in all cells of a particular organ. In addition to the steroid receptors other transcription factors regulating the MMTV LTR have been identified (Lefebre et al., 1991; Yanagawa et al., 1991; Mink et al., 1992). Interestingly, epigenetic heterogeneity in the hormonal response has also been observed in tissue culture. The dose-dependent induction of a transfected MMTV LTR construct in fibroblasts was found to result from changes in the number of responding cells, rather than from changes in the expression level of individual cells (Ko et al., 1990).

The glucocorticoid-dependent transactivation of the MMTV LTR is cell-cycle dependent (Hsu et al., 1992). It is possible that the transcription of the MMTV LTR, and consequently the expression of the c-*erbB-2* protein, might initially be restricted to a small subset of cells. These cells maintain the MMTV LTR in an active configuration, either due to "the formation of a stable transcription complex" (Ko et al., 1990) or through a feedback control exerted by the activated c-*erbB-2* receptor. The expressing cells proliferate and give rise to the hyperplastic phenotype observed in the different tissues.

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