Nucleic Acids Research

B-lymphocyte targeting of gene expression in transgenic mice with the immunoglobulin heavy-chain enhancer

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ABSTRACT

A hybrid gene containing rabbit β-globin structural sequences (-9 to +1650), and a chicken conalbumin gene promoter (+62 to -102) in the place of the β-globin promoter (upstream from -9), was inactive in 5 different transgenic mouse line. Adding the mouse immunoglobulin heavy-chain (IgH) enhancer to this construction specifically stimulated expression in B-cells. These results show that IgH enhancer is specifically active in B-cells. Expression of the hybrid gene was low compared to the endogenous immunoglobulin heavy and light-chain genes. Substituting the mouse immunoglobulin κ light-chain gene (Ig κ) promoter (+4 to -800) for the heterologous conalbumin promoter was not sufficient to restore gene expression to level of the endogenous genes. In addition to the reproducible B cell expression, we also found inheritable unexpected expression in certain tissues, which varied from line to line.

INTRODUCTION

Transgenic mice can be used to study gene regulatory elements, as well as oncogenicity and control mechanisms such as allelic exclusion (Reviews: 1-4). In particular, it has been shown that the rearranged mouse immunoglobulin heavy- and light-chain genes are expressed at high levels in specific tissues in transgenic mice (5, 6). We have asked whether it is possible to target expression of the rabbit β-globin structural gene to lymphocytes with the immunoglobulin heavy chain (IgH) enhancer and promoter sequences from either the chicken conalbumin or mouse κ light-chain genes. The chicken conalbumin promoter element is a weak promoter in several cell-lines and shows no specificity for B-cells in transfection experiments (7 and see below). The human β-globin gene, which is extensively homologous in sequence with the rabbit β-globin gene (8), has sequences downstream from the RNA initiation site which can regulate expression from foreign promoter elements (9, 10). We show here that a hybrid rabbit β-globin gene, in which the chicken conalbumin promoter replaces β-globin promoter elements, is not expressed in five transgenic mouse lines. Addition of the IgH enhancer allows

specific expression in B lymphocytes. Nevertheless, replacing the conalbumin promoter by a Ig κ promoter apparently results in higher specific expression. These results show that in transgenic mice the IgH enhancer is sufficient to specifically stimulate transcription in B→lymphocytes, and suggests that an immunoglobulin promoter element can increase the level of specific expression.

MATERIALS AND METHODS

Construction of recombinants and microinjection.

A 1kb XbaI DNA fragment, containing the mouse IgH enhancer (for sequence, see 11, 12) was inserted into the XbaI site of the M13mp12 polylinker just upstream from the conalbumin promoter of pβCT (13) to give pβCTM. To construct pβκM, a NsiI (trimmed with T4 DNA polymerase and ligated to Hind III linkers)→BglII fragment from the MPC11 κ light→chain gene promoter was used to replace the Hind III (+62) →BamHI (→102) conalbumin fragment of pβCTM. Standard techniques were used (14). Microinjections were performed as discussed in Brinster et al. (15).

B and T→cells purification.

B and T→cells were purified from spleens of the 71 and 106 pβCTM trans→ genic mouse lines as described in Mishell and Shiigi (16). A single cell suspension from 2 spleens from each line was treated with 15mM Tris→HCl pH 7.2, 0.14M NH4Cl to lyse red→blood cells and adherent cells were removed by attachment to plastic culture flasks. Remaining cells were verified for via→ bility with trypan blue and divided into aliquots. One aliquot was enriched for T→cell by panning out B→cells with anti→mouse Ig (Biosys). The yield of T→cells was about 15%, and no more than 5% of these cells were Ig positive (as determined by immunofluorescence). B→cells were purified from another aliquot by selectively removing T→cells with anti thyl.2 (rat IgG 2b, Becton Dickinson) and rabbit complement (Gibco). The yield of B→cells was about 15% and 90% of the cells were Ig positive.
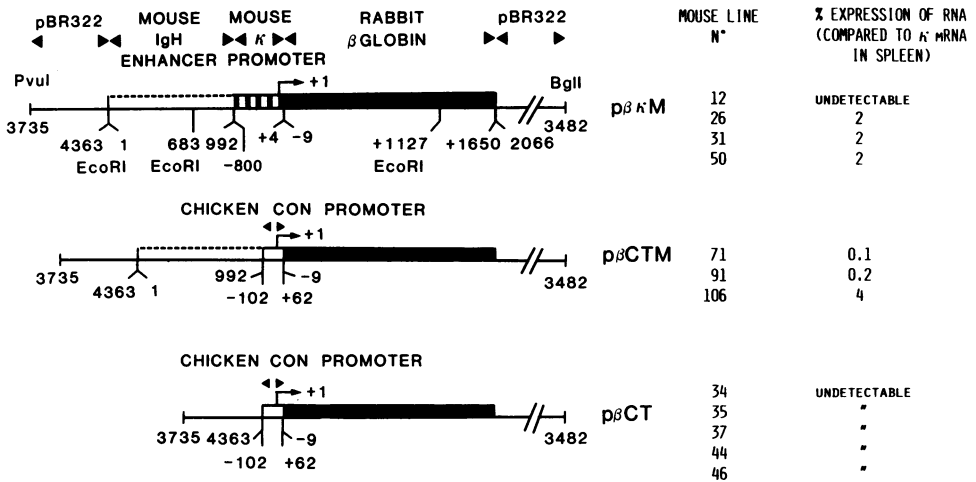
RNA analysis.

Total RNA was isolated from tissues with LiCl and urea (17) and from isolated B and T→cells with hot phenol (18). RNA was analysed by quantitative S1 nuclease mapping as described previously (19).

RESULTS

Establishment of transgenic lineages

Fig. 1 illustrates the various heterologous promoter elements which have been linked upstream from nucleotide →9 of the rabbit β→globin gene: MPC11 Ig

	MOUSE LINE N°	% EXPRESSION OF RNA (COMPARED TO κ mRNA IN SPLEEN)
pβκM	12	UNDETECTABLE
	26	2
	31	2
	50	2
pβCTM	71	0.1
	91	0.2
	106	4
pβCT	34	UNDETECTABLE
	35	"
	37	"
	44	"
	46	"

Fig.1. Structure, integration and expression of hybrid genes. The recombi-
nants contain rabbit β-globin structural gene (-9 to +1650) downstream from
either 1) an active mouse κ light-chain gene promoter from MPC11 myeloma
cells (-800 to +4) and the mouse IgH enhancer (pβκM) or 2) the chicken conal-
bumin gene promoter (-102 to +62) and the IgH enhancer (pβCTM), or 3) just
the chicken conalbumin promoter (pβCT). PvuI-BglI DNA fragments of pβκM,
pβCTM and pβCT were injected into fertilized mouse eggs. The names of the
mouse lines which had integrated the recombinants is given in the first
column. The quantity of specific stable RNA produced in the spleens of these
lines relative to κ light-chain gene mRNA is given in the last column.

κ (+4 to -800) or chicken conalbumin gene (+62 to -102) promoter elements
together with the mouse IgH enhancer (XbaI fragment, 1-992; pβκM and pβCTM
respectively, see Materials and Methods) or just the conalbumin gene promoter
element without an enhancer (pβCT). Purified linear PvuI-BglI DNA fragments
were microinjected into fertilized eggs (about 200 copies/egg) of C57/Bl6xSJL
hybrid crosses. About one thousand surviving eggs were allowed to develope in
pseudopregnant recipients and 191 newborns were analyzed for retention of the
microinjected DNA by dot hybridization of tail DNA (20). Twelve transgenics
were found and bred with hybrid mice (C57/Bl6xSJL) to establish mouse lines.

DNA blot analysis of both genomic DNA (mouse n° 12,26,31 and 50 for
pβκM; 71,91 and 106 for pβCTM and 34,35,37,44 and 46 for pβCT) and recombi-
nant plasmids, digested with EcoRI (compare fig.1 and 2), suggests that the
microinjected DNA is integrated head to tail, in tandem, with different copy
numbers at a unique and different site within each mouse genome, except for
mouse 37 which integrated only part of the pβCT fragment (not shown). The
copy numbers reported in fig.2 were estimated by compairing dot hybridiza-
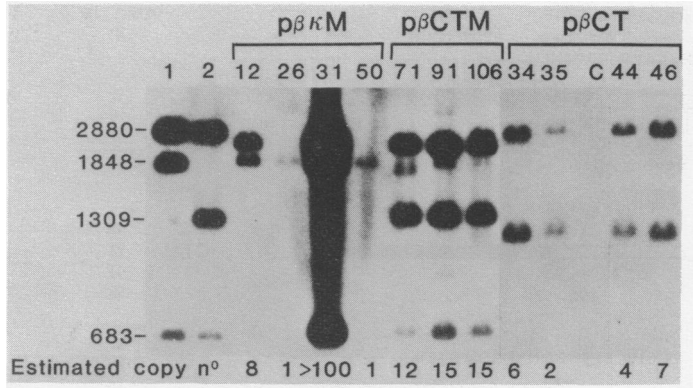
Fig.2. Southern blot analysis of the integrated genes is transgenic mice. Tail DNA (10 μg), prepared from the indicated mice, was digested with EcoRI, electrophoresed on a 1% agarose gel, blotted and hybridized at high stringency with [^{32}P] labelled pβCTM (PvuI→BglI fragment). Mouse DNA samples digested with EcoRI and electrophoresed either alone (lane C) or mixed with 15 μg of the EcoRI digests of the pβκM (lane 1) or pβCTM (lane 2) plasmids were used as controls and molecular weight markers. The numbers at the bottom of each lane gives the approximate integrated copy numbers.

tions of various quantities of transgenic genomic DNA with known amounts of the injected fragments mixed with mouse DNA. Breeding experiments illustrated that the transgenes were transmitted in a normal mendelian fashion except for mouse line 106 (injected with pβCTM) which, for 11 crosses between heterozygous transgenics, was shown to have a retarded fertility and be homozygous lethal.

The IgH enhancer activates specifically the chicken conalbumin promoter in B-cells

RNA was extracted from tissues of heterozygous descendants of five independent lines of transgenic mice containing the chicken conalbumin→rabbit β→globin hybrid gene (pβCT) and analyzed for expression of the transgene by S1 nuclease mapping. A 5'[^{32}P] labelled 170 bp BamHI fragment, extending from +62 to →102 of the conalbumin promoter, was used to quantitate RAN initiated from the conalbumin promoter. The expected S1 nuclease resistant product is labelled CON in figs.3 and 4. Control of tissue contamination by mature B→lymphocytes was systematically performed on the same RNA preparations using a 5'[^{32}P] labelled 381 bp BanII→AvaII probe (see fig.5) which detects correctly spliced Ig κ messenger RNA. However this is only an indirect measure, because the IgH enhancer is active in B cell development before the Ig κ gene is transcribed (21), and hence κmRNA concentration may not be directly
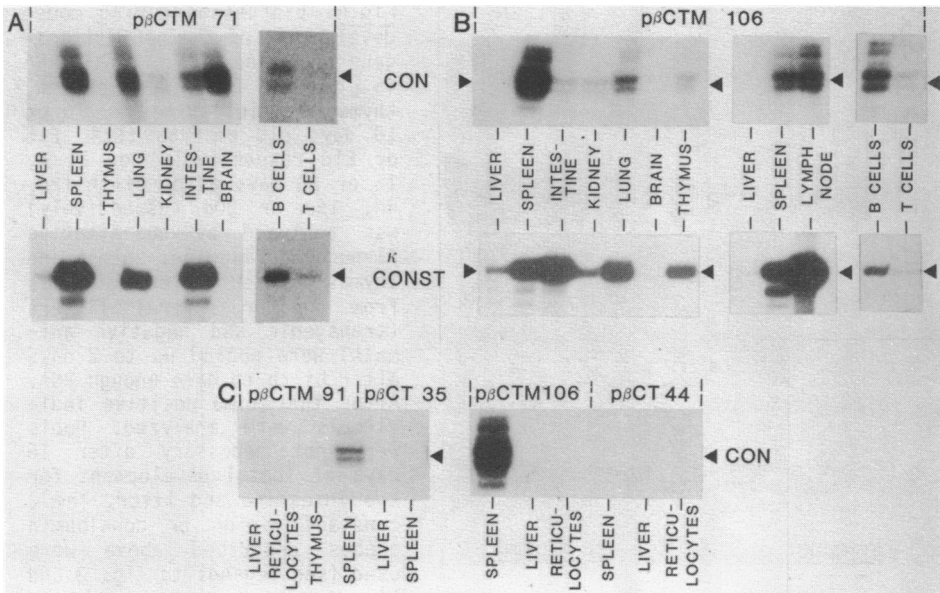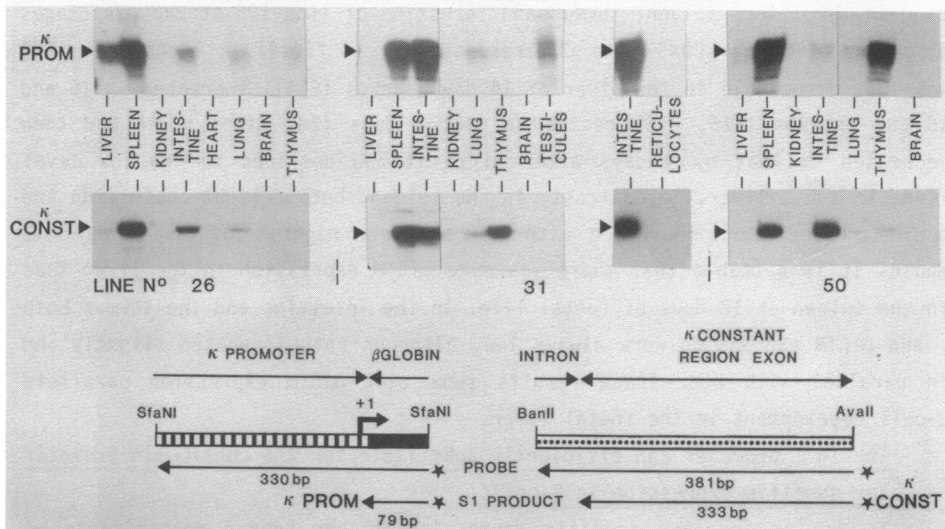
Fig.3. S1 nuclease analysis of RNA from pβCTM and pβCT transgenic mice. 5-15 µg total RNA from tissues, or splenic B or T-cells, was analyzed by quantitative S1 nuclease mapping using single-stranded 5' [^{32}P] labelled conalbumin (+62 to -102, 19) or κ-constant region (see fig.5) probes. Arrow-heads indicate bands corresponding to RNA initiated at the conalbumin start site (CON) or spliced κ light-chain message (κ CONST). A) pβCTM line 71; B) pβCTM line 106; C) pβCTM line 91 compared to pβCT line 35 and pβCTM line 106 compared to pβCT line 44.

proportional to the number of cells with IgH enhancer activity. pβCT is not detectably expressed in several different tissues nor in reticulocytes of the 5 mouse lines containing the hybrid gene (see fig.3C for examples with mouse lines 35 and 44). In addition to the tissues shown, there was no detectable expression in the following tissues: line 34, liver, spleen, reticulocytes; line 35, heart, intestine, brain and reticulocytes; line 37, liver, spleen, kidney, brain, heart, thymus, testes and reticulocytes; line 44, brain, thymus, kidney, intestine; line 46, liver, spleen, intestine, kidney, testes, brain and reticulocytes (results not shown). The recombinant containing in addition the IgH enhancer (pβCTM) was expressed specifically in organs containing lymphoid cells, in particular the spleen (see lines 71, 106 and 91 in fig.3A, B and C) and mesentreric lymph-nodes (see line 106, fig.3B). It should be noted that there was no specific expression in the heart of line 106, and in the intestine, kidney, brain, heart and lung of line 91 (results
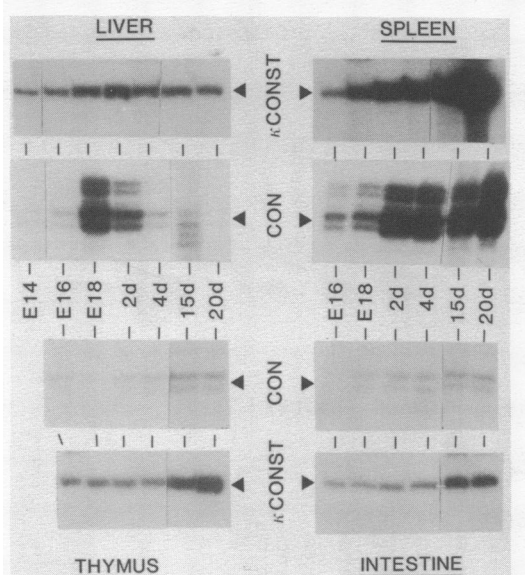
Fig.4. Expression during mouse development of the pβCTM transgene in line 106. Total RNA (5 μg) from the liver, spleen, thymus and intestine 14, 16 or 18 days old embryos (E14, E16 or E18 respectively) or 2, 4, 15 or 20 days after birth (2d, 4d, 15d or 20d respectively) was analyzed by quantitative S1-nuclease mapping. Spleen and thymus of sufficient animals from one or several litters (transgenic and negative animals) were pooled up to 2 days after birth to have enough RNA. After that time positive individuals were analyzed. Pools were not necessary after 16 days of foetal development for the intestine and liver. The κ constant region or conalbumin probes described above were used (see legends to fig. 3 and 5).

not shown). Nevertheless, expression was variable and ranged from high (line 106) to weak (lines 71 and 91). The levels of expression cannot be directly compared between lines in fig.3 because autoradiograms were exposed for different times to highlight the variation of expression in different tissues. Using conalbumin and κ constant region probes of known specific activity, we found that there was about 25 times (line 106) or about 500 to 1000 times (lines 71 and 91) less specific RNA transcribed from pβCTM compared to the endogenous κ light-chain gene RNA (fig.1, last column and data not shown). Expression in other organs, in general, followed B-cell contamination (compare κ CONST for lines 71 and 106 in fig.3A and B) except for the brain of mouse line 71, in which expression was at least as high as in the spleen. The low expression of pβCTM in the thymus, which can be correlated with κ light-chain gene expression, and high expression in spleen (lines 71 and 106) and lymph nodes (line 106) suggests that pβCTM is expressed preferentially in B-cells. To test this directly, we purified splenic B and T-cells (see Materials and Methods). For both lines 71 and 106, greater than 90% of the purified B-cells were surface Ig positive cells (tested by immunofluorescence, data not shown). These results were supported by quantitative S1 nuclease mapping of RNA with the κ constant region probe (compare κ CONST for B and T-cells in fig.3A and B). pβCTM expression was

Fig.5. S1→nuclease analysis of RNA from pβκM transgenic mice. 5→15 μg total RNA from different tissues of 3 transgenic lines (26, 31 and 50) was analyzed by quantitative S1 nuclease mapping using single→stranded $5'[^{32}P]$ labelled DNA probes to measure globin RNA chain initiation from the κ promoter (κ PROM) and κ light→chain (κ CONST) messenger RNA respectively. Autoradiograms were exposed for different times to allow comparison between different tissues with a given probe, hence the intensities of the bands for different probes cannot be compared directly (see last column fig.1). Bands of the expected size, corresponding to RNA initiated at the correct κ start site (κ PROM), or correctly spliced κ light→chain messenger RNA (κ CONST) are indica→ted by arrow→heads.

much higher in the B→cell preparation, showing that the IgH enhancer specifi→cally stimulates expression in B→cell [the low level of transcription of pβCTM in T→cells is that expected from the slight B→cell contamination (compare CON and κ CONST in B and T→cells in fig.3A and 4B)]. This result was further supported by culturing splenic cells from mouse line 106 in the pre→sence of either concanavalin A (ConA) or bacterial lipopolysaccharides (LPS) for several days. Ten times more κ light→chain mRNA and specific pβCTM trans→cripts were detected in LPS compared to ConA stimulated cell cultures (data not shown).

pβCTM is transiently expressed in foetal liver

During mouse ontogeny, B→cell production begins in the liver, where it reaches a peak around 17 days of foetal life and ceases several days after birth. We have measured both κ light→chain mRNA and specific pβCTM transcrip→

tion in the liver, spleen, thymus and intestine of line 106 at various stages of ontogeny (see κ CONST and CON respectively in fig.4). κ light-chain RNA was just detectable in the liver of 14 day embryos (E14), increased in 16 and 18 day embryos (E16, E18) reached a peak 2 days (2d) after birth and then decreased so that by 4 days after birth it had declined to the low level found in adult liver. In contrast, in the spleen, both κ light-chain mRNA and pβCTM transcription increased with age and were highest in the 20 day old mouse. It is noteable that there was more pβCTM expression in the liver than in the spleen at 18 days of foetal life. In the intestine and the thymus both κ and pβCTM expression were always low, although both increased slightly and in parallel with age. These results show that pβCTM expression parallels B-cell development in the foetal liver.

The Ig κ promoter can efficiently substitute for the conalbumin promoter for specific expression in B-cells

To try to increase specific expression of our hybrid recombinants we replaced the conalbumin promoter, which is poorly active in mouse cells, with the mouse Ig κ promoter (pβκM). RNA from transgenic lines was analyzed with a 5'[^{32}P] labelled 330 bp SfaNI DNA fragment (fig.5). RNA initiated from the κ promoter results in a S1 resistant product labelled κ PROM (fig.5). From four transgenic lines, three preferentially expressed the transgene in the spleen (fig.5). The fourth line (n°12) did not show any expression in the tissues tested. In the three positive mouse lines no expression at all was detected in kidney, brain and reticulocytes. Expression in the lung can be correlated with the contamination of this tissue by lymph-nodes as it parallels that of the κ light-chain gene. However, expression in the intestine, which contains numerous lymph follicules in its wall, was highly variable from line to line (high, intermediate or very low in lines 31,26 and 50 respectively). Unexpected expression, different for each line, was obtained in liver (line 26), testes (line 31) or thymus (line 50), which is clearly not correlated with κ light-chain RNA transcription. The amount of transgenic RNA in the spleen was measured relative to κ RNA using κ promoter and κ constant region S1 nuclease probes of known specific activity. In all three positive lines there was about 50 times less pβκM RNA than endogenous κ light-chain gene mRNA (results not shown, see fig.1 last column), which is a higher level of expression than was found with the conalbumin promoter (see above). These results suggest that the Ig κ promoter increases the efficiency of specific gene expression.

DISCUSSION

The IgH enhancer is specifically active in splenic B-cells in transgenic mice

The hybrid gene consisting of a chicken conalbumin promoter element (+62 to -102) and the rabbit β-globin structural gene (pβCT) gave no detectable mRNA in a variety of tissues, including liver and reticulocytes, in 5 transgenic mouse lines. This result was not predictable for a number of reasons. pβCT is specifically transcribed, albeit at low level, after transfection in several mouse cell-lines (22; B. Wasylyk and C. Wasylyk, unpublished results). Transfection studies in mouse erythroleukemia cells show that sequences downstream from the translation initiation codon can regulate expression from foreign promoter elements (9, 10). In transgenic mice, 48 bp of 5' flanking sequence are sufficient for specific β-globin expression (23) and the chicken conalbumin gene has been reported to be specifically expressed in the liver of transgenic mice (24). Our results suggest that the rabbit β-globin structural gene and the conalbumin promoter element, when linked in pβCT, are poorly active when reintroduced into the mouse genome.

The IgH enhancer in pβCTM specifically stimulated transcription in B-cells. Recently Adams et al. (25) showed that the c-myc oncogene linked to the IgH enhancer specifically induced malignancy in B-cells. Although in this case the oncogene might have contributed to the B-cell specificity, taken together with our results, we can conclude that the IgH enhancer is most active in B-cells. This agrees with some experiments using mouse cell-lines. Mason et al. (21) found in transfection experiments that the IgH enhancer showed no detectable activity in several T-cell lymphomas. Ephrussi et al. (26) observed, using dimethyl sulphate modification of DNA in chromatin, that certain guanine residues in the enhancer region exhibited altered reactivities in cells of the B-cell lineage, but not in non-lymphoid cell-lines or or in several T cell-lines. In contrast, in some T cell-lines, sterile transcripts have been observed initiated from cryptic promoters in the germline heavy-chain locus (27, 28) and in transgenic mice, the introduced rearranged IgH gene is efficiently expressed in both the thymus and in Lyt2+ T-cells (6). We did not detect IgH enhancer activity in either the thymus or in splenic T-cells (50% of which are normally Lyt2+) showing that the IgH enhancer is inactive in most T-cells. It seems likely that in the rearranged IgH gene, promoter sequences outside the enhancer are responsible for the transcriptional activity in T-cells. These promoter sequences may lie upstream from the RNA initiation site or perhaps in the D-J region in which

sterile transcripts initiate in both pre B and T-cells (27, 29).

The transgenes are less efficiently expressed than the endogenous Ig genes

pβCTM, containing the IgH enhancer and the conalbumin promoter, was expressed at a variable level in the spleen of three transgenic lines, at either 4%, 0.2% or 0.1% of the κ light-chain mRNA (see fig.1 last column). Assuming 7500 κ mRNA molecules per spleen cell (30) this represents 300, 15 or 8 conalbumin-β-globin mRNA molecules per cell. When we substituted the Ig κ promoter for the conalbumin promoter (pβκM) we found about 150 RNA mole-cules per spleen cell in each of the three transgenic mouse lines. This apparent higher efficiency is consistent with recent reports showing that the κ light-chain promoter is more active in B-cell lines than in fibroblasts (31, 32). However, the amount of stable RNA transcribed from pβκM is lower than when rearranged heavy and κ light-chain genes were introduced into mice, which yield about 700-3000 κ (5, 33) or 700-2000 heavy chain (6, 33) mRNA molecules per spleen cell. Several factors may contribute to the level of expression we observed. i) the transgenic RNA may be less stable than κ mRNA. ii) The pBR322 sequences introduced with the transgenes may inhibit expres-sion (23). iii) The Ig κ promoter in our recom binant is not stimulated by the IgH enhancer. However, in transfection experiments in myeloma cells, the IgH enhancer can efficiently enhance transcription from the Ig κ promoter (B. Wasylyk and C. Wasylyk, unpublished results). iv) There are other elements in the Ig genes which contribute to Ig expression. Interestingly, Grosschedl and Baltimore (34) have shown that μ intragenic sequences contribute to differen-tial IgH expression in lymphoid versus non-lymphoid cells. This suggests that expression of heterologous gene sequences may be improved by introducing a cDNA copy of these sequences (minus the polyadenylation signal) into a complete rearranged gene. In this way unidentified promoter elements are not excluded, and the hybrid message may be stabilized by the IgH RNA sequences.

Unexpected transgene expression in individual mouse lines.

In many of the mouse lines we have observed transgene expression in a tissue which could not be accounted for by B-cell contamination, and which was stably inherited over several generations. This includes the liver of line 26 (pβκM), the testes of line 31 (pβκM), the thymus of line 50 (pβκM), the brain of line 71 (pβCTM) and probably the intestine of line 31 (pβκM). In line 31, expression in the intestine was consistently higher than expected from B-cells contamination (as judged from κ mRNA) especially when compared to lines 26 and 50. Intestinal tissues contain Peyer's patches which comprise

IgA producing lymphocytes. It is surprising that most lines (for pβκM and pβCTM), except line 31, do not express the transgene in the intestine, even though a substantial amount of κ gene transcription is detected. It is conceibable that the IgH enhancer is not active in IgA producing B lympho⁻cytes. Unexpected transgene expression has been observed previousy (4) and may result from integration in a region of the chromosome which is specifi⁻cally active in a given tissue. It may also arise from pBR322 sequences, which are known to influence gene expression in transgenic mice (23). In addition, juxtaposition of control sequences from three different sources (the rabbit β⁻globin sequences, the chicken conalbumin promoter and the mouse IgH enhancer) may have created a new specificity (35). However, whatever the cause, the interesting observation was that aberrant expression was only found when the IgH enhancer was present in the recombinant. This agrees with other studies, which show that the IgH enhancer is active in certain condi⁻tions in non B⁻cells (see 7). Perhaps new tissue specific genes and enhancers could be isolated by cloning host flanking sequences. Mouse line 106 (pβCTM) was found to have a reduced fertility and to be homozygous lethal before the 9th day of embryo development. The flanking host sequences have been cloned and experiments are in progress to study the site of integration.

In conclusion, we have developed a hybrid gene construction in which the specificity of the IgH enhancer can be studied in transgenic mice. A central core of the enhancer used in this study can efficiently stimulate transcrip⁻tion in non⁻lymphoid cell⁻lines, such as mouse fibroblasts (7) and surrounding sequences contain cell⁻type specific transcription inhibitory sequences (Imler et al., in preparation). Various mutants in the IgH enhancer are now being tested, using pβCT type recombinants in transgenic mice, to study the function of these sequences in both tissue specificity and B⁻cell differentiation.

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