factor (or factors) is required for IL-2 gene expression in these Jurkat T cells. Recently, an inducible nuclear factor of activated T cells, NFAT-1, was shown to be involved in the control of IL-2 gene activation in Jurkat T cells (*14*). Since NFAT-1 expression is dependent on de novo protein synthesis in Jurkat cells, it is a candidate for being at least one of these additional factors. We (*20*) and others (*21*) have found that IL-2 gene expression in mitogen-activated peripheral blood lymphocytes is independent of protein synthesis. Although the biochemical basis for this paradoxical result in Jurkat and primary T cells is unresolved, it may reflect biologically important differences in transcription factor production or action (or both) in these cell types.

Our findings support a role for κB-specific DNA binding proteins in the overall regulation of IL-2 gene activation in human Jurkat T cells. That these inducible factors also regulate IL-2Rα gene activation suggests that these proteins contribute to the coordinate induction of these genes, both of which are critically involved in the control of human T cell growth.

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Metastatic Hibernomas in Transgenic Mice Expressing an α-Amylase–SV40 T Antigen Hybrid Gene

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Mice transgenic for a hybrid gene containing the liver promoter of the mouse α-amylase gene (*Amy-1*^{a}) fused to the SV40 tumor antigen coding region unexpectedly developed malignant brown adipose tissue tumors (malignant hibernomas). Expression of the α-amylase gene had previously been thought to be confined to the liver, parotid, and pancreas; however, analysis of white and brown adipose tissue from nontransgenic mice revealed expression of the endogenous *Amy-1*^{a} gene in these tissues. Gene constructs driven by the *Amy-1*^{a} liver promoter thus provide a means of targeting gene expression to the adipocyte cell lineage in transgenic mice. Moreover, the high frequency of metastases in the liver, lungs, spleen, heart, and adrenals of these mice provides an experimental system in which to study the development of disseminated malignancy.

STUDY OF THE MOUSE α-AMYLASE gene *Amy-1*^{a} has provided a valuable model of regulated gene expression during development (*1*). Transcription of *Amy-1*^{a} is controlled by two differentially regulated promoters; a stronger upstream promoter (the parotid promoter) regulates expression in the parotid gland, whereas a weaker downstream promoter (the liver promoter) regulates expression in the liver, parotid, and pancreas. Each promoter controls transcription of an mRNA containing a specific nontranslated leader sequence that is joined by differential splicing to the shared *Amy-1*^{a} coding region. Activation of the two *Amy-1*^{a} promoters is temporally regulated in the developing parotid gland. Expression from the liver promoter is detectable at birth, whereas expression from the parotid promoter does not occur until 14 days postpartum. Adult levels of *Amy-1*^{a} mRNA are not attained until approximately 3 weeks after birth owing to the late activation of the parotid promoter (*1*). Little is known about the mechanisms regulating the tissue-specific expression of the *Amy-1*^{a} gene. Some information has been obtained from analysis of hybrid *Amy-1*^{a} gene constructs transfected into cells in culture and by in situ analysis of *Amy-1*^{a} mRNA expression in the mouse (*1*). However, these studies do not address the issue of which regions of flanking sequences regulate *Amy-1*^{a} expression within the various tissues of the organism. To study the regulation of this gene, we have produced transgenic mice harboring a hybrid gene consisting of the *Amy-1*^{a} liver promoter and the sequence encoding SV40 T antigen (Tag).

An Eco RI restriction fragment of the *Amy-1*^{a} gene was first isolated that contained 438 bp of the liver promoter and flanking region and 162 bp of the liver untranslated region (*2*). This fragment was then subcloned in front of the Hind III–Bam HI fragment encoding SV40 Tag (excluding the 21- and 72-bp repeats) in pBR322 Δ. The resulting plasmid, p600T, was linearized at the Bam HI site and microinjected into the pronuclei of fertilized one-cell stage C57B1/6J mouse embryos (*3*). Injected eggs were transferred into the oviducts of pseudopregnant outbred CD-1 mice and the mice that were born were analyzed for the presence of the transgene by Southern blotting. Seven transgenic mice containing between five and ten integrated copies of the p600T transgene were obtained and stable transgenic lineages were successfully developed from six of these by backcrossing to C57B1/6J mice and then crossing them among themselves (Table 1). One transgenic mouse that developed bilateral subscapular tumors was killed before a lineage could be established.

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Tumors arose at several specific anatomical locations in p600T mice. These tumors developed in the founders or their progeny after a short latency period (4 to 6 months) in line 334 mice, a long latency period (12 to 18 months) in lines 324, 333, 354, and 443, or after a more variable period (4 to 9 months) in line 441. Only two mice, both from line 354, developed tumors in an expected location, the liver, and no mice developed tumors in the parotid or pancreas (Table 1). The tumors that developed in the other mice were invariably found in the brown adipose tissue (BAT) depots, usually at a single location, but occasionally multifocally in interscapular BAT and less frequently in the subscapular, axillary, cervical, paravertebral, mediastinal, perirenal, and periaortic (thoracic and abdominal) BAT depots. Gross and microscopic examination of newborn mouse tissues revealed no abnormalities in any of the transgenic mouse lines; however, all BAT and white adipose tissue (WAT) depots in mice from each p600T lineage developed age- and lineage-dependent pathologic changes including the appearance of pleomorphic cells and lipofuscin deposits (*4*).

Tumors that developed in the BAT depots in each transgenic lineage were well vascularized and composed of relatively well-differentiated brown adipose cells containing an eosinophilic cytoplasm, multiloculated fat droplets, a centrally or eccentrically placed nucleus and abundant and sometimes pleomorphic mitochondria (Fig. 1). Some tumor cells contained large unilocular fat droplets, which are also commonly observed in both normal and neoplastic BAT (*5*). All tumors examined, irrespective of the transgenic mouse line, expressed abundant SV40 Tag. On the basis of anatomical location and morphologic characteristics, these tumors were diagnosed as brown adipose tumors or hibernomas.

Although previous studies had localized α-amylase gene expression to the parotid, liver, and pancreas (*1, 2*), the consistent appearance of hibernomas in our transgenic mice prompted us to test whether adipose tissue in normal mice is a site of α-amylase gene expression. Northern blot analysis was performed on polyadenylated [poly(A)$^+$] mRNA from the BAT, WAT, parotid, liver, and spleen of 6-week-old C57Bl/6J mice (Fig. 2). Both BAT and WAT contained transcripts that specifically hybridized to the *Amy-1*^{a} probe and corresponded in size and quantity to those in the liver. A band of slightly faster mobility, corresponding to the more abundant (100-fold) mRNA transcripts expressed from the parotid type promoter, was seen only in the lanes containing parotid mRNA. The probe did not hybridize to poly(A)$^+$ mRNA from spleen, a tissue which does not express the amylase genes (*2*). This result provides conclusive evidence of transcription of the α-amylase gene in adipose tissues and suggests that expression of SV40 Tag and hibernoma formation in transgenic mice are the consequences of appropriate regulation and expression of the hybrid transgene.

The expression of SV40 Tag in the tissues and tumors of p600T mice was examined by indirect immunofluorescence, a procedure that is sensitive and allows in situ localization to small subpopulations of cells within a tissue. SV40 Tag was detected in the nuclei of abnormal BAT and WAT cells but not prior to the appearance of overt pathology. However, the intensity of SV40 Tag–specific immunofluorescence in abnormal BAT and WAT was much weaker than that of the tumors (Fig. 3a), thus it is possible that SV40 Tag is synthesized in amounts undetectable by this assay in normal BAT and WAT. The constitutive level of transgenic expression in preneoplastic BAT and WAT would be consistent with previous studies in which transcription of the liver promoter was shown to be weak (*1*) and the present finding that endogenous *Amy-1*^{a} mRNA accumulation is relatively low in adipose tissue. The greater abundance of SV40 Tag in the tumors suggests that amplification or stabilization of SV40 Tag expression occurs concomitant with cell transformation.

The liver, pancreas, parotid, brain, submaxillary and sublingual glands, spleen, kidney, heart muscle, lung, intestine, skin, and gonads were also examined for histopatho-

Table 1. Incidence and age of tumor-onset in p600T transgenic mice.

Transgenic line	Mice autopsied*	Mice with tumors (%)†	Age in months at tumor onset	Tumor type		
				Hibernoma	Hepatoma	Fibrosarcoma
324	8	2 (25)	16	2		
333	9	4 (44)	14	3		1
334	29	22 (75)	5	22		
354	30	12 (40)	15	10	2	
440‡	1	1 (NA)	4	1		
441	50	11 (22)	7§	11		
443	13	4 (31)	12	2		2

*Number of mice during or after the period of tumor development. †Number of mice killed with tumors and percentage of tumor incidence. ‡No lineage established. §Age of tumor onset was more variable in this lineage (4 to 9 months).

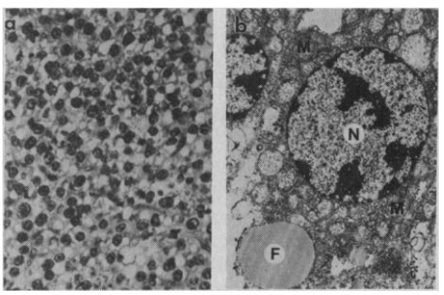

Fig. 1. Hibernoma histology and ultrastructure. (**a**) Section of hibernoma from a 334-line mouse (×150). The nuclear/cytoplasmic ratio is high in the tumor cells and the nuclei are densely packed; lipid vacuoles are abundant. (**b**) Electron micrograph of hibernoma from 334-line mouse (×6100). The cells are relatively well differentiated and contain a centrally or eccentrically placed nucleus (N), multiple round mitochondria (M), and fat droplets (F). For light microscopy, specimens were collected at autopsy, placed in Bouin's fixative for 16 to 18 hours, rinsed overnight in 70% ethanol, embedded in Paraplast, sectioned, and stained with hematoxylin and eosin (H + E) by standard histologic techniques. Specimens for electron microscopy were fixed in cacodylate-buffered gluteraldehyde for 2 hours and processed by standard techniques.

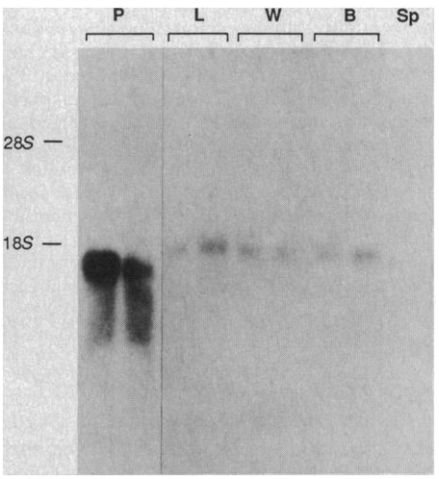

Fig. 2. *Amy-1*^{a} expression in parotid (P), liver (L), WAT (W), BAT (B), and spleen (Sp). Total RNA was extracted from the tissues of 6-week-old C57Bl/6J mice in hot phenol as described (*3*) and passed over an oligo(dT) column. Six micrograms of poly(A)$^+$ mRNA was denatured in glyoxal (*17*) and fractionated on a 1% agarose gel. Northern blotting and hybridization to the nick-translated *Amy-1*^{a} cDNA probe [pMSa 104 (*18*)] was carried out as described (*19*). Positions of the 18*S* and 28*S* ribosomal RNAs are shown. The composite autoradiogram is derived from film exposed for 24 hours to visualize probe hybridization to parotid mRNA and for 72 hours to visualize hybridization to the remaining mRNAs.

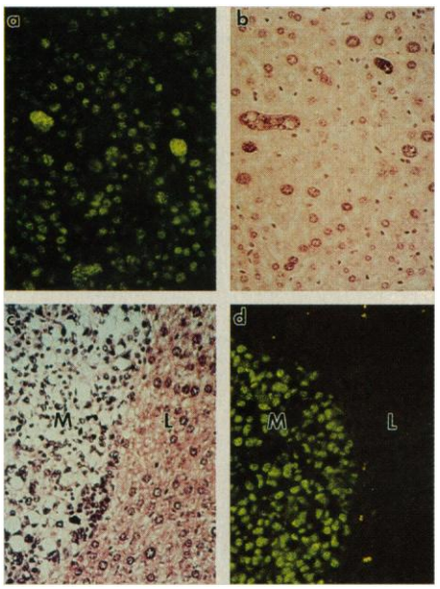

Fig. 3. Detection of SV40 T antigen in hibernomas and a hibernoma metastasis and histology of hepatocellular carcinoma. (**a**) Immunofluorescent detection of SV40 T antigen in a frozen section of a hibernoma from a 334-line mouse. Abundant SV40 Tag is detected in the nuclei of the tumor cells. (**b**) Section of a hepatocellular carcinoma from a 354-line mouse. The liver cells are relatively well organized in this section of the tumor, but the nuclei are extremely pleomorphic and giant vacuolated nuclei are prominent. (**c**) Section of liver showing a focus of hibernoma cells. The tumor cells (M) contain abundant lipid and are morphologically distinct from the surrounding normal, but compressed, liver (L) parenchyma. (**d**) Immunofluorescent detection of SV40 Tag in the liver of a 334-line mouse. Strong immunofluorescence is seen in the nuclei of the metastatic tumor cells (M) but not in the surrounding liver (L) cells. H + E sections were prepared as in Fig. 1. For indirect immunofluorescence, tissue samples were snap-frozen in liquid nitrogen, sectioned at 6 μm on a cryostat, dried, fixed in 2% paraformaldehyde for 30 min, rinsed in phosphate-buffered saline (PBS), and submerged in 0.5% potassium permanganate/0.1% sulfuric acid for 20 s to elute endogenous immunoglobulins. After rinsing in running tap water for 2 min, followed by several changes of PBS, sections were incubated with monoclonal antibody (supernatant from hybridoma clone PAB 101; TIB 117 American Type Culture Collection) to SV40 Tag for 1 hour, rinsed three times in PBS, incubated with suitably diluted fluorescein isothiocyanate–labeled goat anti-mouse immunoglobulin G (Cooper Biomedical) for 1 hour, rinsed in PBS, mounted in aqueous Uvinert (Gurr), and photographed under a Lietz epifluorescence microscope. Final magnification is ×100.

logical changes and expression of SV40 Tag. In the liver, nuclear enlargement and irregularity was seen in scattered parenchymal cells. These changes occurred randomly within the different lineages but were more common in older mice. More rarely, foci of liver cell hyperplasia or hepatocellular carcinoma development (Table 1 and Fig. 3b) were detectable. Low levels of SV40 Tag were detected in the nuclei of the abnormal liver cells whereas those of the hepatocellular carcinomas exhibited uniformly strong fluorescence. Microscopic changes were not detected in the parenchyma or stromal components of other tissues; however, rare pleomorphic SV40 Tag–positive adipocytes could be found in the serosa or mesentery of some organs and in the subcutaneous tissues. Abnormalities of adipocytes in the bone marrow (femur and ribs) were not detected. Although expression of SV40 Tag was not directly assayed in the bone marrow, the absence of pleomorphic cells in the marrow and of abnormalities in hematopoiesis suggests that adipocytes here either did not express the p600T construct or did not show any effects of this expression. Adipose tissue in the bone marrow is important in hematopoiesis and may develop through a different histogenic pathway from that in other locations (*6, 7*). SV40 Tag expression in the preadipocyte 3T3-F442A cell line prevents the appearance of the cells with characteristics of the mature adipocyte (*8*). In p600T mice expression of SV40 T antigen is found in fully-differentiated WAT and BAT.

The malignant nature of these neoplasms is shown by the rapid and expansive growth that leads to the death of the host. Moreover, foci of lipid-containing anaplastic cells, which were readily distinguishable from the surrounding tissue and which reacted strongly with antibody specific to SV40 Tag (Fig. 3, c and d), were detected in a number of tissues of many tumor-bearing p600T mice. These lesions commonly occurred in the liver, lungs, spleen, heart, and adrenals. Although they were observed sporadically in tissue sections of tumor-bearing mice from each lineage, they were found in one or more tissues of virtually all tumor-bearing mice from line 334. The distribution of these focal lesions, which was consistent with a hematogenous route of dissemination, and the lack of multiple hibernomas in the various BAT depots, strongly suggests that they represent metastases of a single primary tumor.

The course of tumor development in 334-line mice and some 441-line mice is similar to that observed in transgenic mice harboring other SV40 Tag–containing hybrid genes (*9, 10*). Despite the fact that abnormalities or preneoplastic changes are common in all BAT and WAT depots, tumor nodules developed focally and in BAT only. Thus it appears likely that secondary events are necessary for tumor formation. The fact that tumors did not arise in all the mice and occurred after a long latency in most transgenic lines (Table 1) suggests that productive secondary events are rare. While pathology and SV40 Tag expression were clearly present in the major WAT depots and livers of some mice, tumors of these tissues were extremely rare. SV40 Tag expression and tissue pathology without tumor development have been previously observed in transgenic mice and have remained largely unexplained (*9, 11, 12*). For example, in mice in which SV40 Tag expression was targeted to the atria of the heart, extensive hyperplasia of the right atrium occurred while no changes were observed in the left atrium (*11*). Differential activation of, or responsiveness to, autocrine or other tumor progression factors was invoked to explain these results (*11*) and may also be involved in tumor development in p600T transgenic mice. The fact that BAT is more richly vascularized and more extensively innervated by sympathetic nerves than WAT (*13*) and retains a greater capacity to proliferate in response to physiologic stimuli (*4, 14*) may contribute to the different tumor ontogeny observed.

Appropriate transgene expression in the livers and adipose tissue of many p600T mice indicates that the regulatory sequences necessary for tissue-specific expression of this promoter are contained within the *Amy-1*^{a}–derived 600-bp region. However, expression of the transgene was not detected in the parotid and pancreas, where the endogenous *Amy-1*^{a} liver promoter is normally active. Expression of the *Amy-1*^{a} liver promoter in the parotid and pancreas may be controlled by a mechanism requiring additional regulatory sequences upstream or downstream of the 600-bp DNA fragment contained within the hybrid gene. Alternatively, cellular sequences or vector sequences flanking the hybrid gene may preferentially suppress transgene expression in these tissues (*15*). Curiously, the tissue sites of expression of the p600T transgene are very similar to those for which amylase expression is controlled by the *Amy-1*^{a} liver promoter in the rat, whereas there was no transcription in the parotid and only slight activity in the pancreas (*15*).

The findings in this report illustrate the utility of extending the study of developmentally regulated genes to the transgenic mouse model. By employing the strategy of targeting oncogene expression in transgenic mice we discovered a new site of *Amy-1*^{a} gene expression and simultaneously developed a model of adipose tissue development and tumor metastasis. The inbred background of the mice, the autochthonous nature of the tumors, and the availability of both early- and late-onset tumor lineages

should favor the use of transgenic mice as models of metastasis and neoplasia.

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Mouse Embryonic Stem Cells and Reporter Constructs to Detect Developmentally Regulated Genes

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A strategy was devised for identifying regions of the mouse genome that are transcriptionally active in a temporally and spatially restricted manner during development. The approach is based on the introduction into embryonic stem cells of two types of *lacZ* reporter constructs that can be activated by flanking mouse genomic sequences. Embryonic stem cells containing the *lacZ* constructs were used to produce chimaeric mice. Developmental regulation of *lacZ* expression occurred at a high frequency. Molecular cloning of the flanking endogenous genes and introduction of these potential insertional mutations into the mouse germ line should provide an efficient means of identifying and mutating novel genes important for the control of mammalian development.

TO GAIN A BETTER UNDERSTANDING of mammalian development, it is essential for one to identify genes and mutations involved in establishing the basic body plan around the time of gastrulation and early organogenesis. The experiments described here are aimed toward this goal, the rationale arising from our earlier results in which the integration of the *lacZ* gene linked to a weak promoter was shown to detect cis-acting elements in the mouse genome that activated β-galactosidase expression in the developing spinal cord and caused a neurological mutation (*1*). Similar developmental activation of *lacZ* constructs was reported in *Drosophila* (*2*) and other transgenic mouse strains (*3*). The use of transgenic mouse lines to detect developmentally regulated genes, however, is limited by the number of integration events that can be readily analyzed. Thus, we have developed a strategy in which mouse embryonic stem (ES) cells (*4*) and two different types of *lacZ* constructs are used to screen many integration events and to rapidly clone the associated genes. ES cells fully retain their pluripotent character after a variety of genetic manipulations in vitro and efficiently form chimaeras after reinjection into blastocysts (*5*). This property allowed us to introduce reporter gene constructs into ES cells and analyze their expression directly in chimaeric embryos without the necessity of generating transgenic mouse lines.

Two types of reporter constructs were used in these studies. In the first, which we term an "enhancer trap" construct (Fig. 1A), the *lacZ* gene was fused in frame to a minimal promoter derived from the mouse heat-shock protein 68 (*hsp68*) gene that provides a TATA box and translation initiation codon but is not sufficient on its own for expression of β-galactosidase in mouse ES cells (*6*). The construct also contained the bacterial *SUIII*$^+$ suppressor gene and the bacterial neomycin resistance (*neo*) gene. In this construct, expression of the *lacZ* gene should depend on cis-acting regulatory elements close to the site of integration that activate the weak *hsp68* promoter.

In contrast to fertilized eggs microinjected with DNA, ES cells permit selection for rare-occurring integration events, which allowed us also to design and use a "gene trap" vector (Fig. 1B). The vector contains

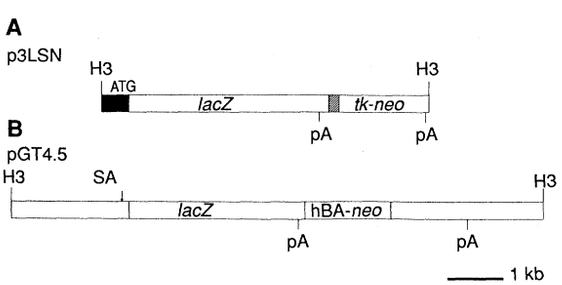

Fig. 1. *LacZ* gene constructs used as reporter genes. (**A**) The enhancer trap construct p3LSN: a 0.5-kbp Stu I–Nco I fragment of the mouse *hsp68* gene (filled-in box) was used as a minimal promoter (*15*). This fragment contains two heat-shock elements upstream of the TATA box and translation initiation codon. To avoid any possible regulatory effects we also made an additional construct, p6LSN, in which the heat-shock elements were deleted. No obvious difference was observed between the two in our studies. The promoter was linked in frame to *lacZ* (pMC1871; Pharmacia). The bacterial *SUIII*$^+$ gene (cross-hatched box) was obtained from piVX (*16*). The *neo* gene under the control of the herpes simplex virus *tk* promoter was taken from pMoTN (*17*). (**B**) The gene trap construct pGT4.5: mouse *En-2* genomic DNA (stippled box), consisting of a 5′ 1.8-kbp Eco RI–Sst I fragment including the homeobox-containing exon (*7*), was joined in frame to the *lacZ* gene. SA indicates the splice acceptor site of the homeobox exon and hBA-*neo* consists of the human β-actin promoter driving *neo* (*9*). A 3′ 2.7-kbp Bgl II–Sst I *En-2* genomic fragment containing the 3′ untranslated region provided the polyadenylation signal for *neo*. The Hind III site within the 5′ *En-2* intron sequences was deleted. Both constructs contain unique Hind III (H3) sites at their ends. Polyadenylation signals (pA) were derived from SV40.

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