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The human lysozyme promoter directs reporter gene expression to activated myelomonocytic cells in transgenic mice

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ABSTRACT **The 5′ region of the human lysozyme gene from −3500 to +25 was fused to a chloramphenicol acetyltransferase (CAT) reporter gene and three transgenic founder mice were obtained. All three transgenic lines showed the same pattern of CAT enzyme expression in adult mouse tissues that was consistent with the targeting of elicited, activated macrophages in tissues and developing and elicited granulocytes. In normal mice high CAT enzyme activity was found in the spleen, lung, and thymus, tissues rich in phagocytically active cells, but not in many other tissues, such as the gut and muscle, which contain resident macrophages. Cultured resident peritoneal macrophages and cells elicited 18 hr (granulocytes) and 4 days (macrophages) after injection of sterile thioglycollate broth expressed CAT activity. Bacillus Calmette–Guérin infection of transgenic mice resulted in CAT enzyme expression in the liver, which contained macrophage-rich granulomas, whereas the liver of uninfected mice did not have any detectable CAT enzyme activity. Although the Paneth cells of the small intestine in both human and mouse produce lysozyme, the CAT gene, under the control of the human lysozyme promoter, was not expressed in the mouse small intestine. These results indicate that the human lysozyme promoter region may be used to direct expression of genes to activated mouse myeloid cells.**

The mononuclear phagocytes are recruited from bone marrow precursors to most tissues of the body, particularly during inflammation or immune stimulation (1). Recent studies with monoclonal antibodies have been useful in visualizing the resident, steady-state macrophages which are not associated with inflammation or injury—evident, for example, in the stroma of hematopoietic tissues such as the fetal liver and the adult bone marrow (2). To date, much of the work on macrophage biology has focused on the functional response of elicited and resident macrophages to the external environment through the expression of cell surface receptors and on the possible role of macrophages in the modulation of that environment through contact or protein secretion (3). As yet, very little is known about the transcriptional control, particularly *in vivo*, of the large repertoire of genes expressed, some of which are specifically or predominantly expressed only in macrophages (4).

We are interested in the transcriptional mechanisms by which certain genes are expressed in macrophages and in finding a promoter region which can be used to target gene expression to macrophage populations *in vivo*. Lysozyme is a major product of human and murine myelomonocytic cells (5) and has been shown to be an inducible marker of activation in macrophages (5–7). In this paper we report that 3500 bp of the human lysozyme 5′ region are sufficient to target granulocytes and activated resident macrophages and to respond to immune and inflammatory signals in transgenic mice.

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MATERIALS AND METHODS

DNA. A 3.5-kb *Bgl* II–*Hin*cII fragment of human lysozyme 5′ region from a λEMBL3 human genomic library was subcloned between the *Bam*HI and *Hin*cII sites of pUC19. The *Hin*cII site is at +25 in the 29 bp which separate the transcriptional and translational start sites of the human lysozyme gene. The fragment was excised with *Eco*RI and *Hin*cII, the *Eco*RI site was filled in with Klenow polymerase I, and the fragment was subcloned into the *Sma* I site of the chloramphenicol acetyltransferase (CAT) expression vector pUC119OCAT (a gift from P. Lamb, Sir William Dunn School of Pathology), to make p3500hLZM-CAT. pUC119OCAT contains the CAT gene and simian virus 40 splice and poly(A) sites removed as a *Sma* I–*Bam*HI fragment from pMSG-CAT (Pharmacia) and ligated into the equivalent sites in pUC119. The transgene was excised as a *Sac* I–*Bam*HI fragment from p3500hLZM-CAT and recovered from low-melting-point agarose (Sigma) using a Geneclean kit (Bio 101) according to the manufacturer's instructions. DNA manipulations, restriction enzyme digestions, and DNA ligations were performed by standard methods (8).

Transgenic Mice. PO (Pathology Oxford) mice, bred from a Swiss mouse strain, in the Sir William Dunn School of Pathology, Oxford, were used as recipients for the transgene. Microinjection of eggs and transfer of embryos were performed by standard methods as described (9). Mouse genomic DNA was prepared from tail biopsies as described (9) and transgenic founders were identified by Southern hybridization using the CAT coding region as a probe or by PCR analysis. To determine copy numbers, genomic DNA (5 μg) from heterozygous mice was hybridized to two probes: (*i*) −518 to +25 of the human lysozyme 5′ region and (*ii*) a 266-bp probe (+144 to +410) specific for the endogenous M lysozyme gene to correct for loading differences. Genomic DNA (5 μg) from the human promonocyte cell line U937 was used as the copy number standard and 5 μg of DNA from a nontransgenic mouse was used to correct for background. The hybridization signals were analyzed using a PhosphorImager (Molecular Dynamics).

CAT Assay. Levels of CAT activity were determined according to the butyryl-CoA organic phase extraction method (10) in the CAT buffer described by Pothier *et al.* (11). Organs, tissues, or cells in buffer were immediately assayed or rapidly frozen in dry ice and stored at −70°C before assay. Cells were disrupted by three freeze–thaw cycles. Organs or tissues in 1 ml of CAT buffer (11) were homogenized for 20 sec with a Polytron homogenizer. Extracts were heated at 70°C for 20 min to inactivate endogenous acetylases. Protein content was measured spectrophotometrically against bovine serum albumin with the Bradford protein assay dye reagent (Bio-Rad). Fifty micrograms of protein was used per assay except when it was necessary to dilute the lysate to retain reaction linearity.

Abbreviations: CAT, chloramphenicol acetyltransferase; BCG, bacillus Calmette–Guérin; FACS, fluorescence-activated cell sorting.

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Incubation was at 37°C for 3 hr and was repeated for 10 hr to detect low levels of activity. [^{14}C]Chloramphenicol (Amersham) was the substrate and CAT (Promega) was the standard. The activity is expressed as arbitrary units. One unit of CAT enzyme activity is equivalent to 10^{-4} unit of the Promega CAT enzyme standard.

Cell Lines and Transfections. Mouse cell lines RAW264.7, P388D1, and L929 and human lines HeLa153, U937, and HepG2 were obtained from the Sir William Dunn School of Pathology cell bank. Colon carcinoma cell lines HT29 and Ls174t were acquired from the American Type Culture Collection. Transfections were performed with Lipofectin (GIBCO/BRL) according to the manufacturer's instructions. The lysoplate assay was as described (12).

Elicited and Activated Cells. One milliliter of sterile thioglycollate broth was injected into the peritoneal cavity of 12-week-old mice. The cells were recovered 18 hr or 4 days later by washing the cavity with 5 ml of sterile phosphate-buffered saline (PBS). Recovered cells were either kept on ice, quickly centrifuged, and assayed immediately or cultured on tissue culture plastic in RPMI medium with 10% fetal calf serum. Nonadherent cells were removed by four washes with warm medium. For bacillus Calmette–Guérin (BCG) infection, frozen BCG (Pasteur strain) stocks were obtained from G. Milon (The Pasteur Institute, Paris) and about 10^7 live organisms in sterile PBS were injected into the peritoneal cavity.

Fluorescence-Activated Cell Sorting (FACS). Bone marrow cells were obtained by extruding marrow plugs with PBS using a syringe fitted with a 19-gauge needle. Single cell suspensions were prepared by repeated pipetting and passage through a 19-gauge needle and syringe. Thioglycollate-elicited cells were prepared as above. Particulate matter was removed by passage through a fine grid. Approximately 10^8 cells in PBS were labeled with the rat anti-mouse granulocyte-specific monoclonal antibody 7/4 (13) for 60 min at 4°C followed by detection with a fluorescein isothiocyanate-conjugated anti-rat antibody (Sigma) for 30 min. The sorter was a Becton Dickinson FacStar Plus.

RESULTS

Generation of Transgenic Mice. The human lysozyme gene with about 10 kb of 5′ region was cloned from a λEMBL3 human genomic library. Transient transfection assays with a transcription reporter gene plasmid, p3500hLZM-CAT (see *Materials and Methods*) constructed with 3.5 kb of the 5′ region and the CAT gene, showed that there was expression in mouse monocyte/macrophage cell lines RAW264.7 and P388D1 and in the human line U937, but not in the human epithelial line HeLa153 or the mouse fibroblast line L929 (data not shown). Consequently, we used this same construct to determine if myeloid specificity was retained *in vivo* in transgenic mice. Three founder mice (from 22 born) were produced, all of which stably transmitted the introduced DNA through the germ line. There was no noticeable abnormality in the development or behavior of the founders or their progeny.

Copy numbers were estimated from quantitation of Southern blots using DNA from the human promonocyte cell line U937 as a standard. The haploid copy numbers of the three lines, referred to as the Blue, Green, and Yellow lines, were approximately 18–20, 11–13, and 3–4 copies, respectively. Heterozygous mice of all three lines were assayed for reporter gene expression.

The Human Lysozyme Promoter Directs CAT Expression in Myeloid-Rich Tissues. The CAT activity in tissues of representative 12-week-old mice from each of the three lines is shown in Fig. 1*A*. The bone marrow, spleen, and lung showed the highest levels of activity and there was no detectable CAT enzyme activity in kidney, liver, and brain. The bone marrow always had the highest activity, followed by spleen and lung. There was some activity in the hearts of 12-week-old mice and this activity increased slightly as the mice became older. The pattern of enzyme activity in the three lines was alike, suggesting that there was no effect on tissue specificity from the integration position of the transgene. The animals used were a randomly bred strain kept under normal conditions and we did observe individual differences in the level of CAT activity in the positive organs, but never in the pattern of expression. Positive organs, such as the lung, always showed CAT enzyme levels well above background, while the liver, kidney, and brain were always at background level. The enzyme activity in tissues of mice that did not carry the transgene was also at the background level of the CAT enzyme assay, indicating that there was no endogenous CAT enzyme activity in mouse tissues.

Since the variable level of CAT activity, in the bone marrow, spleen, and lung, observed among individual mice, could have arisen from variation in the recruitment and activation signals from the environment, and individual responses to those signals, we measured the CAT activity in the thymus, an organ which has no granulocytes and in which the macrophage population should be less influenced by signals from outside the animal. Thymic macrophages of neonatal and young mice

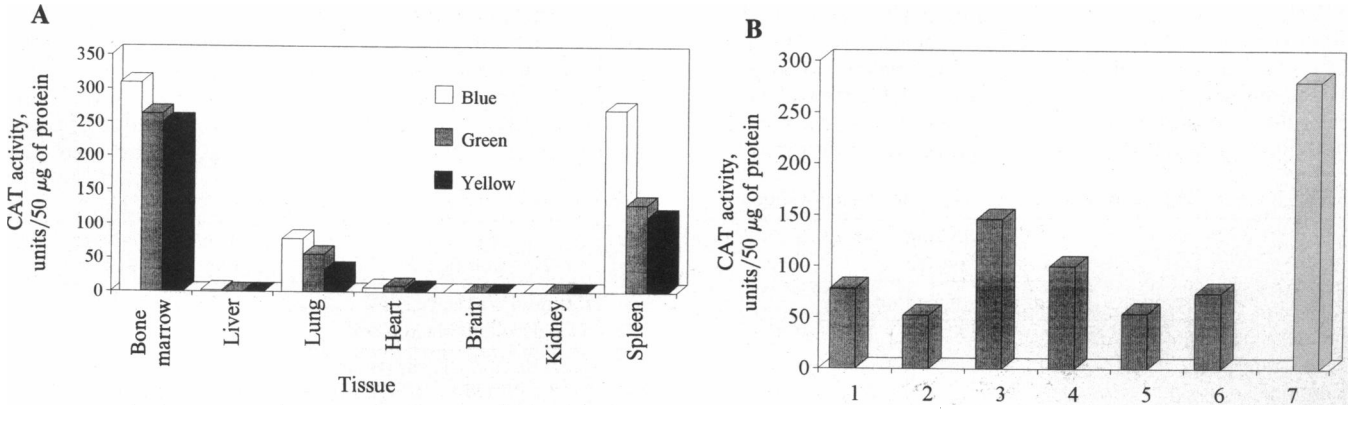

FIG. 1. The CAT gene is expressed in myeloid-rich tissues. Individual organs were homogenized and 50 μg of lysate was assayed for CAT activity. (*A*) The activity in the organs of a representative 12-week-old individual from each of the three transgenic strains is shown. The bone marrow, spleen, and lung were able to express the CAT gene and there was little or no activity in liver, heart, brain, or kidney. The expression pattern in all three lines was identical. (*B*) The thymus from each of six 21-day-old mice from the same Blue litter was analyzed for CAT enzyme activity. A 3-fold individual variation in CAT enzyme levels was observed. The CAT enzyme activity in the median spleen of the six animals is shown for comparison (column 7).

are able to make lysozyme mRNA (6) and the thymus has lysozyme activity (14). Therefore, we would expect to find CAT activity in the thymus of a young mouse. Fig. 1*B* shows the CAT activity in six mice from the same litter at 21 days of age. The human lysozyme promoter directed CAT enzyme expression in the thymus and, as we have observed in all positive organs, there was about a 3-fold variation in the level of activity among individuals of the same line. The spleens of these same animals had a range of activity similar to that observed in older mice. The activity in the median spleen of the six is shown, indicating that the level of expression in the thymus was less than half that of the spleen.

Other tissues which had little or no activity in healthy mice were skeletal muscle, testes, adrenal gland, submaxillary gland, stomach, duodenum, and large intestine. Tissues which tested positive for CAT activity were mesenteric lymph nodes and the pancreas. Although only a small amount of lysozyme mRNA has been found in the pancreas (14), the pancreas of the three transgenic mice lines expressed the CAT gene at a level comparable to that of the lung. F4/80-positive macrophages, in the mouse, have been shown to be associated with the pancreatic sinuses and to have extensive membrane processes which extend between the acinar cells (15). We checked for the presence of macrophages in the pancreas of the PO transgenic mice with the macrophage-specific monoclonal antibody F4/80 (3). The pancreas looked normal and the numbers of macrophages were about the 2–5% previously reported (15). Although it is possible that the human lysozyme promotor is very active in pancreatic macrophages, *in situ* hybridization must be used to determine whether or not nonmyeloid cells in the pancreas are producing CAT enzyme. Except for the pancreas, the pattern of CAT gene expression observed in the transgenic mice is consistent with previously reported work on the expression of the lysozyme gene in myelomonocytic cells in mice (5–7, 14). Because there was individual variation in the level of CAT enzyme activity observed, shown particularly well in Fig. 1*B*, it was not possible to relate transgene activity to copy number.

Lack of CAT Expression in the Intestine. In the mouse, the Paneth cells of the small intestine are a rich source of lysozyme (5, 6). The mouse has two lysozyme genes, the M gene specific for myeloid cells and the P gene expressed in Paneth cells (16). Humans have only one lysozyme gene, which is expressed in both myeloid and Paneth cells (17). Given this difference in the regulation of human and mouse lysozyme, it was interesting to determine whether or not the mouse Paneth cells would be able to use the human lysozyme regulatory region in the transgenic mice.

Table 1 shows the activity in six transgenic mice of the Blue line. There was a low level of CAT enzyme activity in the small intestine, but it was only about 5% of that observed in the lung. Based on previous work in our laboratory (5, 6), in which *in situ* hybridization and mRNA levels were used to examine lysozyme expression, we would have expected that, if there was positive expression of the CAT gene in the Paneth cells, the level of activity in the small intestine would be at least equivalent to that in the lung. Animals from the two other transgenic strains also expressed low levels of CAT in the small intestine (not shown), making it unlikely that the lack of expression is caused by a position effect. The duodenum and large intestine of the three strains were negative.

Table 1. The CAT gene is not expressed in the small intestine

Mouse no.	CAT activity, units/50 μg of protein	
	Lung	Small intestine
1	62.4	4
2	120	3.6
3	111	2.4
4	54	3
5	150	12
6	118	2.5

The CAT activity in the small intestine and lungs of six 5-month-old Blue strain individuals is shown. The CAT activity in the small intestine is only about 5% of that in the lung.

Table 2. The CAT gene is expressed in granulocytes

Sample	CAT activity, units in 10^6 cells	
	Granulocytes	Others
Bone marrow	270	12
Peritoneal cells	225	17

Bone marrow cells and peritoneal exudate cells elicited 18 hr after Blue strain mice were injected with thioglycollate broth were sorted by FACS using the granulocyte-specific monoclonal antibody 7/4 (13), which labeled approximately 45% and 70% of the nucleated cells in each preparation, respectively. Cells (10^6) from each fraction were assayed for CAT activity. In both cases the 7/4-positive, granulocyte fraction expressed high CAT activity and the negative cells expressed very low activity.

Since there are no human or mouse Paneth cell lines, we were not able to test whether or not the human lysozyme promoter could direct *in vitro* expression. We did, however, examine the expression of our human lysozyme-CAT construct in several human, lysozyme-positive, nonmyeloid cell lines. HT29, an epithelial cell line from a human colonic adenocarcinoma, secretes lysozyme into the culture medium (18). HepG2, a human hepatoma cell line has lysozyme mRNA (17). In transient transfection assays, the construct used to generate the transgenic mice expressed the CAT gene in HepG2 cells, but not in the HT29 cells. Ls174t, another colon adenocarcinoma line (19), also failed to express the human lysozyme CAT reporter plasmid. All three cell lines were positive for lysozyme secretion by the lysoplate assay (data not shown).

Expression in Defined Myeloid Cell Populations. The human lysozyme promoter directed high levels of CAT expression in two granulocyte populations. Bone marrow cells and cells recruited to the peritoneal cavity 18 hr after injection with thioglycollate broth, when the population is mostly granulocytes (20–22), were sorted by FACS using the granulocyte-specific monoclonal antibody 7/4 (13). As shown in Table 2, expression was high in the granulocyte fraction and very low in the negative fraction in both cases. Immature granulocytes are known to make lysozyme in the bone marrow and store the enzyme in granules (ref. 23 and references therein). Therefore, CAT expression in the bone marrow and peritoneal granulocytes of the transgenic mice indicates equivalent control of the transgene and endogenous promoters in granulocytes.

Freshly isolated resident peritoneal cells have near-background lysozyme mRNA levels (7), yet cultured resident peritoneal macrophages continuously secrete lysozyme (24)

Table 3. CAT expression in peritoneal macrophages

Cell type	CAT activity, units/50 μg of protein
Cultured resident cells	136
Thioglycollate-elicited cells	252

Resident peritoneal cells (Blue strain), which were ≈30% macrophages, were cultured in RPMI medium, washed after 2 hr to remove nonadherent, nonmacrophage cells, and assayed after 10 hr in culture when >95% of the cells were macrophages. Thioglycollate-elicited cells (79% macrophages, 2% granulocytes) were harvested from the peritoneal cavity 4 days after injection and assayed immediately. There was high CAT activity in 50 μg of cell lysate from both cell preparations. Freshly isolated, uncultured resident peritoneal cells had only 5 units of activity in 50 μg of protein.

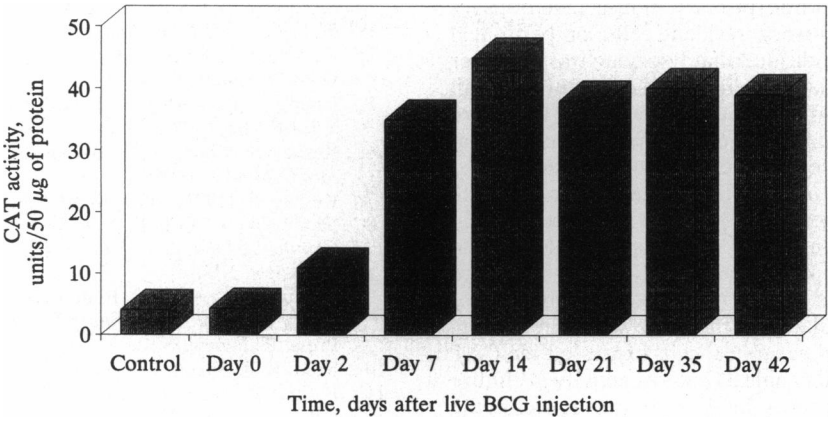

FIG. 2. CAT enzyme activity was induced in the liver of mice infected with BCG, starting 2 days after injection of $\approx 10^7$ organisms into the peritoneal cavity. Transgene activity continued for 6 weeks.

and make high levels of lysozyme mRNA (5, 6) in culture medium in the absence of exogenous stimulation. Freshly isolated resident cells from transgenic mice had only 5 or less units of CAT activity per 50 μg of protein. When the resident cells were cultured, the adherent cells, which were >95% macrophages, constitutively produced CAT enzyme (Table 3), indicating the same lack of requirement for specific extracellular signals. The enriched peritoneal macrophage population recruited 4 days after thioglycollate broth injection produced high levels of CAT enzyme (Table 3). Counts of the cell types in a cytospin sample showed 79% macrophages, 16% lymphocytes, 2% granulocytes, and 3% unidentified. However, when the cells were cultured on tissue culture plastic and washed to remove the nonadherent, nonmacrophage cells, the CAT enzyme activity declined. Activity dropped by 20–30% after 1 hr and by 90% after 3 hr. There was no CAT activity in the nonadherent cells. Thus the human lysozyme promoter region used, in contrast to that of the endogenous mouse gene (5, 6, 24), does not retain the induced expression in thioglycollate-elicited cells upon culture.

The Human Lysozyme Promoter Targets Macrophages Recruited After Pathogen Infection. It has been shown that systemic infection with an intracellular pathogen such as *Mycobacterium bovis* (BCG strain) leads to the proliferation of bone marrow monocytes followed by recruitment and accumulation of activated macrophages in granulomas of liver, spleen, and lungs (25–27). Normally the mouse liver does not contain lysozyme or its mRNA (6, 14), whereas the liver of a BCG-infected mouse has lysozyme mRNA and lysozyme-positive macrophages (6). CAT enzyme activity in the liver of the transgenic mice was near background levels (Fig. 1*A*), indicating that the human lysozyme promoter like the endogenous mouse M lysozyme promoter was not transcribed. If the transgene and the endogenous gene were regulated in the same way, we would expect the CAT gene to be switched on in the livers of BCG-infected transgenic mice. Fig. 2 shows that over a time period of 6 weeks following infection, the transgenic liver exhibited readily detectable levels of CAT enzyme activity. The livers also acquired typical macrophage-rich granulomas. This is evidence that the human lysozyme promoter is responding, in the mouse, as an inducible marker of macrophage activation.

DISCUSSION

There are now >20 cloned genes with myeloid specificity reported in the literature (4) and a number of these have been studied to determine the DNA sequence elements which confer tissue-specific gene expression. Five of these genes—chicken lysozyme (28), human c-fps/fes (29), human gp91-phox (30), human cathepsin G (31), human CD11b (32, 33), and human scavenger receptor (34)—have been analyzed in transgenic mice. It is not clear which cis regions are responsible for chicken lysozyme, c-fps/fes- or cathepsin G-directed expression, for in each case the genomic clone with 5′ and 3′ sequences was used as the transgene (28, 29, 31). The gp91-phox promoter was inactive in most phagocytic cells, including granulocytes, in which the endogenous mouse gene was expressed (30). Although CD11b is a myeloid-specific differentiation marker, it can also be expressed on subsets of B and T cells. The myeloid-specific promoter sequences may not yet be defined since the reports of transgene expression patterns showed conflicting results (32, 33). Human scavenger receptor promoter cis elements targeted macrophages in mouse atherosclerotic lesions, spleen, and testes but not other organs which contain macrophages, at least when measured at the whole organ level.

An advantage of the use of the human lysozyme promoter, as the results in this report suggest, is the specific targeting of activated macrophages in tissues and developing granulocytes in the bone marrow, including cells which can be recruited to sites of inflammation. The CAT transgene was expressed in the lung, spleen, thymus (Fig. 1), and mesenteric lymph nodes, all of which have phagocytically active resident macrophages and lysozyme mRNA (6, 7, 14). The liver, kidney, brain, skeletal muscle, stomach, large intestine, adrenal gland, heart, submaxillary gland, and testes had little or no lysozyme mRNA (6, 7, 14) and did not express the CAT transgene. The pancreas was the one organ tested which has resident macrophages and expressed the CAT transgene but has no known source of lysozyme or its mRNA (14). Otherwise, the pattern of CAT transgene expression in organs reflects the pattern of endogenous mouse lysozyme gene expression in myeloid-rich tissues.

Granulocyte expression probably accounts for the high activity in the bone marrow (Fig. 1*A*) since, when the marrow cells were sorted, almost all activity remained in the 7/4-positive, granulocyte fraction (Table 2). It is likely that much of the CAT activity in the spleen (Fig. 1*A*) was from recruited myelomonocytic cells since lysozyme mRNA levels in spleens of healthy mice were equivalent to those of the thymus, yet the lysozyme activity was 16-fold higher (14). Granulocytes with CAT enzyme activity were also recruited to the peritoneal cavity after injection of thioglycolltate broth (Table 2).

Induced expression in activated macrophages was clearly indicated after BCG infection, in which the liver, which normally did not express the transgene (Fig. 1*A*), acquired CAT enzyme activity (Fig. 2). This expression after pathogen infection makes the human lysozyme promoter an excellent tool for the study of pathogen control by activated macrophages.

In vitro analysis of cultured primary mouse macrophages from bone marrow precursors, resident cells, or peritoneal exudates has in the past indicated that lysozyme was a marker of macrophage differentiation which was constitutive in all cultured macrophages (24). However, whole organ (14) and *in situ* tissue measurement of lysozyme mRNA (6, 7) showed that many differentiated resident tissue macrophages did not express the lysozyme gene *in vivo*. In the transgenic mice the human lysozyme promoter was expressed at a low level in freshly isolated resident uncultured peritoneal cells but the CAT gene was highly expressed after only 10 hr in culture medium (Table 3). In this respect the transgene and endogenous promoter were regulated in the same way. The finding that thioglycollate-elicited peritoneal cells expressed the CAT gene when freshly isolated (Table 3) but lost activity in culture indicates regulatory differences. Support for differences in the regulation of lysozyme secretion by human and mouse peritoneal macrophages comes from a study of macrophages from patients with peritonitis (35). In this case, lysozyme secretion by cultured macrophages could be influenced by immunoregulators and was related to the maturation state of the cell.

The failure to detect high levels of CAT gene expression in the small intestine of the transgenic mice (Table 1) may indicate that the human lysozyme regulatory region used did not contain the recognition regions necessary for expression in human Paneth cells. It may also mean that expression of the gene in mouse Paneth cells requires cis sequences not present in the human lysozyme regulatory region used in these experiments or that mouse Paneth cells do not have the trans factors necessary to transcribe the human gene. Failure of the transgene to express in the human colon carcinoma cell lines HT29 and Ls174t, both of which express the endogenous lysozyme gene, suggests that 3.5 kb of human lysozyme 5′ region does not contain all of the cis regions needed for expression of the lysozyme gene in all lysozyme-positive cells so far reported and may account for the lack of transgene expression in the mouse Paneth cells.

The expression of the transgene in activated macrophages and developing granulocytes, combined with the fact that all three transgenic lines exhibit the same pattern of tissue-specific expression, leads us to believe that the human lysozyme promoter is a reliable and useful tool for the manipulation of phagocytic cell gene expression, especially in pathological conditions. For *in vitro* study, a pure population of primary macrophages can be readily obtained, since resident peritoneal macrophages express the transgene constitutively in tissue culture.

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