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Multiple endocrine neoplasia induced by the promiscuous expression of a viral oncogene

(simian virus 40 large tumor antigen/major histocompatibility complex class I gene enhancer/lymphoid hyperplasia)

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ABSTRACT **There is increasing evidence for the importance of events that govern and influence the interaction between the transformed cell and its host being ultimately responsible for the establishment of the cancer phenotype. To derive an animal model that will allow us to define some of these phenomena at the molecular level, we have chosen to induce the expression of a viral oncogene in all tissue types, with the hope of identifying sites that are more susceptible to malignant transformation. When the gene for simian virus 40 large tumor antigen (T antigen) was placed under the control of a major histocompatibility complex class I gene enhancer, the resulting transgenic mice not only developed choroid plexus papillomas, as seen with wild-type simian virus 40, but also lymphoid hyperplasia and multiple endocrine neoplasias. The development of lymphoid hyperplasia was preceded by an elevated level of expression of T antigen in these tissues at an early age. Surprisingly, the striking thymic hyperplasia has not been observed to progress toward malignancy. The multiple endocrine neoplasias developed later in life and involved the pancreas, pituitary, thyroid, adrenals, and testes. While not preceded by an elevated level of expression of T antigen, once endocrine tumors appeared they quickly progressed toward malignant growth. Although other tissues also exhibited a basal level of expression of the viral oncogene similar to that detected in endocrine tissues, they rarely developed tumors. This transgenic mouse model seems particularly suitable for a molecular understanding of events responsible for certain tissue types being so much more susceptible to neoplastic conversion, with others being so refractory.**

Over the past 2 decades, the major effort to understand the molecular biology of the cancer cell has focused primarily on defining the mechanism of transformation, a process whereby a normal cell is converted in culture to a state of uncontrolled proliferation (1, 2). A large number and variety of oncogenes and protooncogenes have been defined, and while the study of their activities is central to our understanding of the etiology of cancer at the molecular level, the phenomena that govern and influence the interaction between the transformed cell and its host are ultimately responsible for the establishment of the cancer phenotype (3, 4).

The growth of a tumor depends, among other things, on its ability to recruit an adequate blood supply (5), to interact with appropriate growth factors that serve as signals for proliferation (6), and to escape destruction by different immune effector cells (7). These are some of the events that determine whether a transformed cell will be allowed to grow into a tumor and to metastasize. To better define these phenomena at the molecular level, it will be important to derive appropriate animal models.

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We have chosen to deregulate the expression of a viral oncogene in transgenic mice with the hope of identifying sites that are either more privileged or more susceptible to tumor growth. The availability of such a transgenic model should allow us to dissect the molecular events underlying the basis for tumor growth in a setting where cell–cell interactions remain intact.

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 (8).

Analysis of Large Tumor Antigen (T Antigen) Expression. Equivalent amounts of total protein, obtained from different tissues by lysis with a buffer containing 1% Triton-X100/1% sodium deoxycholate/0.1% $NaDodSO_4$, were immunoprecipitated with excess antiserum against the simian virus 40 (SV40) T antigen (9). The immunoprecipitates were fractionated on a $NaDodSO_4$/10% polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was probed with a mouse monoclonal antibody to the 90-kDa T antigen, followed by incubation with ^{125}I-labeled protein A.

RESULTS

A prerequisite for the success of our approach is the selection of an oncogene that has the potential of being able to transform most, if not all, cell types. The SV40 large T oncogene has the unique distinction of encoding a product that can be found both in the nucleus and on the plasma membrane (10). As a consequence, this 90-kDa protein is able to induce both "nuclear" functions such as immortalization and "cytoplasmic" functions such as anchorage independence (11, 12). Indeed, T antigen can efficiently transform a broad spectrum of cell types *in vitro* (13). In addition, specific targeting of this oncogene in transgenic mice has given rise to different neoplasms including pancreatic endocrine tumors (14), pancreatic exocrine tumors (15), and tumors of the lens (16).

Construction of the Chimeric Gene. To deregulate the expression of the SV40 T-antigen gene, which is preferentially expressed in the choroid plexus when introduced into transgenic mice (17, 18), we have selected to replace the viral transcriptional enhancer with that from the major histocompatibility complex class I K^b gene. The K^b gene is known to be expressed in most cell types in the mouse, although the level of expression may vary somewhat from tissue to tissue (19). Deletion mapping studies have localized the enhancer sequence to a region 5′ of the transcriptional start site (20). This control element is composed of a 16-base-pair (bp) repeat located immediately upstream of the interferon responsive sequence (Fig. 1*A*).

Abbreviations: T antigen, large tumor antigen; SV40, simian virus 40.

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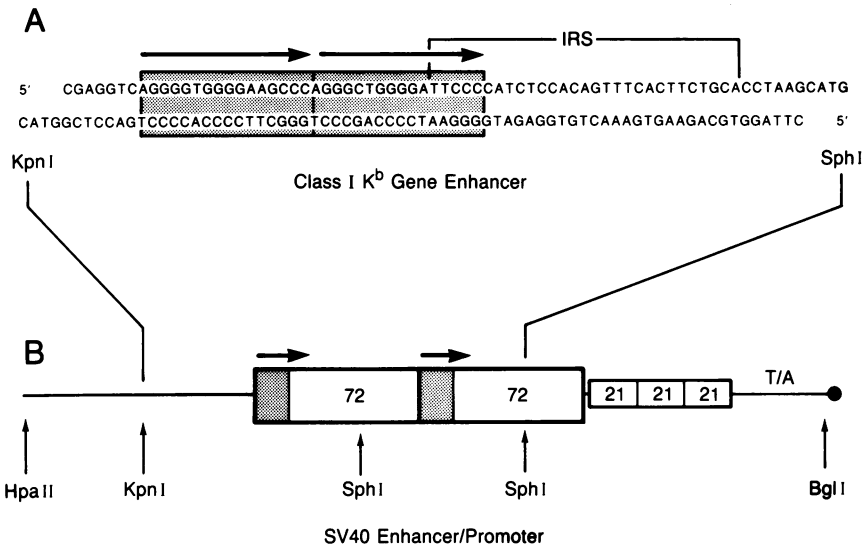

FIG. 1. Features of the plasmid containing the SV40 large T oncogene under the control of the class I K^b gene enhancer. (*A*) Sequence of the synthetic class I K^b gene enhancer with the 5' *Kpn* I and 3' *Sph* I cloning sites. Shaded area represents the 16-bp repeats, which constitute the functional enhancer. The position of the interferon-responsive sequence (IRS) is also indicated. (*B*) Scheme of the SV40 enhancer and promoter. Relative positions of the 72-bp repeats, the 21-bp repeats, TATA sequence, and RNA start site (●) are indicated. Shaded areas represent the core sequences of the transcriptional enhancer.

To replace the viral enhancer, we first cloned the entire SV40 genome at its unique *Bam*HI site into pUC13. The resulting construct was then digested jointly with *Hpa* II, which cuts once on the 5' side of the enhancer sequence, and with *Sph* I, which cleaves only one time within each of the 72-bp repeats (Fig. 1*B*). In doing so, a 166-bp stretch of DNA containing both of the core enhancer sequences has been deleted. In its place, a synthetic 73-bp fragment containing the K^b enhancer and the interferon responsive sequence was inserted (Fig. 1*A*). The resulting chimeric construct would have the authentic SV40 promoter (including the 21-bp repeats) and transcriptional start site, but otherwise would be under the control of the K^b gene enhancer.

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 digestion with *Hpa* II (or *Msp* I), which cuts only once within the SV40 genome and 13 times within pUC13. The resulting 3.0-kilobase (kb) fragment was purified and microinjected into fertilized mouse eggs.

Genomic DNA from the tail tissue of each offspring was digested with *Msp* I. The presence of an authentic 3.0-kb fragment in the genomic DNA digest, as revealed by DNA blot analysis, would indicate the integration of at least one intact copy of the transgene. Of the 115 mice born, 21 were found to carry the transgene. This frequency was somewhat less than what we routinely observed for other DNA constructs in our laboratory and suggested possible *in utero* death of some of the transgenic embryos. In support of this was the observation that 8 of the 21 transgenic mice born were runts and died within 2 weeks of undetermined cause.

Tumor Phenotype of the Transgenic Mice. Six of the transgenic mice (designated X8, I1, R1, F5, F2, H4) were allowed to develop to maturity. All six mice died between the ages of 58 and 91 days (Table 1). While all of the males (X8, F5, H4) were fertile, none was able to transmit the transgene. The females (I1, R1, F2), on the other hand, were able to transmit but were often too sick to care for their litters.

At autopsy, all six mice had obvious brain tumors. In each case, that was the most likely cause of death. A typical histological analysis is shown in Fig. 2*A*. The section shows a portion of normal cerebral cortex (top right) and a portion of normal choroid plexus (top left), being compressed against a mass of tumor cells, which have a papillary pattern of growth (bottom). As compared with the normal choroid plexus, the neoplastic cells have enlarged and hyperchromatic nuclei with numerous mitoses. The fibrovascular connective tissue was often covered with layers of these neoplastic epithelial cells.

These observations were consistent with the diagnosis of choroid plexus papilloma and confirmed previous findings with the SV40 enhancer driving the same SV40 T-antigen gene (20, 21). At least three of the six mice also showed a progression from benign disease to malignancy. The latter was characterized by the striking changes in the morphology of the tumor cells (Fig. 2*B*). Except for a few neoplastic cells with

Table 1. Tumor phenotype of transgenic mice

Mouse	Sex	Age	Br	Lu	He	Li	In	Ki	Mu	Sa	Ov	Pi	Tr	Ad	Pa	Te	Tm	Sp	LN	BM
R3	M	(20)	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0
S7	M	(21)	0	0	0	0	0	0	0	0	—	0	ND	0	1	0	0	0	0	0
K1	F	(22)	0	0	0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0
U6	M	(49)	1	0	0	0	0	0	0	0	—	ND	0	0	0	0	1	1	1	0
M4	F	(58)	2	0	0	0	0	0	0	0	0	0	0	0	0	—	1	1	1	0
X8	M	58	2	0	0	0	0	0	0	0	—	ND	2/3	3	3	2/3	1	1	1	0
I1	F	63	2	0	0	0	0	0	0	0	0	ND	ND	3	3	—	1	1	1	ND
R1	F	69	3	0	0	0	0	0	0	0	0	ND	2/3	3	3	—	1	1	0	0
F5	M	78	2	0	2	0	0	2	0	0	—	ND	2/3	3	3	2/3	1	1	1	0
F2	F	83	3	0	0	0	0	0	0	0	0	ND	ND	3	3	—	1/2	1	1	ND
H4	M	91	3	0	0	0	0	0	2	0	—	3	2/3	3	4	2/3	1/2	1	1	0

Mice were either allowed to mature and die or were sacrificed before any phenotype was obvious. Age at the time of death is shown in days. When mice were sacrificed prematurely, the age when they were analyzed is indicated in parentheses. Part of each tissue or organ removed was fixed in formaldehyde, sectioned, and stained with hematoxylin and eosin. Those tissues that were either not identified or not saved are indicated as not done (ND). The histologic phenotype was scored either as normal (0), hyperplastic (1), benign neoplastic (2), malignant neoplastic (3), or metastatic (4). Br, brain; Lu, lung; He, heart; Li, liver; In, intestine; Ki, kidney; Mu, muscle; Sa, salivary gland; Ov, ovary; Pi, pituitary gland; Tr, thyroid; Ad, adrenal gland; Pa, pancreas; Te, testes; Tm, thymus; Sp, spleen; LN, lymph node; BM, bone marrow.

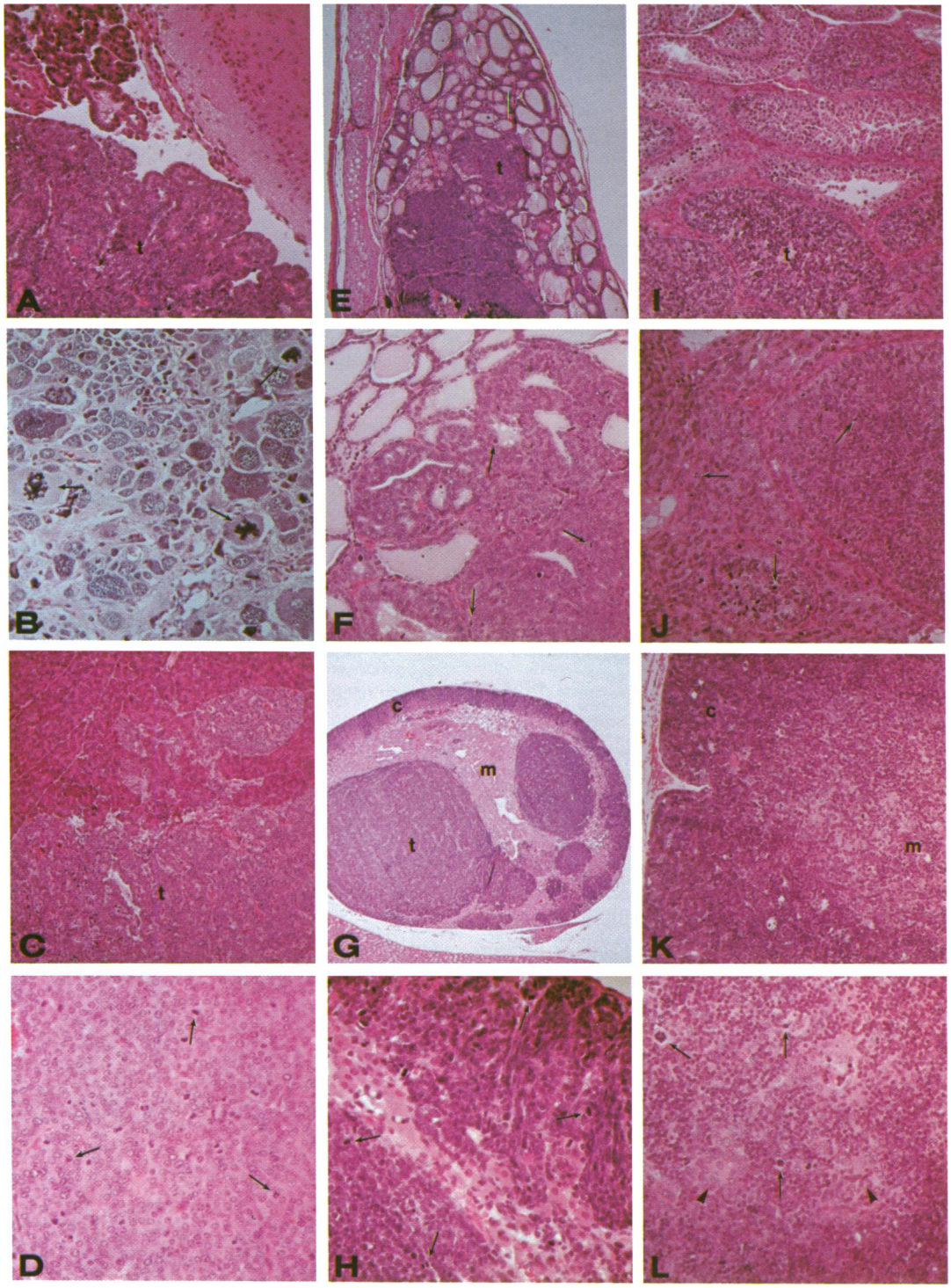

FIG. 2. Microscopic examination of different tissues from transgenic mice. Paraffin-embedded sections were stained with hematoxylin and eosin and examined microscopically. (*A*) Brain from mouse H4. (×85.) (*B*) Choroid plexus tumor from mouse F2. (×200.) (*C*) Pancreas from mouse F5. (×85.) (*D*) Pancreatic tumor from mouse F2. (×200.) (*E*) Thyroid gland from mouse H4. (×85.) (*F*) Thyroid tumor from mouse I1. (×200.) (*G*) Adrenal gland from mouse R1. (×20.) (*H*) Adrenal tumor from mouse R1. (×200.) (*I*) Testis from mouse H4. (×85.) (*J*) Testicular tumor from mouse F5. (×200.) (*K*) Thymus from mouse X8. (×85.) (*L*) Medullary region of thymus from mouse F2. (×200.) Locations of tumor tissues (t) are indicated. Representative mitotic figures are shown by arrows. Clusters of epithelial cells in the thymus are indicated by arrowheads.

nuclei similar in size to those seen in benign lesions (top middle), most of the tumor cells have enlarged and multilobulated nuclei. In addition, many bizarre mitoses were seen (arrows).

Without exception, all six mice had strikingly enlarged thymuses (Fig. 3*A*). For most of these mice (X8, I1, R1, F5), histologic analysis of the thymus showed preservation of the usual architecture and a distinct corticomedullary junction. In comparison to normal littermates, the medullary region appeared disproportionately enlarged. Furthermore, progressive changes in the thymus were seen in the two older mice (F2, H4), where a gradual loss of cortical cells was detected that resulted in the blurring of the corticomedullary junction (Fig. 2*K*). What was perhaps most striking was the apparent increase in the number of epithelioid or nurse cells in the medulla. In some areas, they appeared in clusters rather than being scattered (Fig. 2*L*, arrowheads). Numerous mitoses were also observed, some of which were abnormal. This may

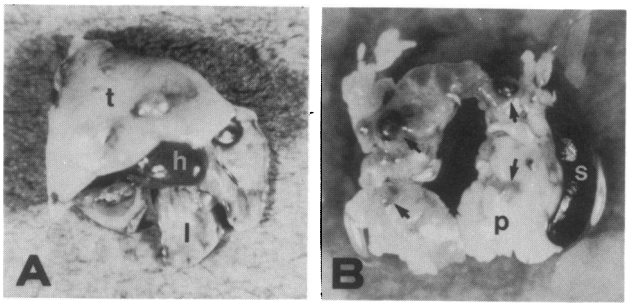

FIG. 3. Gross dissection of tissues from mouse F2. (*A*) Diffusely enlarged thymus (t) overlying the heart (h) and lungs (l). (*B*) Enlarged pancreas (p) with multiple tumor nodules (arrows) lying adjacent to the spleen (s).

be an early sign of clonal expansion and a suggestion of a benign neoplastic disease. Almost invariably, the spleen and lymph nodes also showed evidence of hyperplastic growth, albeit with preservation of normal tissue architecture.

Extensive histologic analyses failed to reveal any consistent pattern of tumor growth in the lungs, heart, liver, intestine, kidneys, muscle, salivary gland, and ovaries (Table 1). However, a sporadic case of a myocardial myxoma and a neuroblastoma was seen in mouse F5, and a rhabdomyosarcoma was seen in mouse H4. All of these tumors were detectable only microscopically and represented small and benign lesions.

Malignant tumor growth was consistently seen in all endocrine tissues analyzed from each of the six mice. Gross dissection of pancreatic tissues routinely revealed discrete nodular masses with prominent vascularity (Fig. 3*B*). Histologic sections through the pancreas showed separate nodules composed of islet-type pancreatic cells (Fig. 2*C*). The two islets shown in the middle were of normal size, whereas the remaining two nodules showed progressive enlargement with a total replacement of normal islet architecture at the bottom. At a higher magnification, numerous mitoses were seen (Fig. 2*D*). The nuclei showed moderate variation in size and shape and have clumped chromatin and small nucleoli.

Microscopic analysis of thyroid tissues revealed the replacement of usual colloid follicles with tumors (Fig. 2*E*). Within the tumor mass was a necrotic center with dark-blue calcium granules (bottom). Very often, tumors appeared to arise independently from different areas. At high magnification, the neoplastic cells were seen to be larger than the follicular cells and have pleomorphic nuclei (Fig. 2*F*). Frequent mitoses were seen throughout.

Bilateral neoplastic involvement of both adrenal glands was seen in all six transgenic mice. Multiple masses of tumor cells often displaced the medullary portion of the adrenal (Fig. 2*G*). The tumors were composed of small round cells with scanty cytoplasm and without distinct cell borders. At high magnification, areas of proliferation in the cortex, which extended into the medulla, were seen (Fig. 2*H*). The cells in the nodules were identical to cells at the proliferating cortex. This suggested that the tumors were derived from the endocrine portion of the adrenal glands and were not the result of metastases from other sites.

Histologic examination of the testes revealed both normal and abnormal seminiferous tubules in all three male mice (X8, F5, H4). The abnormal tubules were distended and filled by neoplastic cells with enlarged hyperchromatic nuclei (Fig. 2*I*). An unusual feature was that normal tubules were found adjacent to neoplastic ones. This may reflect that proliferative events were occurring throughout, but that only a few succeeded in becoming tumors. Neoplastic changes were also seen among the interstitial (perhaps Leidig) cells, in addition to the seminiferous tubule cells; the separation of two tubules by proliferating interstitial cells was clearly seen (Fig. 2*J*). The interstitial cells had a spindle-shaped appearance with more abundant cytoplasm than the tubule cells. Mitoses were frequent in both compartments. The involvement of the testes would explain the inability of male mice to transmit the transgene.

With the extensive tumor involvement of the choroid plexus, it was often difficult to locate the pituitary glands in these mice. However, with mouse H4, a pituitary tumor was detected.

Expression of the Transforming Protein in Transgenic Mice. To explain the observed phenotype, we determined the expression of SV40 T antigen in the various tissues in each of the six transgenic mice. A typical experiment is shown in Fig. 4*A* with mouse H4. High levels of expression of the 90-kDa transforming protein were detected in the brain, pituitary, thyroid, adrenal, pancreas, and testes. This was consistent with the tumor phenotypes seen in these same tissues (Table 1).

High levels of expression were also observed in the thymus, which was grossly hyperplastic but not malignant. A somewhat lower amount was found in the spleen and in lymph nodes, consistent with these tissues also being histologically hyperplastic. Both metastatic tumors, one located in the inguinal area and the other in the abdomen, also expressed high levels of the transforming protein.

Despite the absence of any tumor phenotype, a low but detectable level of expression of T antigen was observed in most other tissues (Fig. 4*A*). In fact, among the six transgenic mice, no tissue that has been analyzed was found to be spared of expression of the transgene.

To address the possibility that increased levels of expression of the transgene in endocrine tissues was responsible for the preferential development of multiple endocrine tumors and that a delay in expression in lymphoid tissues was

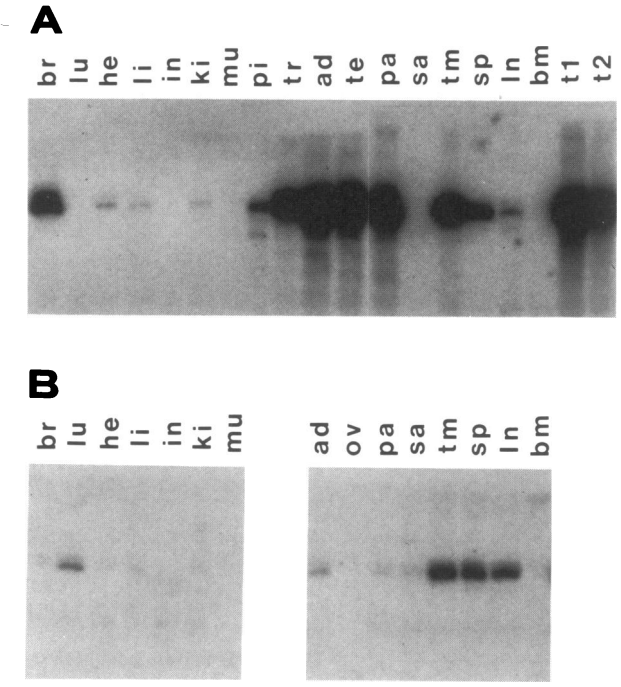

FIG. 4. Tissue distribution of T antigen in transgenic mice. Expression of T antigen was detected by immunoprecipitation followed by immunoblot analysis. Tissues analyzed included brain (br), lung (lu), heart (he), liver (li), intestine (in), kidney (ki), muscle (mu), pituitary (pi), thyroid (tr), adrenal gland (ad), testis (te), pancreas (pa), salivary gland (sa), thymus (tm), spleen (sp), lymph node (ln), bone marrow (bm), inguinal tumor mass (t1), and abdominal tumor mass (t2). (*A*) Mouse H4, which died at 91 days of age. (*B*) Mouse K1, which was sacrificed at 22 days of age.

responsible for their achieving hyperplastic but not neoplastic growth, we turned to the analysis of transgenic mice before they had a chance to develop tumors. Recognizing that if allowed to mature, the mice would all develop tumors and die between 9 and 13 weeks, we opted to sacrifice three transgenic animals (R3, S7, K1) when they reached the age of 3 weeks. Except for mouse S7, which had somewhat enlarged islets in the pancreas, none of the mice showed any pathology in any of their tissues analyzed (Table 1). Between them a low but detectable level of expression of the transgene was seen in all of the tissues. As with mouse K1, all mice showed relatively high levels of expression in each of the three lymphoid tissues (Fig. 4*B*). At this age, expression in the endocrine tissues was not any more pronounced than in other tissues. These observations were consistent with the suggestion that the lymphoid tissues, but not the endocrine tissues, had a head start in the expression of the transforming gene.

To further this investigation, we sacrificed two other transgenic mice (U6, M4) between 7 and 9 weeks old. These two mice not only had elevated levels of expression of T antigen in their lymphoid tissues, but also had extensive thymic hyperplasia detected both at the gross and microscopic levels (Table 1). In none of the mice were endocrine tumors observed at the histologic level. The extent of expression of the transgene in endocrine glands remained comparable to those in other nonlymphoid tissues (data not shown). This has led to the suggestion that the endocrine tissues were inherently more susceptible to progression to neoplasia and that this predisposition did not require an elevated basal level of expression of the transforming gene.

DISCUSSION

Introduction of the SV40 large T oncogene under the control of a major histocompatibility complex class I gene enhancer gave rise to transgenic mice with a distinct predisposition to multiple endocrine neoplasia. Every mouse that was allowed to grow to maturity came down with malignant tumors in the pancreas, pituitary gland, thyroid gland, adrenal glands, and testes. In addition, these same mice also had extensive lymphoid hyperplasia with the involvement of the thymus, spleen, and lymph nodes.

As early as 3 weeks of age, analysis of these mice had revealed a low but detectable level of expression of the transgene in virtually all body sites and an elevated level of expression only in the lymphoid tissues. The preferential expression of T antigen in the thymus was accompanied by extensive hyperplasia detected even during gross examination of mice that were sacrificed at 7–8 weeks of age. Despite the apparent head start, in none of the mice did the hyperplastic growth progress to malignancy, even at the time of death between 9 and 13 weeks. It was not clear, however, that had the mice survived longer, they would not succumb to lymphoid malignancies. It is interesting to note that the involved cell types in the thymus appeared to be the epithelial nurse cells, which were of neuroendocrine origin.

What was most surprising was the multiple endocrine neoplasms detected in each of the mice. This is not the result of preferential expression of T antigen in endocrine tissues. The class I enhancer is no more active in these tissues than in other sites (data not shown), and the analysis of mice sacrificed as late as 7–8 weeks of age had failed to reveal an elevated level of expression of the transgene in any of the endocrine tissues. The high level of expression of the transforming gene in all of the endocrine tumor tissues most likely reflected clonal expansion of the involved cells, rather than an elevation within the individual transformed cells. We have failed to correlate the elevated expression to any alteration in DNA methylation or to the amplification of the transgene in tumors (18). Nevertheless, once "activated" the tumors all progressed rapidly to malignancy.

The suggestion that endocrine tissues, once transformed by the appropriate agent, served as preferred sites for the derivation of malignant growth was supported by the observation that nonendocrine tissues, such as lung, heart, liver, intestine, kidney, muscle, and salivary gland, all expressed T antigen at a similar level but rarely developed tumors. This was not because T antigen could not induce tumors in these sites; tumors had been observed sparingly in the heart, kidney, ureter, and muscle. Most likely, they were less susceptible to neoplastic conversion.

In the present study, we have derived a transgenic mouse model that seems appropriate for a molecular definition of events responsible for tumor growth. One can now begin to ask relevant questions based on our observations. Why are endocrine tissues more susceptible to neoplastic changes in this mouse model? We have noted that most of the endocrine tumors, as well as the choroid plexus tumors, were highly vascular. It is possible that endocrine tumor cells are more capable of secreting growth factors that can induce angiogenesis. Endocrine tissues have also been known to be targets for autoimmunity, such as diabetes mellitus and autoimmune thyroiditis. If such disorders are the result of heightened immune recognition of otherwise immunologically "privileged" sites, then transformed cells derived from such tissues would be more capable of escaping immune detection. Why, despite extensive hyperplastic growth, do the lymphoid tissues not undergo malignant conversion? Unlike endocrine cells, which are terminally differentiated and cannot "tolerate" cell division, lymphoid cells are inherently able to divide and are perhaps more permissive to sustained proliferation without triggering a neoplastic state of growth.

Since most of the suggestions raised here can be tested, we believe that the availability of an appropriate transgenic model will undoubtedly allow us to better define those cell–cell interactions that are crucial for the derivation of the malignant phenotype. In addition, this transgenic mouse model resembles in many ways the human multiple endocrine neoplasia (MEN) syndromes (21). As in our animal model, human MEN syndromes involve inherited proliferative disorders of endocrine glands and neuroendocrine cells. Our mouse model should be particularly useful in defining the biochemical basis underlying this human disorder.

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