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Transgenic mouse model for human gastric carcinoma

(human adenovirus type 12/adenocarcinoma/adenosquamous carcinoma)

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ABSTRACT **To understand the pathogenesis that may be induced by human adenovirus type 12 (Ad12), we have generated transgenic mice carrying the Ad12 early region 1 under control of the mouse mammary tumor virus long terminal repeat. Eleven of 11 male founder mice, but only 2 of 12 females, died between 3 to 4 mo of age. Death was associated with presence of tumors at or near the squamocolumnar junction of the stomach. Microscopically, these multifocal tumors appeared to arise from hyperplastic epithelium and showed features consistent with adenocarcinoma or adenosquamous carcinoma. High levels of expression of both the Ad12 *E1A* and *E1B* genes were seen in the tumor-bearing stomach. Various levels of expression were also detected in other tissues, although the stomach was the only organ with detectable pathology. These observations suggest an organ-specific action of the Ad12 early region 1 gene products. This transgenic mouse model provides an experimental system for studying the development of human carcinomas at sites of transition from squamous to columnar epithelium.**

Human adenoviruses can transform a variety of cultured rodent cells, including fibroblasts from mice, rats, hamsters, and rabbits (1–4). Oncogenic transformation of these cells requires the continued expression of the left terminal 11% of the adenovirus genome (5). This segment contains early region 1 (*E1*), one of four regions of the adenovirus genome transcribed in the early phase of lytic infection. *E1* consists of two transcriptional units, *E1A* and *E1B*, which complement each other both in terms of expression and transforming activity (6). Products of *E1A* genes are required to activate the expression of *E1B* genes (7). Cells transformed by E1A protein alone show only morphological transformation, grow to low-saturation density, and are unable to induce tumors when injected into nude mice (8). Although E1B protein cannot transform primary cells by itself (9), it can cooperate with E1A protein to fully transform cells, which grow to high-saturation density and form tumors when injected into either nude mice (10) or immunocompetent syngeneic rodents (3).

Oncogenesis is a multistep process that involves not only uncontrolled cell proliferation but also acquisition of an adequate blood supply, interactions with appropriate growth factors, evasion of immune detection, and derivation of a malignant and metastatic state of growth (11). The concept that multiple genes may be involved in oncogenic transformation is supported by the *in vitro* finding that v-*myc* and *HRAS* oncogenes can cooperate in oncogenic transformation of primary cells (12). In transgenic animal studies, c-*myc* and v-Ha-*ras* genes have also been shown to act synergistically in inducing tumorigenesis (13). However, it is not known whether each oncogene plays a separate role in the process of oncogenesis *in vivo* or whether the two genes are additive for the same pathway. Because adenovirus *E1A* and *E1B* genes are known to cooperate in transformation *in vitro*, introduction of these viral genes into the germ line of mice may provide a useful model to study their function and cooperativity in oncogenesis *in vivo*.

In this study, we replaced the control region of the adenovirus type 12 (Ad12) *E1* genes with the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) to prevent lethality induced by early expression during embryogenesis. Transgenic mice carrying both *E1A* and *E1B* genes, but not those with only one of the two genes, developed gastric tumors near the squamocolumnar junction and showed features of adenocarcinoma or adenosquamous carcinoma.

MATERIALS AND METHODS

Generation of Transgenic Mice. The purified DNA fragment was microinjected into fertilized eggs from superovulated CD-1 females that had been mated with (C57BL/6 × DBA/2) F_1 males. The manipulated eggs were then implanted back into pseudopregnant CD-1 female mice. The procedure used was essentially as described (14).

Northern (RNA) Blot Hybridization. Total RNA was obtained from tissues by the isothiocyanate–cesium chloride procedure. Fifteen micrograms of RNA was fractionated in 0.9% agarose–formaldehyde gel. The RNA was then transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled single-stranded DNA probes (15).

RESULTS

Because human adenoviruses can transform embryonic rodent cells (3) and also infect embryonal carcinoma cells in culture (16), introduction of the entire Ad12 *E1*, under its own regulatory control, into the germ line of mice may induce early developmental abnormalities that will result in lethality of the embryos. This suggestion may explain our inability, despite repeated attempts, to derive transgenic mice using the proximal 11% of the Ad12 genome.

Construction of the Chimeric pMTVAd12 Construct. To restrict the expression of the *E1* genes, we replaced the enhancer and promoter of the *E1A* gene with the MMTV 5′ LTR (17). Expression of the MMTV-LTR is restricted during early development and is inducible by glucocorticoids in adults. Transcription of the *E1B* gene has been shown to depend on the E1A protein, both in lytically infected cells (7) and in transformed cells (18). In the absence of E1A proteins, the rate of transcription from *E1B* is <8% of that in wild-type infected cells (7), and E1A protein is known to regulate *E1B* gene expression by means of the TATA sequence within the *E1B* promoter (19). Therefore, replacement of the *E1A* control region by the MMTV-LTR was designed to place the expression of both *E1A* and *E1B* genes under control of a

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Abbreviations: Ad12, adenovirus type 12; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; E1, early region 1; E1A, E1 subunit A; E1B, E1 subunit B.

regulatory element not active during embryonic development. It has been shown that MMTV-LTR-regulated genes are expressed in various adult tissues, in addition to the mammary gland, in transgenic mice (20, 21).

To replace the *E1A* enhancer and promoter, we first changed the *Nar* I site located between the transcriptional start site and the translational initiation codon in the plasmid pAd12RIC (22) to a unique *Xho* I site (Fig. 1). The resulting plasmid, designated pAd12RICXho, was then digested jointly with *Xho* I and *Eco*RI; the latter enzyme cleaves once downstream of the *E1B* polyadenylylation site. Separately, the MMTV-LTR-containing pMSG plasmid (Pharmacia) was digested jointly with *Xho* I, which cuts once at the multiple cloning site downstream of the transcription start site of the MMTV-LTR, and with *Eco*RI, which also cleaves once within the vector (Fig. 1). The *Xho* I–*Eco*RI fragment of pAd12RICXho was then ligated to the *Xho* I–*Eco*RI fragment of pMSG. In the resulting chimeric plasmid, designated pMTVAd12, expression of Ad12 *E1A* is under control of the MMTV-LTR, whereas *E1B* retained its own promoter (Fig. 1).

Production and Screening of Transgenic Mice. To facilitate integration and efficient expression in target tissues, we excised the entire transcriptional unit from bacterial plasmid sequences by combined digestion of pMTVAd12 with *Nde* I and *Eco*RI (Fig. 1). The resulting 6.7-kb fragment, containing both *E1A* and *E1B*, was purified and microinjected into fertilized eggs.

Genomic DNA from the tail tissue of each offspring was digested with *Hin*dIII, which cuts three times within the microinjected fragment and releases a 3.3-kb fragment containing the entire MMTV-LTR, *E1A*, and part of the *E1B* gene, and analyzed by Southern blot hybridization. Of the 87 mice born, 31 (≈36%) carried the transgene. The intensity of the positive hybridizing bands varied among individual transgenic mice, and the estimated copy numbers varied from <1 to 10.

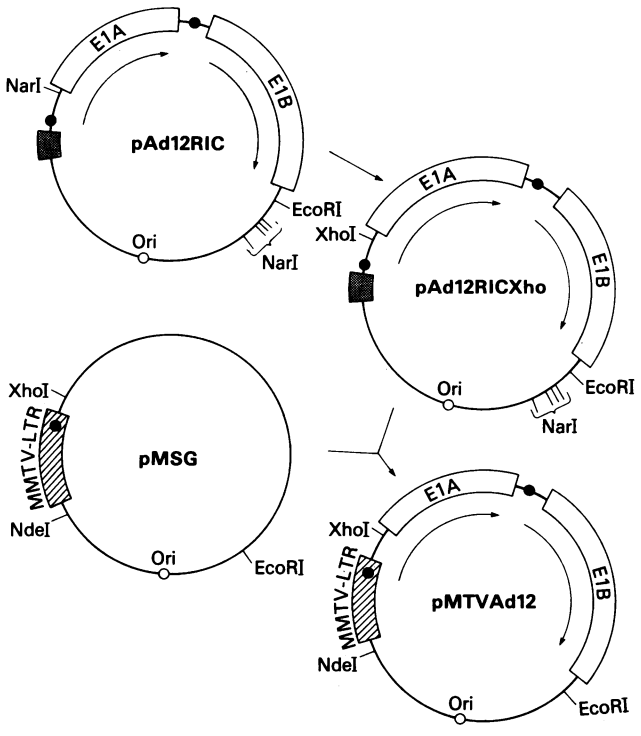

FIG. 1. Construction of the plasmid pMTVAd12. One of five *Nar* I sites, located 7 base pairs (bp) upstream of the *E1A* initiation codon in pAd12RIC, was changed to an *Xho* I site. The *Xho* I–*Eco*RI 5-kilobase (kb) fragment of the resulting plasmid pAd12RICXho was ligated with the *Xho* I–*Eco*RI fragment of pMSG, which contained the MMTV-LTR. In the final construct, pMTVAd12, the enhancer and promoter of the Ad12 *E1A* gene have been replaced by the MMTV-LTR, whereas the *E1B* gene retains its own promoter. Transcriptional enhancers are indicated by shaded (*E1A*) or hatched (MMTV) boxes, and black dots represent the transcriptional promoters. Arrows inside the plasmids indicate expected transcripts.

Tumor Phenotype of the Transgenic Mice. Of the 31 transgenic mice, 2 mice were runts and died within 2 weeks of age, 4 mice had incomplete copies of the transgene, and 4 mice were sacrificed. Twenty-three of the transgenic mice developed to maturity. All 11 male mice (designated B2, B4, F1, F3, F4, G3, N1, O1, O2, O5, and O6) died between the ages of 87 and 117 days. Of 12 female mice (designated B3, C1, F2, G5, J4, J8, L10, L13, M6, M8, N2, and N8), only 2 mice died between 81 and 89 days of age.

At autopsy, every mouse that died of "natural" causes had distended loops of bowel, suggesting the presence of intestinal obstruction; sepsis was most likely the cause of death in each case. In addition, 9 of the 11 deceased male mice, as well as the 2 deceased female mice, had grossly visible lesions in the stomach. The gastric mucosa had a papillary or multinodular appearance with the lesions extending intraluminally from the adjacent squamous mucosa. Anatomically, the squamous epithelium lining the forestomach changes to columnar epithelium lining the glandular stomach in the middle of the mouse stomach (Fig. 2*A*). All the gastric lesions in the transgenic mice arose from the forestomach near the squamocolumnar junction.

On histologic examination, squamous hyperplasia was frequently noted overlying or adjacent to an infiltrating neoplasm. The epithelial hyperplasia was characterized by thickening of the mucosa with marked irregularity of the basement membrane and nuclear atypia with increased mitotic activity (Fig. 2*B*). Extending into the underlying muscularis were nests of squamous cells with keratin pearl formation (Fig. 2 *C* and *E*). Often a second component of the lesions was also apparent. Within the expanded squamous submucosa, nests of atypical cells forming glandular structures were noted (Fig. 2*D*). The origin of these glandular hyperplastic spaces is unknown because pathology was not seen in control mice. Interestingly, the adjacent columnar epithelium showed no neoplastic or hyperplastic features; therefore, the neoplastic glandular component was thought to arise within the squamous mucosa. Clear evidence of infiltration of the submucosa and muscularis was seen by the gland-forming tumors (Fig. 2*D*), which were composed of sheets of pleomorphic cells with high nuclear-to-cytoplasmic ratio and scattered spaces lined by columnar cells with apical cytoplasm (Fig. 2*F*). Mitotic figures were abundant.

Tumors of nine mice were primarily composed of glandular elements that warranted a diagnosis of adenocarcinoma (Fig. 2 *D* and *F*). In addition, the infiltrating gastric tumors in the remaining two mice had both squamous and glandular components, consistent with a diagnosis of adenosquamous carcinoma (Fig. 2 *C* and *E*). Although the squamous element appeared histologically malignant on the basis of invasion and pleomorphism, only the glandular component of the tumors fulfilled the absolute criteria of malignancy, with metastases to lymph nodes and lungs in most cases. In two of the male mice (F1 and F3), no tumors were detected by either gross or microscopic examination, although both mice had intestinal obstruction. All male mice with gastric carcinoma were sterile and were unable to transmit the transgene.

Expression of *E1* Genes. To explain the observed phenotype, we determined the expression of Ad12 *E1A* and *E1B* genes by Northern (RNA) blot hybridization. Total RNA was isolated from selected tissues including brain, salivary gland, thymus, heart, lung, liver, stomach, intestine, spleen, kidney, and testis. A typical result is shown in Fig. 3 from mouse B2. High levels of expression of both *E1A* gene (Fig. 3*A*) and

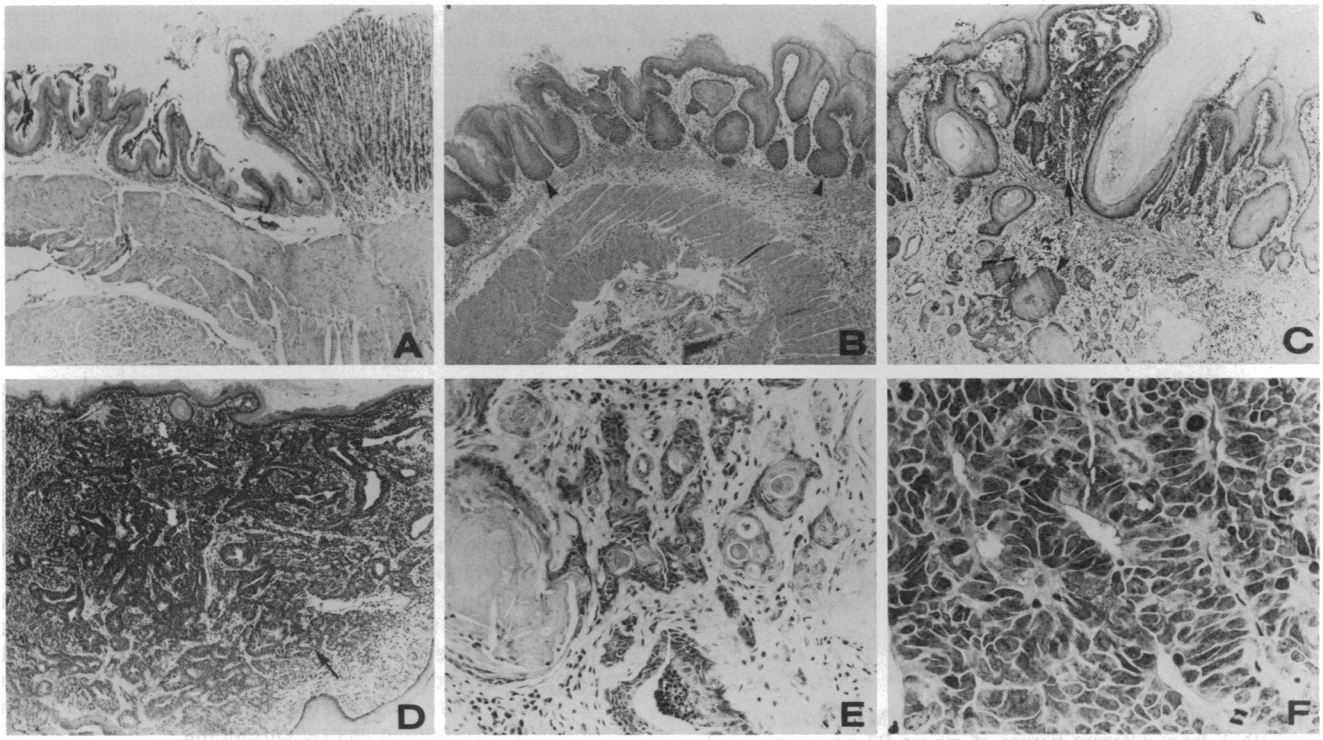

FIG. 2. Microscopic examination of stomach tissues from transgenic mice. Paraffin-embedded sections were stained with hematoxylin/eosin and photographed. (*A*) Stomach from a normal CD-1 mouse. (×45.) Junctional area of squamous epithelium (left) and glandular epithelium (right) is shown. (*B*) Hyperplastic squamous epithelium of the stomach from mouse B2. (×45.) Note thickening of the mucosa with marked irregularity of the basement membrane (arrowheads). (*C*) Gastric tumor from mouse B2. (×45.) Note the adenocarcinoma cells forming glandular structures (arrow) and atypical squamous cells extending into the underlying muscularis (arrowheads). These findings are consistent with a diagnosis of adenosquamous carcinoma. (*D*) Gastric tumor from mouse B4. (×45.) Note the differentiated adenocarcinoma underlying the normal squamous epithelium. (*E*) High magnification of mouse B2 (×145) showing squamous components with keratin pearl formation existing beyond the lamina propria. (*F*) High magnification of mouse B4 (×290) showing tumor cells with large polymorphic nuclei and numerous mitotic figures.

E1B gene (Fig. 3*B*) were detected in stomach, in testes, and in the metastatic adenocarcinoma in the abdomen. A low but detectable level of expression was also seen in brain. The transgenic E1A transcripts are somewhat larger in size than those from wild-type Ad12-transformed cells and are consistent with their being initiated within the MMTV-LTR, which provides 270 additional nucleotides at the 5′-untranslated region. As expected, the transgenic E1B transcripts are identical in size to the 22S RNA detected in wild-type Ad12-transformed cells. High levels of expression in stomach and the metastatic lesion were compatible with tumors being present in these tissues. However, brain and testes showed no evidence of neoplasia.

In another male mouse, F4, various levels of expression of *E1* genes were detected in brain, salivary gland, lung, stomach, kidney, testes, seminal vesicles, muscle, bone marrow, and metastatic tumor. Despite this expression, no neoplastic features were seen except for the stomach and metastases of the primary tumor. High levels of expression were detected in the testes in five of six male mice tested, but no histologic abnormalities were seen. Undoubtedly a biochemical abnormality existed because these mice were all infertile. We could not obtain sufficient breast tissues to examine the expression in the two female mice with gastric carcinoma. Female mice without a phenotype (C1 and F2) showed no expression in any tissue. In summary, all gastric carcinomas and metastatic tumors showed high levels of expression of both *E1A* and *E1B*. Various levels of expression were detected in testes, seminal vesicles, brain, and several other organs without tumor phenotype.

Expression of Major Histocompatibility Complex (MHC) Class I Genes. The Ad12 *E1A* gene can down-regulate the expression of the MHC class I genes *in vitro*, and this suppression of class I antigens is thought to be responsible for the escape of Ad12-transformed cells from immune detection (11, 23, 24).

To define the relevance of class I gene expression in *in vivo* tumorigenesis by the Ad12 *E1* genes, we determined the expression of class I transcripts in our transgenic mice. In Fig. 4*B*, identical amounts of RNA from liver, stomach, and testis of a transgenic mouse B2 and a normal littermate were analyzed by hybridization with a class I cDNA probe (designated 8D), derived from the 3′-noncoding region, which is known to detect nondiscriminately the class I *K*, *D*, and *L* genes (25). The stomach with carcinoma (lane 3) shows a considerable decrease of class I gene expression compared to a normal littermate (lane 4), whereas RNAs from liver (lanes 1 and 2) and testis (lanes 5 and 6) show no difference in the levels of expression between the transgenic mouse and the normal littermate. At the same time, testis showed a high level of Ad12 *E1A* gene expression (Fig. 4*A*, lane 5). Because the RNA was isolated from the gastric carcinoma as well as from the adjacent normal stomach, the levels of class I expression may be much lower if only tumor cell RNA could be isolated.

Transgenic Mice with Either *E1A* or *E1B*. To determine whether both *E1A* and *E1B* genes are required for the development of gastric carcinoma, transgenic mice carrying either *E1A* or *E1B* under control of the MMTV-LTR were generated.

The MMTV-LTR-driven *E1A* gene was derived from pMTVAd12 (Fig. 1) by the deletion of the *E1B* gene. Two transgenic mouse lines have been generated, and F_1 mice were analyzed for the expression of E1A transcripts. No E1A mRNA was detected in either the salivary gland or the stomach by Northern blot hybridization. Because expression

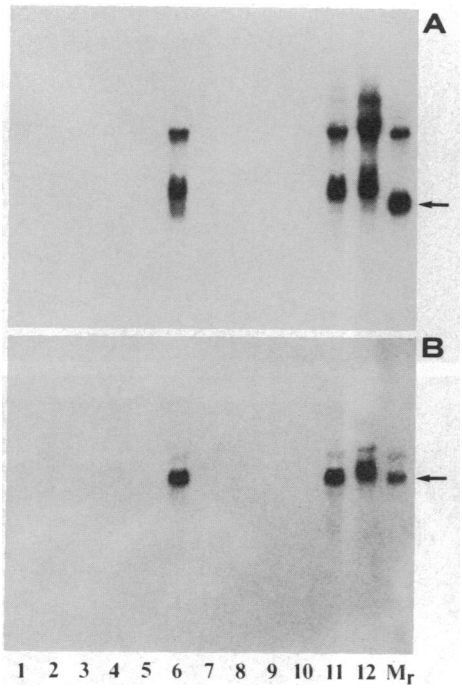

FIG. 3. Tissue-specific expression of the MMTV-Ad12 *E1* genes. Total RNA from different tissues of mouse B2 was compared by Northern blot hybridization using ^{32}P-labeled DNA probes derived from either the *E1A* (*A*) or *E1B* (*B*) gene. Lanes: 1, brain; 2, salivary gland; 3, lung; 4, heart; 5, liver; 6, stomach; 7, intestine; 8, spleen; 9, pancreas; 10, kidney; 11, testis; 12, metastatic tumor in the abdomen; M_r, a control from Ad12-transformed mouse fibroblast cell line C57AT1. Arrows indicate the E1A and E1B transcripts from C57AT1 cells.

of *E1* was not detected even in transgenic mice carrying both *E1A* and *E1B* genes in the stomach before development of gastric carcinoma, it was not surprising that the *E1A*-containing mice showed no detectable expression of E1A transcripts. However, i.p. injections of dexamethasone resulted in marked induction of *E1A* gene expression in both salivary gland and stomach (data not shown). Gastric carcinoma has not been detected in these transgenic mice, either with or without treatment with dexamethasone, over a period of >12 mo.

To derive a MMTV-LTR-driven *E1B* gene, we have removed the *E1A* gene from pMTVAd12 by combined digestion with *Xho* I and *Acc* I—the latter enzyme cleaved 56 bp into the *E1B* coding region—and inserted a 65-bp synthetic oligonucleotide that reconstituted the entire coding sequence for E1B. Northern blot hybridization of RNA from F_1 mice from all three transgenic mouse lines showed no detectable expression of the E1B transcript in either the salivary gland or the stomach. However, i.p. injection of dexamethasone induced the expression of E1B transcripts (data not shown). As with mice with only the *E1A* gene, the *E1B*-containing mice also did not develop gastric carcinoma, either with or without treatment with dexamethasone, over a period of >1 yr.

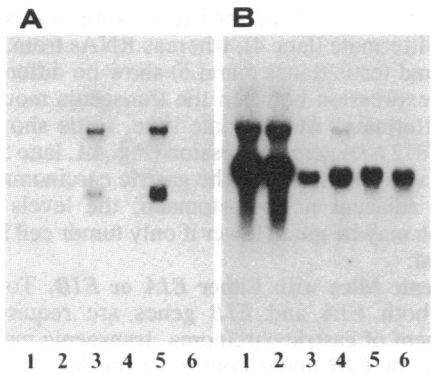

FIG. 4. MHC class I gene expression in tissues from the transgenic and normal mice. RNA from liver (lanes 1 and 2), stomach (lanes 3 and 4), and testis (lanes 5 and 6) were hybridized with either the E1A probe (*A*) or a class I cDNA probe (*B*). Samples in lanes 1, 3, and 5 were from the transgenic mouse B4 that had gastric carcinoma, and samples in lanes 2, 4, and 6 came from a normal littermate.

DISCUSSION

Mice transgenic for the Ad12 *E1A* and *E1B* genes developed gastric neoplasms. High levels of accumulation of both E1A and E1B mRNAs in the tumors indicated a close relationship between expression of the two viral oncogenes and development of the malignancy. Histological findings showed tumors of different cell types and differentiation, including benign squamous hyperplasia, adenosquamous carcinoma, and adenocarcinoma.

There is a clear male preponderance in the development of gastric tumors in the transgenic mice. Although at least 9 of 11 males developed a malignancy in the stomach, only 2 of 12 females developed the neoplastic phenotype. It is interesting to note that there is a similar sexual dimorphism in the development of human gastric carcinoma.

Analyses of the expression of the Ad12 *E1* genes in the transgenic mice suggest that the restricted pattern of tumor formation is due to the specificity of action of the two viral oncogene products and not the tissue-specificity of the transcriptional control element. While a high level of expression was also found in testes, together with low but detectable levels in several other organs, neoplasms arose only in the stomach. Specific subcellular targets, present only in certain cell types and on which the Ad12 E1 products act, may be required for inducing tumorigenesis. However, it is also possible that gastric epithelial cells are more susceptible to transformation than cells in other tissues and organs, leading to the development of tumors at 2–3 mo of age. Other types of tumors might develop could the mice survive longer.

It was not anticipated that MMTV-LTR-regulated Ad12 *E1* genes would selectively induce tumors in the stomach because the MMTV-LTR has been considered active in the mammary gland. However, recent studies with transgenic mice carrying MMTV-LTR-controlled chimeric genes have shown that tissue-specificity of the MMTV-LTR is much broader than it was once considered, and expression has been detected not only in the mammary gland, but also in other tissues, such as the salivary gland, testis, brain, lung, kidney, intestine, thymus, and prostate gland (20, 21). Expression in stomach was not mentioned in previous studies. The constitutive expression in all these tissues and organs cannot be explained by glucocorticoid induction of the MMTV-LTR. Recently, besides the glucocorticoid response element, another regulatory region located in the 5′ end of the LTR has been reported to have a significant role in controlling MMTV expression *in vivo* (26). Based upon these observations, it is not surprising that the stomach supports the expression of MMTV-LTR-regulated Ad12 *E1* genes. We could not obtain sufficient material to determine the expression of the *E1A* and *E1B* genes in the mammary gland because both female mice that had gastric tumors died before they could be bred.

The tissue-specificity of the Ad12 E1 products in transgenic mice appears different from the spectrum of tumors induced by infection of animals with Ad12. In newborn hamsters inoculation of Ad12 intrathoracically or subcutaneously induced tumors only at the site of injection. Because the virus did not seem to be disseminated to every organ of

the mouse (27, 28), it is not appropriate to argue the tissue-specificity of the Ad12 E1 products in those studies. In tissue culture studies adenoviruses can also efficiently transform different cell types, such as baby rat kidney cells (23), rat embryo brain cells (3), rat liver epithelial cells (29), and human embryonic retinoblasts (30). However, unlike transformation *in vitro*, tumorigenesis *in vivo* involves not only uncontrolled growth but also other factors such as the escape from immune detection (11). In this regard it is interesting to note that while high levels of expression of the *E1A* and *E1B* genes were detected in both stomach and testes, suppression of MHC class I gene expression and development of malignancy were detected only in the stomach and not in testes. The down-regulation of class I gene expression in the gastric tumors may play an important role in tumorigenesis induced by the viral oncogenes in the transgenic mice.

Another point of interest in these transgenic mice is that the stomach neoplasms demonstrated numerous histological variations. From our preliminary observation, transgenic mice harboring either the *E1A* or the *E1B* gene alone did not induce gastric carcinoma. This finding is consistent with the suggestion that both *E1* genes are required to induce tumors, which appear to progress through multistages involving benign squamous hyperplasia to malignant adenocarcinoma or adenosquamous carcinoma.

In mouse the border of the squamous and columnar mucosa lies in the middle of the stomach, while in humans this border exists at the distal end of the esophagus at the esophagocardiac junction. One feature of the gastric involvement in our transgenic mice is that the adenocarcinoma invariably arose from the squamous mucosal area near the squamocolumnar junction of the stomach. Several human neoplastic diseases, such as carcinoma of the uterine cervix (31) or adenocarcinoma in Barrett esophagus (32), also have an origin in the area of the squamocolumnar junction. Barrett esophagus is a condition in which the squamous mucosa that normally lines the human distal esophagus is replaced by a columnar epithelium resembling that in the stomach (32). This concept is clinically important because the risk of esophageal cancer in patients with Barrett esophagus is estimated to be increased by 30- to 40-fold over that of the general population (32). In addition, cervical cancer is known to arise in the transformation zone of the cervix, an area of active metaplasia between the mature squamous epithelium of the exocervix and the columnar epithelium of the endocervical canal (31).

The finding that our Ad12 *E1*-carrying transgenic mice developed only gastric carcinomas, in spite of the expression of the viral oncogenes in many other tissues, is suggestive that the epithelial junctional area may be a "hot spot" for carcinogenesis. Therefore, our transgenic mice may be a valuable model system in which to investigate the carcinogenic events involved at the squamocolumnar junction, such as occurs in Barrett esophagus and with cervical neoplasms. In addition, our transgenic mouse model provides an *in vivo* system to test the recent suggestion obtained from the study of tissue culture cells that the E1A protein acts by binding to the product of the retinoblastoma gene (33).

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