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Targeting of transgene expression to monocyte/macrophages by the gp91-phox promoter and consequent histiocytic malignancies

(myeloid differentiation/cis-regulatory elements/neutrophil cytochrome *b*)

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ABSTRACT **A component of a heterodimeric cytochrome *b*, designated gp91-phox, is required for the microbicidal activity of phagocytic cells and is expressed exclusively in differentiated myelomonocytic cells (granulocytes; monocyte/macrophages). In an attempt to identify cis-elements responsible for this restricted pattern of expression, we produced transgenic mice carrying reporter genes linked to the human gp91-phox promoter. Immunohistochemical and RNA analyses indicate that 450 base pairs of the proximal gp91-phox promoter is sufficient to target reporter expression to a subset of monocyte/macrophages. Mice expressing simian virus 40 large tumor antigen under control of the gp91-phox promoter develop monocyte/macrophage-derived malignancies with complete penetrance at 6–12 mo of age and provide an animal model of true histiocytic lymphoma. As these transgenes are inactive in most phagocytic cells that express the endogenous gp91-phox-encoding gene, we infer that additional genomic regulatory elements are necessary for appropriate targeting to the full complement of phagocytes *in vivo*.**

The microbicidal activity of phagocytic cells depends on the function of a membrane-associated NADPH–oxidase complex. Through study of patients with chronic granulomatous disease (1) critical components of this system have been identified, and the cDNAs encoding these polypeptides have been cloned (2–4). In X chromosome-linked chronic granulomatous disease, the most common variety of the disorder, patients harbor defects in the gene encoding gp91-phox, a subunit of an unusual heterodimeric cytochrome *b* (1, 5). This gene is normally expressed only in terminally differentiating cells of the myelomonocytic lineage.

Although insight has been gained recently regarding control of gene transcription in the erythroid (6) and lymphoid (7) hematopoietic lineages, regulatory mechanisms operative in myelomonocytic cell development are largely unknown. In an effort to address this general problem, we have sought to identify cis- and trans-elements responsible for expression of the human gp91-phos-encoding gene. In view of the low transfection efficiency of transformed myelomonocytic cell lines, we have used transgenic mice as an assay system for the identification of cis-regulatory elements. This strategy avoids the potential artifacts inherent in studying gene expression in transformed cell lines and has proven highly reliable in defining important regulatory elements within an intact animal (8). Recently, appropriate regulation of chicken lysozyme (9) and human c-*fps/fes* (10) transgenes in monocytic cells of mice has been achieved by the introduction of the corresponding genomic loci. However, the specific cis-elements and trans-factors that mediate this transcriptional specificity remain to be defined.

Here we report that the promoter of the gp91-phox gene directs expression of two different reporter genes in myelomonocytic cells in transgenic mice. Four hundred and fifty base pairs of the 5'-flanking region are sufficient for this activity, and sequences between −138 and −450 bp are necessary. Rather than directing expression to all myelomonocytic cells, however, constructs bearing the gp91-phox promoter are active only in a subset of mononuclear phagocytes. Targeted expression of simian virus 40 (SV40) large tumor (T)-antigen with the gp91-phox promoter results in monocyte/macrophage-derived malignancies in transgenic mice, which constitute an animal model of true histiocytic lymphoma. Overall, our studies suggest that subsets of myelomonocytic cells use distinct cis-regulatory signals within the gp91-phox gene, and that sequences required for appropriate transcriptional regulation in the full spectrum of phagocytic cells will ultimately be found to reside elsewhere in the gene.

MATERIALS AND METHODS

Transgene Construction and Introduction into Mice. Construction of gp91-phox promoter/human growth hormone (GH) and gp91-phox promoter/SV40 early region plasmids are described elsewhere (11, 12). Transgene constructs were excised from the vectors, and purified fragments were microinjected into fertilized ova generated from crosses of (C57BL/6Mr × C3H/HeJNCr)F_1 mice, which were then introduced into pseudopregnant mice. Offspring were analyzed for the presence of the transgene by Southern analysis (13) of tail DNA. A transgenic line carrying ≈70 copies of the SV40 T-antigen construct was obtained by breeding a founder animal with wild-type and sibling animals, and transgenic offspring were routinely identified by PCR amplification of SV40 sequences from tail blood as described (14).

Analysis of RNA. Total RNA from mouse tissues was prepared by the single-step guanidinium isothiocyanate-phenol-chloroform extraction method (15). Peritoneal phagocytes were recovered from animals free of obvious tumor at

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Abbreviations: CDP, CCAAT displacement protein; SV40, simian virus 40; T antigen, large tumor antigen; GH, growth hormone.

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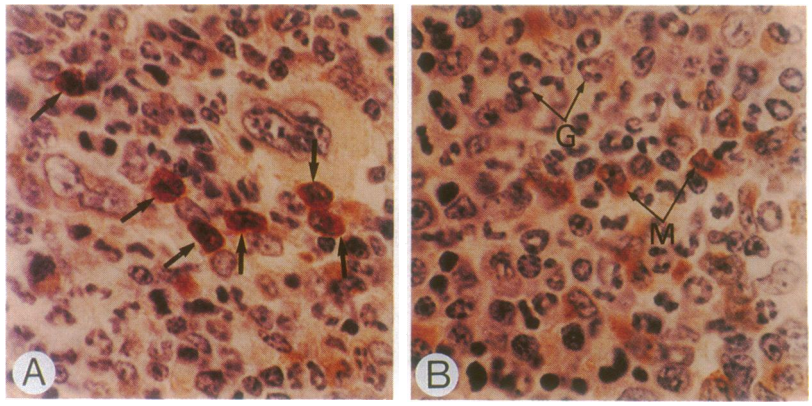

FIG. 1. Targeting of reporter gene expression by the gp91-phox promoter. Immunohistochemical detection of human GH in a section of spleen red pulp (*A*) and bone marrow (*B*) from a 1541-bp gp91-phox/GH transgenic animal. Tissue sections were prepared and analyzed as described. Positive cells exhibit reddish-orange cytoplasmic staining (arrows in *A*). G, granulocyte; M, monocyte. (×250.)

various times after a 1-ml i.p. injection of 2% (wt/vol) thioglycollate broth. Red blood cells were removed from peritoneal samples by lysis with 5 mM $NaPO_4$, pH 8.0. RNase protection assays were done as described (16) by using 10 μg of total RNA. The gp91-phox, SV40 T antigen, and γ-actin messages protect probe fragments of ≈400 bases, 275 bases, and 80 bases, respectively. Northern (RNA) analysis was done essentially as described (16). The murine gp91-phox cDNA clone was provided by Jason Fisherman, National Cancer Institute. The γ-actin plasmid was provided by M. Celeste Simon, Children's Hospital, Boston.

Histology and Immunohistochemistry. Mouse tissue was fixed in Bouins' solution or 10% (vol/vol) buffered formalin, dehydrated in graded alcohols and xylenes, embedded in paraffin, and 3- to 4-μm sections were prepared and stained with Harris' hematoxylin/eosin (17).

For immunohistochemistry experiments, tissue was embedded in OCT freezing solution and sectioned by cryostat. Tissue for human GH immunohistochemistry was fixed in 2% (wt/vol) paraformaldehyde for 10 min, preincubated with 10% preimmune goat serum, incubated with polyclonal rabbit anti-human GH antibody (DAKO, Carpinteria, CA) for 30 min, then incubated with reagents from a Vectastain ABC-Peroxidase kit (Vector Laboratories) for rabbit IgG primary antibody. For SV40 T-antigen immunohistochemistry, tissue was fixed in 2% paraformaldehyde for 10 min and then in methanol for 10 min, incubated with monoclonal mouse anti-SV40 T-antigen (18) for 30 min and then in horse serum for 5 min, and finally incubated with reagents for mouse IgG primary antibody. For hematopoietic lineage-specific immunohistochemistry, tissue was fixed in 2% paraformaldehyde for 10 min; then incubated with monoclonal antibody M1/33 (19), M1/70 (20), F4/80 (21), MOMA1 (22), MOMA2 (23), B220 (24), or Thy1.2 (25), and finally incubated with reagents for mouse IgG primary antibodies as described above. Lineage-specific antisera were proved by Lester Kobzik, Harvard School of Public Health. Staining for nonspecific esterase was done as described (26).

RESULTS

The Promoter of the gp91-phox Gene Targets Reporter Gene Expression to Monocyte/Macrophages. The tissue distribution of reporter gene expression in transgenic founder mice carrying a 1541-base-pair (bp) gp91-phox promoter/human GH construct (1541 bp gp91-phox/GH) was determined by immunohistochemistry. Human GH is detected in a subset of cells in the red pulp of the spleen and bone marrow (Fig. 1*A* and *B*, respectively). No reporter expression is detected in the white pulp of the spleen, the cortex of the thymus, or in nonhematopoietic cells, and no significant staining is detected when nonimmune serum was substituted for the primary antibody (data not shown). This distribution is consistent with appropriate targeting of transgene transcription to myelomonocytic cells. Tissue-specific transgene expression was seen in 6 of 12 founder animals analyzed (Fig. 2), whereas the transgene was transcriptionally inactive in the other six animals. Variable levels of GH expression in founder animals is presumably due to chromosomal position effects on transgene expression.

Surprisingly, although the tissue distribution of transgene transcription appears consistent with appropriate targeting to myelomonocytic cells, no expression is detected in granulocytes (Fig. 1*B*), cells that express the highest level of gp91-phox. As a control, other myeloid-specific proteins were detected in murine granulocytes by immunohistochemistry with p22-phox (Mary Dinauer, Children's Hospital, Boston) and M1/70 (20) antibodies (data not shown). Failure to detect transgene expression in granulocytes suggests that cis-elements required for appropriate expression in the full complement of myelomonocytic cells are absent from the 1541-bp gp91-phox construct. Based on tissue distribution and morphology, we conclude that expression of the trans-

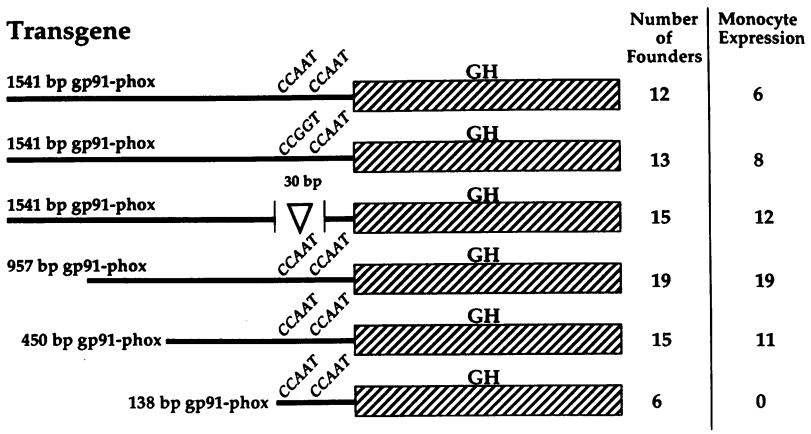

FIG. 2. Localization of cis-elements necessary and sufficient for monocyte/macrophage-specific transgene expression. Construction of transgene plasmids and mutagenesis of the proximal gp91-phox promoter region has been described elsewhere (11). Length of gp91-phox promoter linked to the reporter gene is indicated at 5' end of each construct. Number of founder animals analyzed for each transgenic construct and number exhibiting monocyte-specific transgene expression are indicated in columns at right. GH indicates the reporter gene.

gene *in vivo* is restricted to monocyte/macrophages. These results indicate that cis-elements within the 5′-flanking region of the gp91-phox gene are sufficient to target reporter gene expression to monocyte/macrophages and further suggest that subsets of myelomonocytic cells exhibit distinct requirements for tissue-specific gene expression.

Analysis of additional transgene constructs indicates that 957 or 450 bp of the gp91-phox promoter contain cis-elements sufficient for transcription in monocyte/macrophages, although no transgene expression is detected when only 138 bp of gp91-phox 5′-flanking sequences are present (Fig. 2). Thus, sequences between −138 and −450 bp of the gp91-phox promoter are necessary for transgene transcription. Expression of gp91-phox promoter/GH transgenes is also detected in scattered cells at other body sites consistent with the distribution of tissue macrophages, such as the liver, skin, brain, and gut (data not shown).

Elsewhere we report that two DNA-binding proteins compete *in vitro* for overlapping binding sites (−84 to −132 bp) in the gp91-phox promoter (11). A CCAAT-binding factor indistinguishable from CP1 (27) is apparent in nuclear extracts prepared from cells expressing the gp91-phox gene, whereas a CCAAT displacement protein (CDP) (28) blocks the binding of CP1 in nuclear extracts prepared from nonexpressing cells. To assess the potential significance of these interactions we have examined the expression of transgene constructs with selected mutations in the promoter. A deletion (−102 to −133 bp) that abolishes CP1 and CDP binding does not qualitatively affect expression of the 1541-bp gp91-phox/GH transgene, as cells expressing the reporter are detected by immunohistochemistry in the same distribution of tissues as in animals carrying the wild-type promoter/GH transgene (Fig. 2). However, founder animals carrying the construct lacking the CP1/CDP element are on average, 30% larger than nontransgenic animals or animals carrying the wild-type gp91-phox/GH transgene. Increased body size presumably reflects elevated reporter expression. These results are consistent with tissue culture transfection experiments that revealed derepression of this mutant promoter in cells that do not express the endogenous gp91-phox gene, such as erythroleukemia and uninduced myelomonoblastic cell lines (11). Furthermore, mutation of the distal CCAAT box to CCGGT abolishes CP1 but not CDP binding *in vitro*, yet this mutation does not affect transgene expression (Fig. 2). From these findings, we infer that CP1 binding is not obligatory for transgene expression in the subset of monocyte/macrophages in which the wild-type construct is active *in vivo*. In addition, as is consistent with a proposed role of CDP as a transcriptional repressor (11), removal of the CDP-binding site apparently leads to increased expression *in vivo*.

Targeting of Onco-Transgene Expression to Monocyte/Macrophages. The potential consequences of targeting oncogene expression to monocyte/macrophages *in vivo* were addressed by linking ≈2.6 kilobases (kb) of the gp91-phox 5′-flanking region to the early region of SV40, which encodes the T-antigen oncoprotein. A transgenic line derived from a founder animal carrying ≈70 copies of the T-antigen transgene was studied in detail.

RNA analysis indicated that SV40 T-antigen expression in mouse tissues is coincident with that of the endogenous gp91-phox gene (12). RNA was prepared from infiltrating cells after i.p. injection of thioglycollate to assess relative transgene expression in monocyte/macrophages and granulocytes. Populations enriched (>90%) for each cell type can be obtained by harvesting peritoneal cells at various times after injection. Granulocytes collected 18 hr after thioglycollate injection exhibited no transgene expression as determined by RNase protection assays, whereas transgene transcripts are detected in monocyte/macrophages collected 4–5 days after thioglycolate injection (Fig. 3). As anticipated, the endogenous gp91-phox transcript is detected in both cell fractions. The presence of γ-actin transcripts was used as a control for RNA quantity and integrity.

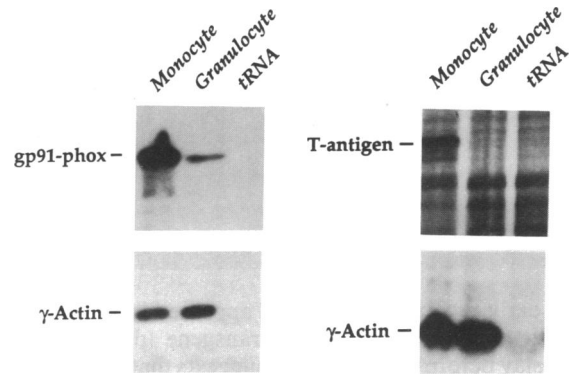

FIG. 3. Restriction of SV40 early region reporter gene expression to a subset of myelomonocytic cells. RNase protection assays of RNA isolated from thioglycolate-induced granulocytes or monocyte/macrophages. Recovery of cells and isolation of RNA are described. The cellular composition of collected samples was confirmed visually by Wright–Giemsa staining.

Restriction of SV40 T-antigen transcripts to monocyte/macrophages is in agreement with immunohistochemical studies that failed to detect human GH reporter expression in granulocytes of mice carrying gp91-phox promoter/human GH transgenes (Fig. 1*B*). Furthermore, immunohistochemical analysis of thioglycolate-induced monocyte/macrophages demonstrates that only a small percentage of nonspecific esterase-positive cells exhibit nuclear staining for T antigen (Fig. 4 *A* and *B*). No significant staining is detected when murine IgG is substituted for the primary antibody (data not shown). These findings indicate that the gp91-phox promoter is sufficient to target expression to a subset of monocyte/macrophages *in vivo*.

Animals carrying the gp91-phox promoter/SV40 early region transgene develop tumors arising with variable gross presentations consistent with a hematopoietic origin. These lesions can arise in subcutaneous tissue, as well as abdominal organs, such as the liver, spleen, or Peyer's patches. Other animals exhibit widely disseminated tumor throughout the abdominal cavity, often associated with malignant ascites (data not shown). These diverse presentations are accompanied by variable tumor invasion in other hematopoietic structures, such as bone marrow, thymus, and peripheral blood.

Hematopoietic tumors occur with complete penetrance at 6–12 mo of age in animals carrying the 2600-bp gp91-phox/

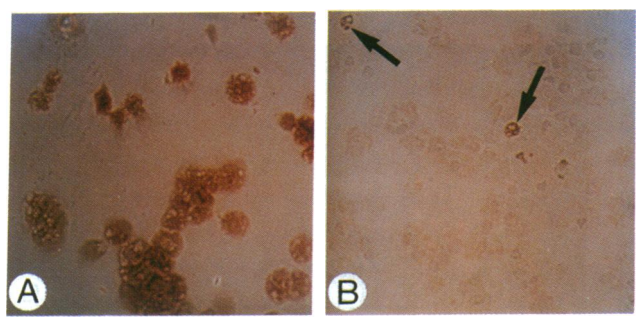

FIG. 4. Restriction of SV40 early region transgene expression to a subset of monocyte/macrophages. (*A*) Nonspecific esterase stain on thioglycolate-induced monocyte/macrophage sample. (×400.) (*B*) Immunohistochemical detection of SV40 T-antigen expression in thioglycolate-induced monocyte/macrophages, as described. (×400.)

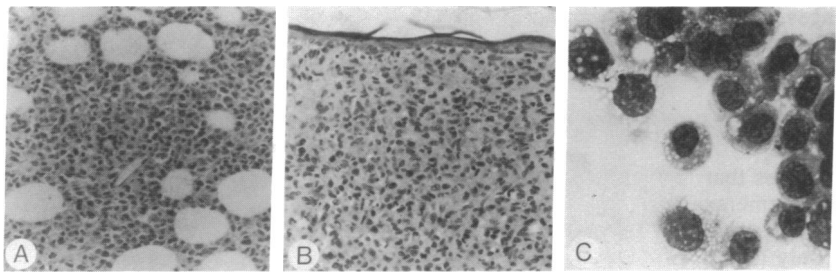

FIG. 5. Histology of histiocytic tumors in mice carrying the 2.6-kb gp91-phox/SV40 early region transgene. Sections were prepared and stained as described. (*A*) Abdominal tumor. Large empty spaces correspond to fat. (×65.) (*B*) Subcutaneous tumor. Top of figure corresponds to skin. (×65.) (*C*) Cells obtained from malignant ascites. (×260.)

SV40 early region transgene and appeared in 15 independent founder animals carrying this transgene (data not shown). The delay before tumorigenesis suggests that somatic events, in addition to SV40 T-antigen production, are required for the development of a malignancy. Male animals generally succumb to an unusual neuroectodermal malignancy before developing the hematopoietic malignancy (12). This lesion appears due to a combinatorial transcription signal created during construction of the transgene (see *Discussion*).

Although hematopoietic tumors arise with diverse gross presentations, they exhibit indistinguishable histology (Fig. 5 *A* and *B*). Both abdominal and subcutaneous tumor cells exhibit large round-ovoid vesicular nuclei with heavy nuclear membranes and one or more prominent nucleoli. Numerous aberrant mitotic figures and scattered pyknotic nuclei are often visible. In addition, cells recovered from malignant ascites are highly vacuolated, suggesting a monocytic origin (Fig. 5*C*). These features are consistent with a pathologic diagnosis of histiocytic lymphoma (29).

Immunohistochemistry was done to establish the cell lineage of origin for these tumors. Subcutaneous tumor exhibits cell-surface staining with the monocyte/macrophage-specific antibody F4/80 (21) (Fig. 6*A*). No significant staining is detected when murine IgG is substituted for the primary antibody (Fig. 6*B*). In addition, expression of both the SV40 early region transgene and the endogenous murine gp91-phox gene is detected by Northern analysis in subcutaneous tumors, cells isolated from malignant ascites, and abdominal tumors (data not shown). Additional immunohistochemistry experiments indicate that subcutaneous and abdominal tumors express an indistinguishable set of lineage-specific markers that define these lesions as true histiocytic lymphomas. Cells from both tumor presentations stain with the myeloid lineage-specific antibodies F4/80 (21), MOMA1 (22), MOMA2 (23), M1/33 (19), and M1/70 (20) but are negative for B-cell (B220) (24) and T-cell (Thy1.2) (25) markers (data not shown). These results confirm that hematopoietic tumors arise secondary to appropriate targeting of onco-transgene transcription to monocyte/macrophages expressing the endogenous gp91-phox gene and are consistent with the observed tissue distribution of expression for human GH and SV40 early region reporter genes.

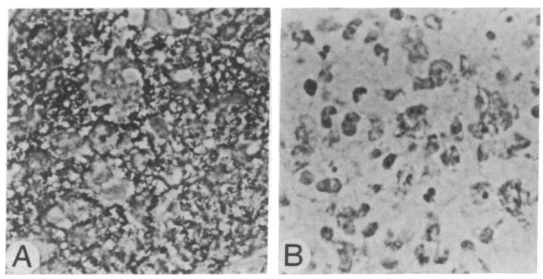

FIG. 6. Biochemical characterization of histiocytic tumors. Immunohistochemical detection of a monocyte/macrophage-specific cell-surface antigen with antibody F4/80 (21) (*A*) and with murine IgG (negative control) (*B*). Pigmentation visible in the negative control corresponds to counterstained nuclei. (×360.)

DISCUSSION

Targeting of Transgene Transcription to Monocyte/Macrophages. In this report we demonstrate that the promoter of the gp91-phox gene directs expression of two different reporter genes to a subset of monocyte/macrophages in transgenic mice. Four hundred fifty base pairs of the gp91-phox promoter are sufficient for this activity. Furthermore, sequences between −138 and −450 bp of the gp91-phox promoter are required, whereas those lying within a duplicated CCAAT box region (−102 to −133 bp) are dispensible for expression. However, transgenes are not transcribed in granulocytes (neutrophils), cells that normally express the highest level of endogenous gp91-phox. Hence, it would appear that transcription in most myelomonocytic cells that express the endogenous gp91-phox gene requires additional genomic regions not contained within the 2.6-kb promoter sequence tested.

We have previously noted competition between a ubiquitous CCAAT-binding factor (CP1) and a CDP for binding *in vitro* to the region between −84 and −132 bp of the gp91-phox promoter (11). CDP DNA-binding activity decreases during myelomonocytic cell development, in association with the onset of gp91-phox expression (11). Hence, we have proposed that CDP acts as a transcriptional repressor of the gp91-phox promoter. We envision that downregulation of CDP DNA-binding activity is a necessary, although not sufficient, step in the induction of gp91-phox transcription. Additional lineage-specific factors presumably act to direct tissue-specific transcription of the gp91-phox gene in the absence of CDP DNA-binding activity. Tissue-culture transfection experiments that reveal inappropriate transgene expression when the CDP site is removed (18), and the increased size of mice bearing a gp91-phox promoter/human GH transgene with a deletion of the CDP-binding site, are consistent with this model.

Complex arrays of regulatory elements are often responsible for control of eukaryotic gene transcription. Subsets of nonoverlapping cis-elements may independently mediate expression in response to different signals. As a reflection of this, different sets of DNase I hypersensitive sites neighboring the chicken lysozyme gene are present in macrophages as compared with hormonally induced tubular gland cells of the oviduct (30). Likewise, restriction of gp91-phox promoter/reporter gene transcription to a subset of monocyte/macrophages suggests that similarly complex elements operate within subsets of myelomonocytic cells. Nonetheless, cells expressing gp91-phox transgenes are found at multiple sites characteristic of monocyte-derived cells, such as the brain, liver, skin, gut, bone marrow, spleen red pulp, peripheral white blood cells, and thioglycolate-induced peritoneal macrophages. Hence, it is unclear what features distinguish this subset of monocyte/macrophages.

Pathologic Consequences of Onco-Transgene Expression in Monocyte/Macrophages. The proximal gp91-phox promoter permits targeted expression of reporter genes in a subset of monocyte/macrophages. To develop a model of tumorigenesis in the monocytic lineage, we examined the consequences of directed expression of the SV40 T-antigen oncoprotein *in*

vivo. Oncogene activation has been implicated in numerous naturally occurring hematopoietic malignancies (31). A causal relationship between many of these genetic alterations and tumorigenesis has been demonstrated by creation of animal models of disease through the introduction of activated oncogenes into the germ line of transgenic mice, or reconstitution of irradiated mice with infected hematopoietic progenitor cells (32, 33).

Transgenic mice carrying the 2600-bp gp91-phox promoter/SV40 early region construct develop an unusual pattern of gender-specific malignancies associated with SV40 T-antigen expression. We previously reported that male animals develop prostate gland-specific neuroblastomas (prostomas) with complete penetrance at 3- to 7 mo of age. These tumors are apparently due to a transcription signal that was generated during construction of the transgene and functions in a unique class of neuroectodermal cells restricted to the prostate gland (12). As prostomas do not express the endogenous gp91-phox gene, these lesions are not due to appropriate tissue targeting of transgene expression by the gp91-phox promoter. Female animals carrying the 2600-bp gp91-phox/SV40 early region transgene do not develop neuroblastoma, presumably due to the absence of the prostate gland and the corresponding neuroectodermal progenitor cells that express the transgene. Instead, female animals develop monocyte/macrophage malignancies (true histiocytic lymphomas) secondary to appropriate targeting of transgene expression to a subset of monocyte/macrophages. Male animals carrying the transgene rarely develop histiocytic lymphoma, as most succumb to prostomas before the age (6–12 mo) at which hematopoietic malignancies occur.

The delayed appearance of gross histiocytic lymphomas suggests that somatic events, in addition to SV40 T-antigen expression, are required for the development of a malignancy; this may reflect the requirement for cooperation between oncogenes for tumorigenesis (8, 34) and the cumulative effects of somatic mutations revealed through study of human cancers (31, 35).

Although the histiocytic lymphomas described here exhibit diverse gross presentations, they are indistinguishable by histological, immunohistochemical, and RNA analyses. These lesions are distinct from the typical form of B-cell-derived "histiocytic" lymphomas found in humans. Though rare in humans, true monocyte/macrophage-derived histiocytic lymphomas give rise to bulky tumors in subcutaneous tissue, bone marrow, and lymph nodes (29, 36), similar to the tumors described in this report. In addition, many mice carrying the 2600-bp gp91-phox/SV40 early region transgene exhibit hepatosplenomegaly and extranodal tumor involvement of multiple tissues, as seen in cases of malignant histiocytosis, an uncommon monocyte/macrophage-derived malignancy that may be a variant of true histiocytic lymphoma (29).

As gp91-phox expression begins at the promyelocyte stage of maturation, tissue-restricted tumorigenesis in the transgenic mice described here suggests that monocyte/macrophages are not postmitotic and are susceptible to SV40 T-antigen-induced malignant transformation at this stage of development. The diversity of gross presentations exhibited by the monocyte/macrophage tumors is provocative. As delayed appearance implies that secondary somatic events are required to induce tumorigenesis, the properties of individual tumors may reflect the characteristics of the monocyte/macrophage in which the initiating tumorigenic somatic event occurred. Similar heterogeneity of tumor properties has been described in malignant histiocytosis in humans (29). Thus, the animal model described here may be useful for defining unique properties of monocyte/macrophage subpopulations, as well as for identifying somatic events required for complete malignant transformation.

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