Transgenic Mice as Probes into Complex Systems

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The transfer of genetic information into mouse embryos to stably alter the genetic constitution of mice is affording new insights into and opportunities in a wide variety of biological problems. Higher eukaryotes are composed of many interacting cells and organs. The properties of individual cell systems are often discernible only by studying natural or induced disruptions in their functions. Transgenic mice represent a new form of perturbation analysis whereby the selective expression of novel or altered genes can be used to perturb complex systems in ways that are informative about their development, their functions, and their malfunctions. The utility of this strategy is illustrated by recent research into immunological self-tolerance, oncogenes and cancer, and development.

THE ABILITY TO STABLY TRANSFER NEW OR ALTERED GENES into the germ line of a mouse, and thereby produce lineages or families of (transgenic) mice that carry a new genetic program, is providing insight into a number of aspects of the cells that comprise a mammalian organism. Early studies in transgenic mice focused on the control and tissue specificity of gene regulation (*1*) and led to the identification of transcriptional regulatory regions as major determinants of tissue-specific gene expression in the whole organism. However, the ability to express genes in selected cells within the tissues of a mammalian organism with cell type–specific regulatory information has raised even more profound possibilities. We can use these genes to disrupt the function or interactions of complex systems and thereby illuminate the properties of the system rather than of the gene. In some sense one can consider this approach a form of perturbation analysis, in which one partially disrupts a system and then monitors the response, thereby discerning information about that system as a whole.

The purpose of this review is to discuss several examples of genetic perturbation analysis to illustrate both the potential scope of the approach and the biological properties that are being revealed. There have been a number of comprehensive and topical reviews on transgenic mice (*2*–*7*), and I do not intend to cover all areas or to be exhaustive. Rather, three distinct aspects of transgenic mouse research will be considered, as each is either providing new information on a particular biological system or has the clear prospect to do so in the future. The focus will be on the consequences of expressing new genetic information as opposed to the specific replacement or deletion of preexisting genes. In addition, transgenes can also serve as insertional mutagens, which inactivate and thereby identify endogenous genes involved in specific developmental processes [reviewed in (*2, 4*)], and there is clear prospect for engineering site-directed DNA replacements in order to alter or abolish the expression of a gene (*8*).

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Self-Tolerance and Autoimmunity

The immune system of an organism does not normally react to its own cells and tissues. This tolerance toward self can be seen not only as a lack of self-destruction but also by an inability to respond after injection of purified proteins or upon immunization with components of self, for example, skin grafts. Nonreactivity toward self is thought to be established during a "self-learning" period, which coincides with the appearance of the lymphocytes during early life, of which the major classes are the B cells that produce antibodies, the cytotoxic T cells that attack and destroy cellular targets, and the helper T cells that regulate the activities of B cells and cytotoxic T cells. There are extensive data to support the proposition that during their development B and T cells are selected for their ability both to recognize and to respond to foreign antigens and yet to not respond to self-antigens (*9*–*11*).

Transgenic mice are providing insights into the properties of self-tolerance. When new genetic information is added to the genome of a mouse, that gene (or genes) and its gene product are genetically self. Thus one can ask if such new gene products are recognized as self by the immune system, and if so what the characteristics of that nonresponsive condition are. With regard to the effects that self-antigens have on the cells of the immune system, the complexity of B and T cells, as seen in the unique rearrangements of immunoglobulin (Ig) or T cell receptor (TCR) genes that occur in every such cell, render their individual responses to self-proteins difficult to monitor. In order to get around this limitation, it has proved possible to introduce rearranged (monoclonal) Ig or TCR genes that recognize a specific self-protein into transgenic mice and elicit their expression in a significant fraction (even a majority) of the B or T cells (respectively). This allows the response of developing lymphoid cells to a particular self-antigen to be monitored. The following examples serve to illustrate what is being revealed both by introducing new self-antigens into transgenic mice and by making transgenic mice with specific Ig or TCR genes in order to visualize the response of developing B and T cells to self-antigens. The results are summarized in Table 1.

Tolerance by B cells. In one set of experiments, the B cell response toward a new self-antigen was evaluated (*12*). Transgenic mice were

Table 1. Characteristics of self-tolerance revealed in transgenic mice.

Transgene		Self-antigen being analyzed			Immunological consequences	Conclusions	References
Description of gene	Site(s) of expression	Description of antigen	Encoded by *	Form and localization			
Metallothionein promoter/chicken lysozyme gene (Mt-HEL)	Many organs	Chicken lysozyme (HEL)	Tg	Secreted protein found at high levels in serum and bound to ECM	Tolerant to HEL; nonresponsive upon immunization; both B and T cells affected		(*12*)
Anti–chicken lysozyme immunoglobulin (H+L) (anti-HEL IgM)	Primarily in lymphoid tissues	None			High serum titers of anti-HEL Ig; no phenotype reported; anti-HEL IgM or IgD present on 60 to 90% of splenic B cells		(*12*)
Double-transgenic mice: (Mt-HEL) + (anti-HEL IgM)	(See above)	HEL	Tg	(See above)	D-Tg mice tolerant to HEL; no serum titers of anti-HEL Ig; 60% of splenic B cells express anti-HEL Ig as above, but low levels of surface IgM and no response to HEL	HEL induces functional inactivation of anti-HEL B cells	(*12*)
Anti–class I MHC immunoglobulins (anti–H-2K^k IgM)	Primarily in lymphoid tissues	None (in H-2^d mice)			High serum titers of anti–H-2K^k IgM; no phenotype reported; 25 to 50% of splenic B cells express anti–H-2K^k IgM on surface		(*14*)
Anti–class I MHC immunoglobulins (anti–H-2K^k IgM)		H-2K^k protein (in H-2^k mice)	En	Cell surface glycoprotein present on most cells	No serum titers of anti–H-2K^k IgM; no splenic B cells positive for surface anti–H-2K^k IgM	B cells expressing anti–H-2K^k IgM are eliminated in mice of k haplotype	(*14*)
T cell receptor specific for male H-Y antigen	Principally thymus, spleen, and lymph nodes	None (in H-2^b females)			30% of CD8$^+$ T cells respond to male cells in vitro (versus 1 in 10^4 in normal females). H-Y TCR on surface of most CD4$^+$ and CD8$^+$ cells. Abnormally high proportion of CD8$^+$ H-Y T cells	Bias for selection of H-Y TCR in context of CD8$^+$ and H-2^b MHC (CD8 implicated as a coreceptor for this TCR)	(*17*)
		H-Y antigen (in H-2^b males)	En	A ubiquitous antigen present on male cells (a minor histocompatibility antigen)	No T cells response to H-Y antigen in vitro, aberrant distribution of T cell types: many CD4$^-$ CD8$^-$, reduced CD4$^+$ and CD8$^+$, and a new class of cells: CD8 (low)	Most CD4$^+$ and CD8$^+$ H-Y T cells are eliminated (clonal deletion). Remaining CD8$^+$ (low) cells maybe functionally inactivated	(*15*)
Insulin gene promoter/SV40 large T antigen	(i) Pancreatic β cells beginning at embryonic day 10 (developmental onset)	Large T antigen	Tg	Nuclear protein with a cell surface component (processed peptides?)	Tg mice are tolerant to large T antigen	Antigen presentation during development confers self-tolerance	(*23, 24*)
Insulin gene promoter/SV40 large T antigen	(ii) β cells, beginning 10 to 12 weeks after birth (delayed onset)	Large T antigen	Tg	Nuclear protein with a cell surface component (processed peptides?)	Tg mice are *not* tolerant and develop spontaneous autoimmune response	Delayed appearance of antigen not sufficient for self-recognition; nontolerance allows autoimmunity	(*23, 24*)

Table 1 *(continued)*

Transgene		Self-antigen being analyzed			Immunological consequences	Conclusions	References
Description of gene	Site(s) of expression	Description of antigen	Encoded by *	Form and localization			
Insulin gene promoter/ class II MHC genes (Ins-E_α+Ins-E_β)	Pancreatic islets and kidney (no detectable thymic exp)	I-E^b protein in mice otherwise I-E^-	Tg	Cell surface heterodimeric glycoprotein	Tg mice are tolerant to I-E^b expressed only in peripheral tissues	Class II MHC on nonimmune cells (i) does not induce autoimmunity (ii) can induce nonresponsiveness by T cells in thymus and periphery	(*27, 28*)
Insulin gene promoter/ H-$2K^b$ gene	Pancreatic β cells	H-$2K^b$ protein	Tg	Cell surface glycoprotein	Tg mice are tolerant to H-$2K^b$ antigen expressed only on β cells	Only peripheral T cells are nonresponsive; suggests a mechanism for extrathymic induction of tolerance	(*29, 30*)

*Tg, transgene; En, endogenous gene.

engineered to express chicken egg lysozyme [or hen egg lysozyme (HEL)]. These mice were found to be nonresponsive (or self-tolerant) when immunized with purified HEL, which indicates that this new self-protein was recognized as such by the immune system. Separately, transgenic mice were produced that carried rearranged Ig heavy and light chain genes specific for HEL. These mice expressed HEL-specific Igs (anti-HEL Igs) of the IgM class on a majority of their B cells, making it possible to observe the fate of these cells under different circumstances, in particular in the absence or in the presence of the antigen (HEL) they specifically recognize. In the absence of the gene encoding HEL, the anti-HEL IgM transgenic mice produced readily detectable levels of circulating antibodies specific for HEL. The levels of antibodies to HEL increased after immunization of mice with purified HEL, which is one criterion of a competent immune response. The two types of transgenic mice were then mated to produce doubly transgenic mice that carried both the gene expressing HEL and the genes expressing the monoclonal antibody specific for this protein. In these double-transgenic mice, no serum titers of antibodies to HEL were detected, even after immunization with purified HEL. Therefore, the presence of the HEL protein during development of the B cells expressing the anti-HEL Ig transgenes suppressed their subsequent ability to secrete antibody, given that in the absence of the HEL protein fully competent B cells were readily detected. When the B cells were analyzed, it was observed that a majority carried low levels of anti-HEL IgM molecules on their surface, but nevertheless were unable to respond by secreting anti-HEL either in vivo, or after in vitro stimulation with purified HEL. The interpretation of these results is that the large numbers of B cells carrying low levels of anti-HEL Ig on their surface have been functionally inactivated, given that they express the specific Ig but do not respond to antigen (*12, 13*).

A second study examining B cell tolerance also utilized monoclonal antibody genes specific for an antigen. In this case, the monoclonal antibody selectively recognized a particular allele of a class I major histocompatibility gene product (H-$2K^k$) (*14*). The heavy and light chain genes encoding an antibody specific for H-$2K^k$ were first established in transgenic mice of the d haplotype (H-2^d). Mice carrying the H-2^d major histocompatibility complex do not normally express the unique antigenic determinants encoded by the H-$2K^k$ gene. The transgenic mice expressed anti–H-$2K^k$ immunoglobulin (IgM) on the surface of a majority of their B cells, which secreted high levels of antibodies to H-$2K^k$ into the circulation. However, when these transgenic mice were mated to normal mice of the k haplotype, which do express the H-$2K^k$ protein, their transgenic progeny had no circulating antibodies specific for H-$2K^k$. Moreover, there were no detectable B cells with anti–H-$2K^k$ IgM on their surface. These results indicate that the presence of the H-$2K^k$ protein during the development of B cells expressing anti-H-$2K^k$ genes resulted in the elimination of those B cells. Thus, two mechanisms of B cell tolerance to self-antigen have been revealed in these experiments: elimination of self-reactive cells and functional inactivation of such cells (Fig. 1).

T cell differentiation and self-recognition. Transgenic mice are also being used to examine the development of antigen specificity and self-tolerance by T cells. Unlike B cells, which recognize antigens in their intact macromolecular form, T cells primarily recognize degraded fragments of antigens bound to cell surface molecules encoded within the major histocompatibility complex (the class I and II MHC molecules). Two cell surface molecules, CD4 and CD8, are differentially expressed in the major classes of T cells: CD4 is on helper T cells that interact with class II MHC molecules; CD8 is on cytotoxic T cells that recognize class I molecules. T cell development occurs in the thymus and has long been thought to involve two selective events: (i) a positive selection in which T cells carrying a rearranged T cell receptor are only allowed to mature if they are capable of specifically interacting with one or more of their self-MHC molecules, since this ability is critical to T cell function, and (ii) a negative selective event, which removes those T cells that not only recognize but also are activated against those MHC molecules or other self proteins. The use of transgenic mice has directly demonstrated the existence of both of these events, as the following example illustrates.

The development of nonresponsiveness in cytotoxic T cells has been addressed in a series of experiments that also used rearranged immunological recognition genes that are specific for an antigen that can be selectively introduced by mating transgenic mice (*15–17*). In this case, the antigen was a presumptive cell surface protein called the H-Y antigen. The H-Y antigen is encoded on the Y chromosome and is found on virtually all cells in males. Neither the gene nor its product is present in females.

The two genes encoding a rearranged T cell receptor specific for the male H-Y antigen were established in transgenic mice, and the characteristics of T cells expressing the anti–H-Y TCR were evaluat-

ed both in females (H-Y$^-$) and in males (H-Y$^+$). First, consider the consequences of expressing the anti–H-Y TCR in female transgenic mice, who do not carry the H-Y antigen, which is therefore not present during the development of their immune system (*17*). When T cells from the anti–H-Y TCR transgenic females were isolated and mixed with male cells carrying the H-Y antigen, 30% of the CD8$^+$ (cytotoxic) T cells responded strongly to the male cells, which indicated that the transgenic receptor was functioning in a large fraction of the T cells. Although both helper (CD4$^+$) and cytotoxic (CD8$^+$) T cells expressed the anti–H-Y TCR, there was an abnormally high proportion of CD8$^+$ T cells in female mice of the b haplotype (*17*). This particular TCR was originally cloned from a CD8$^+$ cytotoxic T cell that developed in a H-2^b mouse and recognized H-Y antigen on male cells expressing class I MHC molecules. It appears that this differentiation pathway is favored in the transgenic mice with the same H-2^b MHC haplotype, in that there is a marked preference for maturation of CD8$^+$ T cells rather than CD4$^+$ T cells bearing the anti–H-Y T cell receptor. In mice of a different MHC haplotype, the special affinity of this particular TCR for class I MHC and CD8 would seem to be lost, thereby allowing a more balanced development of both CD4$^+$ and CD8$^+$ T cells. Thus, the positive selection for a functional anti–H-Y T cell receptor during T cell development in transgenic female mice is influenced both by the alleles of the MHC complex and by the CD4/CD8 accessory molecules. This conclusion likely reflects a general principle of T cell development, as it confirms and extends previous studies in nontransgenic mice (*18*).

In order to examine the effects of the H-Y self-antigen on the development of T cells expressing the anti–H-Y TCR, transgenic females were mated, and male progeny that carried the anti–H-Y TCR were identified (*15*). As in females, transcription of the anti–H-Y TCR genes could be detected, as could T cells expressing the anti–H-Y TCR on their surface. Yet the male mice evidenced no autoreactivity against self-tissues (which are H-Y$^+$). Moreover, isolated T cells were unable to respond to H-Y–positive cells in vitro, thus confirming their self-tolerance. Contrast this to transgenic females, where 30% of the cytotoxic T cells respond to H-Y–positive cells in vitro. Analysis by cell sorting of the T cell populations revealed that a majority of the cells expected to be expressing the anti–H-Y TCR were instead missing in the males, when compared to the populations seen in female transgenic mice. This result implies that the anti–H-Y–specific T cells died and were eliminated during their maturation in the thymus because of their ability to recognize the H-Y antigen. This elimination of self-reactive cells, which is often referred to as clonal deletion by immunologists, appears to be a general mechanism for conferring nonresponsiveness toward self by T cells. Elimination of self-reactive T cells has also been observed in other transgenic mouse experiments (*19*) and was first recognized in nontransgenic mice in studies with natural polymorphic self-antigens that eliminate all T cells with TCRs containing a particular version of the β-chain gene product of the TCR heterodimer (*20*). These recent studies have together

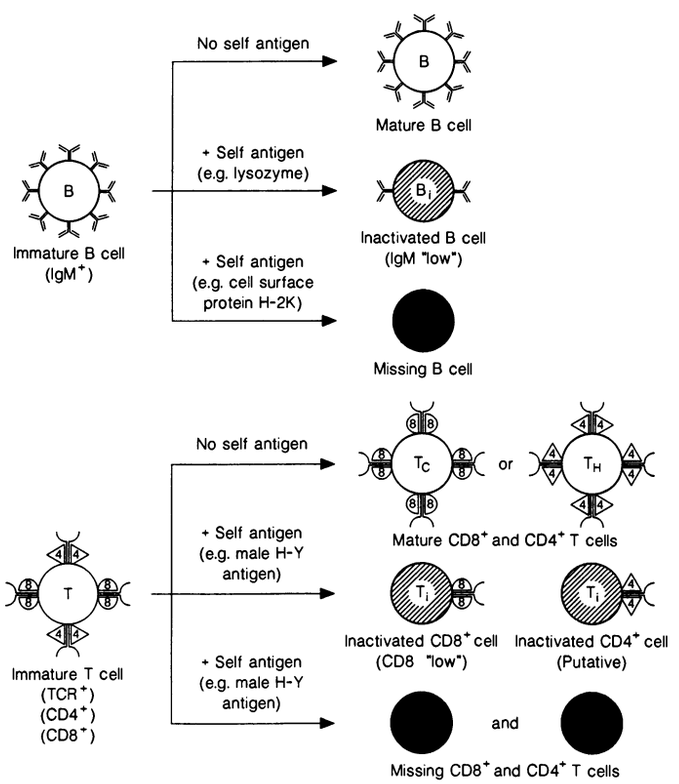

Fig. 1. Two mechanisms for inducing self-tolerance by B and T lymphocytes. The studies on tolerance to specific proteins in transgenic mice have revealed that both cellular elimination (clonal deletion) and functional inactivation of cells that recognize self-antigens can achieve selective nonresponsiveness toward components of one's self. Transgenic mice that carry rearranged immunoglobulin (IgM) or T cell receptor (TCR) genes specific for an antigen but that lack the antigen itself are mated to other mice that do express the antigen, which can be encoded either by another transgene (for example, HEL) or by an endogenous gene (for example, the male H-Y antigen). When B or T cells develop in the presence of this self-antigen, those cells expressing the transgenic IgM or TCR that specifically recognizes the antigen can be missing and are presumed to have died (cellular elimination). Alternatively, B or T cells expressing low levels of the specific receptor can emerge, but these cells are incapable of responding to the self-antigen (functional inactivation). In contrast, transgenic mice lacking the self-antigen develop fully competent B or T cells expressing the antigen-specific IgM or TCR. In the case of T cell development, the CD4 or CD8 cell surface molecules appear to be coselected with the TCR to specify either helper or cytotoxic T cell phenotype.

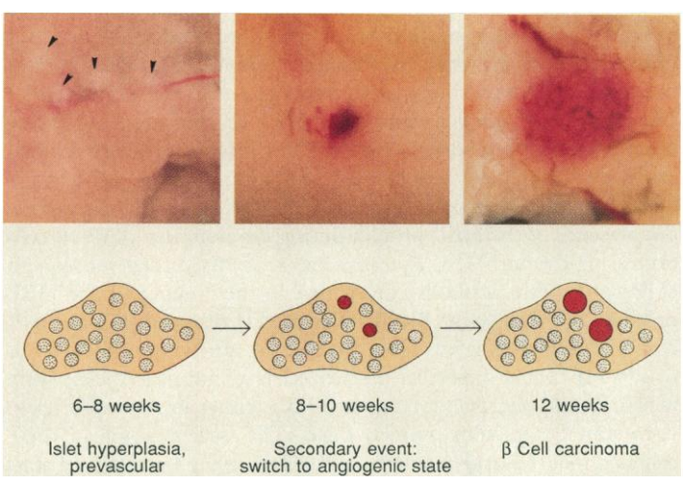

Fig. 2. Implication that induction of angiogenesis is a secondary event in tumorigenesis. The top panels show three distinct pathological stages during tumorigenesis of the pancreatic β cells in transgenic mice. The left photo shows hyperplastic islets that arise during the preneoplastic period in the age-dependent development of pancreatic tumors. The middle photo shows a rare hyperplastic islet undergoing neovascularization, and the right a well-vascularized β cell tumor. The diagram below illustrates the model inferred from studies on oncogene-induced cell proliferation and on the appearance of angiogenic activity during tumorigenesis. Expression of the large T oncoprotein elicits hyperplasia in a majority of the ~400 pancreatic islets (left panel), and yet only a few tumors develop (right panel), which implies that a secondary event (or events) mediates progression to neoplasia. A small fraction of the hyperplastic islets develop angiogenic activity (middle panel), implicating the switch into an angiogenic state as one of the important events in this multistep tumorigenesis pathway.

clearly demonstrated that cellular elimination (clonal deletion) is a significant mechanism for conferring self-tolerance by T cells as well as by B cells, thus confirming the previous interpretations of more traditional and indirect immunological experiments [see, for example (*21*)]. A second mechanism, functional inactivation, was implicated in B cell tolerance to lysozyme (see above) and is again implicated here in that transgenic males do have a minor population of peripheral T cells expressing both the anti–H-Y TCR and low levels of CD8. These anti–H-Y TCR/CD8 (low) cells do not respond to H-Y antigen on male cells in vitro, much as the anti-HEL IgM (low) B cells do not respond to HEL protein in vitro. Thus, functional inactivation appears to be a second general means to achieve nonresponsiveness toward self, as had been suggested previously (*10, 22*). The two mechanisms of self-tolerance by B and T cells that have been demonstrated in these transgenic mouse experiments are illustrated in Fig. 1.

Tolerance to rare self-antigens. Another important aspect of self-tolerance involves the ability of the immune system to recognize and discriminate self and foreign antigens expressed only on rare cell types. The studies discussed above examined the response toward abundant self-antigens, which presumably had ready access to the sites of B and T cell development, the bone marrow and thymus, respectively. With regard to antigens expressed only in dispersed rare cells, one is presented with a dilemma, namely, how is it that the presence of rare self-antigens is conveyed to developing B and T cells, and are they recognized as self at all (*22*). Again, transgenic mice have been utilized to address this issue, by using cell type–specific regulatory regions to direct antigen expression to particular cell types. This approach is exemplified in several studies on the targeted expression of novel antigens in the insulin-producing β cells, which are discussed below and are summarized in Table 1.

In one of the early studies on tolerance toward new self-antigens in transgenic mice, the insulin gene regulatory region was used to direct expression of SV40 large T antigen to the pancreatic β cells (*23, 24*). There were two stable patterns of transgene expression among the several independent lines of transgenic mice and two distinctive immunological responses to the expression of this novel protein. Mice in two of the lines begin to express T antigen during embryogenesis, and these mice recognize this protein as self. Mice in three other lines do not express the protein during development but rather evidence a heterogeneous onset of T antigen synthesis in the β cells of adult mice. The mice with delayed onset of expression are nontolerant, as evidenced by their ability to develop an immune response after immunization with purified large T protein. Thus expression during development is necessary to establish self-tolerance toward this new β cell antigen, a result that is consistent with classical immunological studies implicating a period of self-learning by the developing (immature) immune system.

There is an important consequence to the delayed onset of β cell–specific expression of this new antigen and the failure to establish self-tolerance toward it. Mice in the lines with delayed onset develop an autoimmune response against the large T protein and the β cells that synthesize it. The autoimmune response can be seen in the infiltration of the pancreatic islets with all the major lymphocyte classes and by the appearance of circulating autoantibodies against T antigen. There is a correlation between different haplotypes of the MHC and the frequencies of activating B cells to produce autoantibodies to T (*24, 25*). The MHC linkage is of interest because of provocative correlations between certain MHC alleles and autoimmune diseases in humans (*26*). Here in this transgenic model the autoantigen is known, and the characteristics of the response suggest that the inability to establish self-tolerance can result in the induction of autoimmunity in a process that involves T antigen presentation by MHC molecules.

Two other groups have examined the immunological consequences of expressing novel antigens in the β cells. One was an MHC class II heterodimer (*27, 28*), which is normally expressed only in B cells and macrophages. The second was a particular allele of a class I MHC molecule (*29, 30*). In each case the transgenic antigen was found to be recognized as self by the immune system, in that there was no evidence for an autoimmune response against the antigen or the β cells that synthesized it. However, there were differences in the response of T cells isolated either from the thymus (where they develop) or from the peripheral lymph nodes (where they congregate). T cells in both thymus and periphery were nonresponsive to the novel class II molecule as seen with in vitro assays (*27*). In contrast, the peripheral T cells but not the thymic T cells were nonresponsive to the novel class I molecule (*30*). The class II result supports the consensus that the thymus is the primary site where T cell tolerance is established. However, the latter result with a class I molecule implicates a second mechanism for establishing tolerance, which acts on T cells after they have matured and migrated out of the thymus.

The studies with MHC expression in the β cells of transgenic mice also illustrate another aspect of perturbation analysis in transgenic mice. These experiments were originally designed to test a theory which proposed that inappropriate expression of class II MHC molecules or overexpression of class I molecules on a β cell would be sufficient to induce their destruction by the immune system and the development of autoimmune diabetes (*31*). Three MHC molecules have been expressed under control of the insulin gene regulatory region in transgenic mice: the class II heterodimers I-E (*27*), and I-A (*32*), and the class I molecule H-2^b (*29*). None elicited an autoimmune response, and yet each caused dramatic disruptions in the secretory functions of the β cells, which resulted in a condition of insulin-dependent diabetes. It is likely that overexpression of these cell-surface molecules caused the dysfunctions, since β cells normally express class I molecules, and mice in another transgenic study expressed class II molecules on the surface β cells at levels comparable to those found on lymphoid cells, and yet no physiological diabetes resulted (*33*). Thus attempts to model a theory of disease instead resulted in a novel perturbation that is relevant to β cell function but not to its interactions with the immune system.

Experiments in transgenic mice have provided insight into various aspects of the nature and mechanisms of self-tolerance, as is summarized in Table 1. The expression of new antigens via transgenes has shown that self-tolerance can be established to them and in informative ways. Mechanisms for conferring deliberate nonresponsiveness toward rare antigens as well as to abundant ones appear to exist, and both cellular elimination (clonal deletion) and functional inactivation of T and B cells are utilized for achieving this specific self-tolerance by the immune system.

Oncogenes and Cancer

The epidemiology and pathology of natural cancers both provide strong indications that the development of a tumor is a multistep process (*34*). The two most apparent of these are multiple precancerous (or preneoplastic) lesions, which are not themselves lethal, and the tumors themselves. For example, cancers of the colon, cervix, and breast are often preceded by a preneoplastic stage in which many dispersed small nodules of abnormal cells can be detected (*35*). It is typical that only one primary tumor arises out of these multiple hyperplastic nodules. Thus one is presented with the questions of what are the various stages in tumor development, and what are the events (and the genes) that induce the progression from one stage to the next.

During the last 10 years a number of genes have been isolated through their association with specific tumors or tumor viruses (*36*). Many of these oncogenes affect cell proliferation and are studied (and often initially identified) in assays that involve alterations in the proliferative abilities of cells cultured in vitro. Since cancers are fundamentally diseases of uncontrolled cell proliferation, it is reasonable to suspect that such oncogenes are significantly involved in tumorigenesis. This proposition has been addressed through the introduction of a considerable number of oncogenes into stable lineages of transgenic mice. It is evident from these studies that oncogenes can reproducibly induce tumors that arise out of normal cells in their natural environment. In many cases, every mouse that inherits a particular oncogene from a parent inevitably develops a specific type of cancer. This confirms that oncogenes are centrally involved in carcinogenesis, since many of the transgenic oncogene–induced tumors are unprecedented, in that the incidences in normal mice are low or undetectable. Several recent reviews have described in detail the specific characteristics of the various oncogenes expressed in transgenic mice (*5, 7, 37*), and Table 2 summarizes a representative subset of these studies.

A common pattern of preneoplastic lesions. Although the various transgenic models in which tumors heritably arise involve a plethora of oncogenes and cell types, a common pattern can be observed in almost every case that has been examined in depth. This characteristic is the development of proliferative hyperplasias before the appearance of histologically distinct solid tumors. Preneoplastic stages are evident in c-*myc*–induced tumors of B cells and mammary glands, in the bone lesions of mice carrying the *fos* oncogene, in SV40 large T antigen–induced tumors of the exocrine and endocrine pancreas, in mice that carry the bovine papillomavirus genome and develop skin fibrosarcomas, and so on (Table 2).

This pattern of the development of preneoplastic hyperplasia is remarkably similar to that which characterizes many human cancers (*35*). The fact that oncogenes identified by or associated with cell proliferation in vitro reproducibly elicit proliferative hyperplasias in transgenic mice suggests that the abrogation of growth controls and the activation of cell proliferation is in fact a major part of the roles they play in tumorigenesis. The study of the development of these proliferative conditions in vivo may provide an important complement to the studies on oncogene action in vitro. For example, one might imagine that transcriptional activation of an oncogene would result in immediate cell proliferation. Yet the results of two studies suggest that this is not necessarily the case. Although c-*myc* is classified as an oncogene capable of immortalizing primary cells to

Table 2. Transgenic oncogenes as cancer-inducing agents.

Oncogene			Induced cancers				References
Onco-protein	Characteristics	Regulatory region	Proliferative preneoplastic stage?	Tumor type(s)	Incidence	Comments	
c-myc	Nuclear protein	Ig	Yes	B and pre–B cell lymphomas	High	Tumors clonal, by Ig rearrangements	(*38, 41, 65*)
		MMTV LTR*	Yes	Mammary adenocarcinomas in females	High	Tumors arise in 1 of the 10 mammary glands after second or third pregnancy	(*42, 66*)
		Whey acidic protein (WAP)	Yes	(as above)	Moderate	(as above)	(*67*)
c-fos	Nuclear protein, transcription factor	Human metallothionein promoter, FBJ LTR 3′ region	Yes	Osteosarcomas	Low	Widespread hyperplasia of osteoblasts; infrequent tumors	(*68*)
SV40 large T antigen	Nuclear protein (binds Rb tumor suppressor and p53 oncoprotein)	SV40 early promoter	Yes	Choroid plexus tumors	High	Includes transcriptional activation of latent transgenome	(*69*)
		Elastase	Yes	Pancreatic acinar cell tumors	High	Discernible stages of hyperplasia; focal tumor nodules	(*43, 70*)
		Insulin	Yes	Pancreatic islet (β) cell tumors	High	Typically 1 to 2% of the islets progress to tumors	(*39, 45, 46, 71*)
		αA-crystallin	Yes	Lens cell tumors of the eye	High	Transformation of a cell type not associated with natural cancers	(*72*)
		Atrial natriuretic factor	Yes	Heart tumors	Moderate	Asymmetrical hyperplasia and tumors (in right atrium only)	(*73*)
H-ras	21-kD GTP-binding protein associated with cell membrane	MMTV LTR	Yes	Mammary adenocarcinomas (males and females)	High	Transformation of a mammary epithelial progenitor (in males and females)	(*74*)

*MMTV LTR, mouse mammary tumor virus long terminal repeat; FBJ-LTR, FBJ murine osteosarcomavirus long terminal repeat; Py-mT, polyomavirus middle T protein; HSV,

continuous growth in culture, pre–B cells in young transgenic mice expressing the c-*myc* protein under control of an immunoglobulin promoter are not immortal and in fact die more rapidly than normal, nontransgenic B cells (*38*). Yet after a period of time, immortalized pre–B cells can be identified by their ability to grow continuously in vitro. This study suggests that expression of c-*myc* does not immediately produce a condition of continuous growth, but rather requires a period of time to deregulate the cell (and perhaps allow or induce genetic or epigenetic changes). This conclusion is supported by a study on the development of abnormal proliferation of pancreatic β cells expressing the SV40 large T antigen. Mice in several lines begin to express large T antigen in β cells during embryogenesis, yet abnormal cell proliferation does not develop until ~7 weeks later, when in young adults a proliferative hyperplasia can be detected (*39*).

Thus one can summarize the current perspective on oncogene action in transgenic mice with the proposition that: (i) oncogenes subvert the cellular regulation of proliferation; (ii) different oncogenes are particularly influential on specific cell types; and (iii) the activities of oncoproteins do not necessarily elicit immediate cell proliferation but rather require time and perhaps cellular change to manifest their intentions.

Progression to neoplasia. The consistent pattern of proliferative hyperplasias induced by transgenic oncogenes and the subsequent development of one or a few solid tumors implies that these are separated stages in a multistep process, much as has been inferred from studies on human cancers. One approach to distinguishing the stages of tumorigenesis is to employ cytogenetic techniques to assess the possibility that genetic changes accompany progression. There is increasing evidence that specific cytogenetic changes characterize many (if not all) human tumors. These changes are seen not only as gross chromosomal rearrangements (for example, translocations) but also as deletions or mutations at specific chromosomal loci. The latter changes are inferred to include the inactivation of tumor suppressor genes, the products of which restrict cell proliferation or other aspects of tumor growth (*40*).

Genetic change has been specifically implicated in the progression from hyperplasia to neoplasia in three distinct tumor types in transgenic mice, as is summarized below. Every B and pre–B cell lymphoma that arises in transgenic mice expressing c-*myc* under control of either Ig gene (*41*) or mouse mammary tumor virus (MMTV) (*42*) regulatory regions is clonal, as judged by unique rearrangements in the endogenous immunoglobin genes that occur during B cell development, and hence identify individual cells. The early hyperplastic B cells are not clonal, whereas in the intermediate stage the immortal B cells are typically oligoclonal and occasionally monoclonal (*38*), thus inferring a progressive selection for a particular transformed state.

Table 2 (*continued*)

Oncogene			Induced cancers				References
Onco-protein	Characteristics	Regulatory region	Proliferative preneoplastic stage?	Tumor type(s)	Incidence	Comments	
		Elastase	Yes	None; lethal neonatal hyperplasia	High	ras involved in exocrine development?	(*75*)
int1	(Secreted protein?)	MMTV LTR	Yes	Mammary and salivary-adenocarcinomas (males and females)	High	Typically, a tumor in 1 of the 10 mammary glands	(*76*)
c-myc + H-ras	(see above)	MMTV LTR	Yes	Mammary adenocarcioma	High	Accelerated tumor formation, still focal	(*74*)
neu	Mutant form of (a) rat c-*neu*, an EGF-R like membrane tyrosine kinase	(a) MMTV LTR	?	Mammary adenocarcinomas	Very high	Multifocal tumors in every mammary gland (one step; or rapid progression?)	(*77*)
		(b) MMTV LTR	Yes	(as above)	High	Sporadic tumors, typically in a few separate glands (minor differences in construct from "one-step" version	(*78*)
Py-mT	Associated with c-*src* and cytoplasmic membrane	Py early region	?	Endothelial cell tumors	Moderate	Endothelial cells highly susceptible to transformation by middle T	(*80*) (*79*)
		HSV-TK promoter	?	Endothelial cell tumors and chrondrosarcomas	Variable	(as above)	
tat	Nuclear protein (transactivator)	HIV LTR	Yes	Skin sarcomas and hepatomas	Moderate	Similar to Kaposi's sarcoma in AIDS patients	(*81*)
tax	Nuclear protein (transactivator)	HTLV-I LTR	Yes	Neurofibrosarcomas	Moderate	Implicates HTLV-I in human neurofibromatoses	(*82*)
BPV	Cell surface protein (E5) and nuclear protein (E6)	BPV	Yes	Skin fibrosarcomas	Moderate	Specific cytogenic changes in progression to neoplasia	(*44, 83*)

herpes simplex virus; HIV, human immunodeficiency virus; HTLV-I, human T cell leukemia virus type 1; BPV, bovine papillomavirus type 1.

In tumorigenesis of the exocrine pancreas, focal nodules can be detected within the more generalized hyperplasia, and these are followed by larger, histologically distinct tumors (*43*). Each of these histological stages was found to be distinct in its DNA content: the general hyperplasias were diploid (2N), the hyperplastic nodules approximately tetraploid (4N), and the tumors specifically aneuploid (2.4N to 2.8N). This implies that the tumors are clonal and that specific chromosomal combinations are being selected during the formation of a mature tumor. In fact, a study on the karyotypic stages of fibrosarcoma development in transgenic mice carrying the bovine papillomavirus genome has revealed specific cytogenetic abnormalities (*44*). Early hyperplasias were essentially diploid and more advanced hyperplasias, nonspecifically aneuploid. In contrast, the fibrosarcomas were characterized by one or both of two cytogenetic abnormalities: a gain or internal duplication of chromosome 8; or a loss or translocation of chromosome 14. Thus progression from hyperplasia to neoplasia was accompanied by specific cytogenetic changes, which therefore appear to be important for manifesting the complete tumor phenotype. In this regard transgenic tumorigenesis appears to be modeling similar changes in human cancers and may direct the investigation into the molecular genetic changes that underlie these necessary chromosomal abnormalities.

With regard to the nature of the secondary events that are necessary for the development of solid tumors, the reproducibility of transgenic tumorigenesis is providing an opportunity to identify and characterize them. One biological process that has recently been implicated as a rate-limiting secondary event is the induction of an angiogenic activity that elicits the growth of new blood vessels. The correlation of angiogenesis with tumorigenesis has come from studies of transgenic mice that heritably develop tumors of the endocrine pancreas. Expression of SV40 large T antigen in the insulin-producing β cells induces the development of β cell tumors at age-dependent rates, which are highly reproducible within several independent lines of mice (*45*). The β cells are localized into about 400 focal clusters of cells (the islets of Langerhans), a situation that naturally subdivides the population of β cells into a collection of separate small nodules. In one particularly well-studied family (RIP1-Tag2), hyperplastic islets with a high index of proliferating β cells can be detected beginning at 4 to 6 weeks. By 9.5 weeks, 50% of the islets are hyperplastic (*39*). Histologically distinct solid tumors appear between 10 and 12 weeks. However, only 2 to 10 tumors arise, despite the fact that there are at least 200 focal nodules of proliferating cells. This implicates a secondary event that is necessary for progression from hyperplasia.

Both in vivo histological analyses and an in vitro assay for angiogenic activity were used to assess the ability of the distinct stages in the tumorigenic process to elicit the growth of new blood vessels (*46*). Every tumor was found to be highly vascularized and capable of inducing angiogenesis, whereas normal islets and transgenic islets in young mice were not angiogenic. During the preneoplastic period, a few hyperplastic islets become angiogenic. The

Table 3. Dominant approaches to developmental mechanisms.

Concept	Example	Transgene	Results	Comments	References
Dominant interference	Collagen fibril biosynthesis	Mutant mouse α1(I) collagen gene	Perinatal lethality	Models human genetic disease	(*49*)
Cell ablation	By diphtheria toxin (Dip-A)	Elastase promoter/Dip-A gene	Pancreas does not develop	Penetrance is low	(*51*)
		Growth hormone promoter/Dip-A gene	Somatotrophs and lactotrophs depleted	Infer a developmental interrelation between prolactin and growth hormone cells	(*52*)
	By ricin (Ricin A) (or by Dip-A)	Lens αA-crystallin promoter/ricin-A chain	Disorganization and death of lens cells of the eye	Penetrance is low	(*53, 54*)
Inducible cell ablation	By HSV-TK plus gangcyclovir	Ig promoter/HSV-TK gene	95% of B cells and ~60% of T cells destroyed; regeneration occurs when drug removed	Allows controlled destruction and regeneration; requires proliferating cells in order to act	(*55, 56*)
		Growth hormone promoter/HSV-TK	Ablation of somatotrophs and lactotrophs	Lactotrophs arise out of a growth hormone progenitor cell	(*57*)
		Prolactin promoter/TK	No ablation of lacotrophs	Prolactin expressed in postmitotic cells	(*57*)
Hybrid genes with marker proteins	Development of endocrine pancreas	Insulin promoter/SV40 T antigen gene	Coexpression in all four islet cell types.	Infer a developmental lineage with multipositive progenitor cells	(*58*)
	Development of spermatids	Protamine promoter/ growth hormone gene	GH mRNA and protein in all four spermatids	Phenotypic equivalence of sperm with genotypic differences	(*60*)
Directed expression of growth and differentiation factors	Effects of growth hormone on development	Metallothionein promoter/hGH gene	Increased growth ("big mice")	Growth hormone can regulate body size	(*61, 62*)
	Role of NGF in peripheral neuron development	Insulin promoter/NGF gene	Selective hyperinnervation of the pancreatic islets by sympathetic neurons	NGF can influence developmental innervation	(*64*)

frequency of angiogenic islets was 3 to 4%, which correlates well with the incidence of tumors (about 2% of the islets). In contrast, the frequency of oncogene-induced hyperplasia was much higher (more than 50% of the islets). This statistical analysis suggests that the induction of angiogenesis is an important event in tumor development, a proposition that is illustrated in Fig. 2.

The ability to induce angiogenesis and effect continuing neovascularization is increasingly well established to be a quality common to most tumors (*47*). Transgenic mice can provide a reproducible model with which to address the importance of this capability for initial tumor development. For example, transgenic mice expressing angiogenesis factors could be mated to other mice expressing oncogenes to determine whether the combined actions of the two increase the frequency (and rate) with which hyperplasia progresses into neoplasia. Thus the genetic basis of the transgenic approach provides a way to test the causality of this and other events, which are suspected to mediate the transformation of a normal cell into a cancer. In the future, we may be able to control every event in the process of tumorigenesis both to confirm the necessity of each and to provide models with which to develop methods for preventing those necessary changes and the tumors that arise out of them.

Development

The study of mammalian development and the genes that specify it has been hampered by the size and complexity of the organisms and the relative difficulty both in screening for developmental mutants and in rendering those mutations accessible. Transgenic mice are beginning to provide new insights into mammalian developmental genetics. Again the approaches can be divided into ones that involve either change or inactivation of preexisting genes or the addition of new genetic information. With regard to dominant changes (expression of new information), there is clear prospect that one can perturb developing systems in informative ways using transgenic mice, as is illustrated in the examples summarized in Table 3 and described below.

Dominant interference. Dominant interference is based on the fact that many proteins are multimeric, in that their active form is composed of a number of subunits. It is possible to disrupt the function of some protein complexes by expressing defective subunits that are able to associate with normal subunits so as to produce an inactive multimeric complex (*48*). This approach has recently been used in transgenic mice, in a study that addresses not only this concept but its implication as a mechanism in human disease (*49*). A point mutation was introduced into the murine pro-α1(I) collagen gene, and the mutant gene was established in transgenic mice (*49*). Expression of the mutant transgene at levels of only 10% of the endogenous collagen genes caused neonatal death and clear aberrations in development, apparently as a consequence of distorted collagen fibrils that had incorporated the mutant protein. This result supports the hypothesis that the same point mutation is responsible for a human disease known as osteogenesis imperfecta (type II) (*50*) and exemplifies an approach that is likely to be applied to various multimeric proteins, including (in the longer term perspective) transcription factors that control development decisions.

Genetic ablation of developing cells. A second new approach to questions of mammalian development involves the selective destruction (or ablation) of specific cell types as a means to address their functional necessity and interrelations with other developing cells. Three variations on genetic ablation have been demonstrated in transgenic mice, and two of these have used toxin genes. The diphtheria toxin A gene was engineered to prevent its export and then placed under control of several gene regulatory regions: that of the pancreatic elastase gene (*51*), the pituitary growth hormone gene (*52*), and the γ-crystallin gene of the eye (*53*). A subset of the transgenic mice born carrying the elastase-dipA gene had no exocrine cells or recognizable pancreatic organ and died shortly thereafter. When dipA was instead expressed in the developing pituitary under control of the growth hormone gene, both somatotrophs (expressing growth hormone) and lactotrophs (expressing prolactin) were depleted, a result that implies that the two cell types are interrelated developmentally. The dipA gene was also linked to a lens crystallin gene promoter and used to ablate the lens cells in the developing eye. A second toxin, ricin, has also been used to ablate lens cells in the eye by a similar strategy (*54*). These results demonstrate the possibility of cell ablation as a developmental tool, although all four studies revealed a problem of penetrance of the phenotype, in that only a small fraction of the transgenic mice carrying these toxin genes evidenced significant cell ablation (and toxin gene expression).

A second method for genetic ablation of cells is conditional in that toxic effects are produced only when a drug is administered (*55, 56*). The method uses the herpes simplex virus thymidine kinase (HSV-TK), which is uniquely capable of converting certain nucleoside analogs (for example, acyclovir and gangcyclovir) into conformations where they are incorporated into replicating DNA and thereby effect cell death. In one application of this method, an Ig gene regulatory element was used to target HSV-TK expression to lymphoid tissues in transgenic mice (*56*). When gangcyclovir was administered to the Ig/HSV-TK mice, there was substantial depletion of B cells (to less than 5% of control) and appreciable depletion of T cells. When the drug was removed, the immune system regenerated itself over a 7-day period. Thus this method not only allows the selective destruction of specific cell types but also the opportunity to study regenerative processes. In a second study, the growth hormone gene promoter linked to HSV-TK was found to ablate both somatotrophs (growth hormone cells) and lactotrophs (prolactin cells) upon treatment of embryos with gangcyclovir, while a prolactin gene–TK construct ablated neither (*57*). The results indicate that the prolactin cells arise out of growth hormone progenitor cells, which do not activate prolactin gene expression until they have become postmitotic.

Transgenes for visualizing developing cells. Hybrid genes have been widely used in transgenic mice to direct the expression of marker proteins to selected cell types in order to examine the specificity of expression of the regulatory elements being utilized (*2, 4*). Although not necessarily a perturbation, hybrid marker genes are also being used to address certain aspects of development. For example, a study on the regulation of hybrid insulin genes during β cell development revealed that the transgene was also transiently expressed in each of the three other endocrine cell types of the pancreatic islets: the α, δ, and PP cells (*58*). The results suggest that during their development the islet cells go through a "multi-positive" progenitor stage, much as is well established for stem cells of the immune system (*59*).

A second example of the marker protein approach utilized the sperm-specific protamine gene regulatory region to direct human growth hormone to the cells undergoing spermatogenesis (*60*). As a result of meiosis, only 50% of the spermatids received the chromosome carrying the transgene, since the male mice were hemizygous (that is, only one of the two homologous chromosomes carried the transgene). Despite the fact that the sperm were genetically distinct, every spermatid had human growth hormone mRNA and protein. This result reveals an interchange of mRNA (and protein) between developing spermatids and suggests that all haploid sperm are phenotypically equivalent despite genetic differences.

Targeted expression of growth and differentiation factors. Another method for studying developing systems examines the effects of

putative growth and differentiation factors on the development of specific cells and organs or even on the whole organism. Again the basic strategy utilizes hybrid genes so as to direct expression of such factors to cell types where their effects can be monitored. The prototype for this approach is the now classic production of "big mice" (*61*). The metallothionein gene promoter was used to control expression of human growth hormone (hGH). Since growth hormone is normally expressed only in the pituitary, the use of this broad specificity promoter resulted in widespread expression of hGH in transgenic mice and the consequent development of unusually large mice. These experiments confirm a role for growth hormone in controlling organismic growth, a conclusion that has been supported by the transfer of the hybrid hGH gene into mice with a genetic growth deficiency ("little mice"), in which the transgene increased their growth to near normal size (*62*).

Another recent example of this approach has examined the effects of nerve growth factor (NGF) on the development of the peripheral nervous system. NGF has long been implicated as a differentiation factor for neurons (*63*). To further explore the influences of NGF on the developing nervous system, NGF expression was targeted to the pancreatic islets, which are normally innervated by sensory, sympathetic, and parasympathetic neurons. The constitutive synthesis of NGF in the islets of transgenic mice resulted in their selective hyperinnervation by one subtype of sympathetic neurons (*64*). The other classes of peripheral neurons were unaffected. The results demonstrate that NGF can influence developmental innervation of target tissues and, moreover, that it can show selectivity for the type of neurons it influences. These two examples serve to illustrate a general strategy for perturbing developing systems through the directed expression of growth and differentiation factors.

Conclusion

In this review I have sought to convey the scope with which transgenic mice can be applied to address problems in mammalian biology. The use of transgenic mice to study self-recognition by the immune system is providing us with important information about the properties of tolerance toward self and the mechanisms by which it is achieved, which in turn reflects upon the nature and development of the immune system. Regarding the biology of cancer, the introduction of oncogenes into transgenic mice has confirmed their causality in tumorigenesis and revealed consistent patterns of widespread cell proliferation (tissue hyperplasia) that precede tumor formation. This result appears to model many human cancers and may provide a means to define the characteristics of the individual stages in tumorigenesis and the genes that elaborate them. Finally, the studies on development in transgenic mice have thus far provided intriguing observations but less profound insight into their underlying mechanisms. Yet one can see in these examples the potential for dominant approaches to the genetics of mammalian development. The perturbations afforded by transgenic mice will undoubtedly be applied in the future to many complex interacting systems, of which one of the most challenging will be the nervous system.

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Research Article

Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda

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A novel bacteriophage lambda vector system was used to express in *Escherichia coli* a combinatorial library of Fab fragments of the mouse antibody repertoire. The system allows rapid and easy identification of monoclonal Fab fragments in a form suitable for genetic manipulation. It was possible to generate, in 2 weeks, large numbers of monoclonal Fab fragments against a transition state analog hapten. The methods described may supersede present-day hybridoma technology and facilitate the production of catalytic and other antibodies.

MONOCLONAL ANTIBODIES HAVE BEEN GENERATED THAT catalyze chemical transformations ranging from simple acyl transfer reactions to the energetically demanding hydrolysis of the peptide bond in the presence of metal cofactors (*1, 2–11*). Initially, it was widely held that antibodies would be most useful for catalysis where their predominant role was to overcome entropic barriers that occur along the reaction pathway. The basis of this hypothesis was that the chance occurrence of amino acid side chains capable of acid base catalysis in proximity to the reaction center was unlikely. However, for some reactions, study of the *p*H rate profile has revealed the participation of monobasic residues. Other studies have focused on placing appropriate charges on the antigen to induce specific binding interactions by complementary charged amino acid side chains on the antibody (*9, 12, 13*). Such functionalities might participate as a general acid, base, or nucleophile in the reaction under study.

Apart from the validity of the design of the mechanism based antigen, the probability of finding antibodies where particular amino acid side chains participate in catalysis also depends on the number of different antibodies assayed. Because current methods of generating monoclonal antibodies do not provide for an adequate survey of the available repertoire, we have been devising methods to clone the antibody repertoire in *Escherichia coli* and have described the preparation of a highly diverse immunoglobulin gene library (*14*). Given the difficulty of expressing both heavy and light chains together, we initially considered the construction and expression of libraries restricted to fragments of the variable region of the immunoglobulin (Ig) heavy chain V_H (*14*). In fact, a recent report describes the construction of a plasmid expression library in *E. coli* in which V_H fragments with affinity for keyhole limpet hemocyanin (KLH) and lysozyme have been isolated (*15*). However, the use of isolated V_H fragments as antibody mimics may be limited because (i) the available crystal structures of antibody-antigen complexes show considerable contact between antigen and V_L (light chain

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