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Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival

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Oct-2, a POU homeo domain transcription factor, is believed to stimulate B-cell-restricted expression of immunoglobulin genes through binding sites in immunoglobulin gene promoters and enhancers. To determine whether Oct-2 is required for B-cell development or function, or has other developmental roles, the gene was disrupted by homologous recombination. *Oct-2$^{-/-}$* mice develop normally but die within hours of birth for undetermined reasons. Mutants contain normal numbers of B-cell precursors but are somewhat deficient in IgM^{+} B cells. These B cells have a marked defect in their capacity to secrete immunoglobulin upon mitogenic stimulation in vitro. Thus, Oct-2 is not required for the generation of immunoglobulin-bearing B cells but is crucial for their maturation to immunoglobulin-secreting cells and for another undetermined organismal function.

[*Key Words*: Oct-2; POU homeo domain; B-cell development; immunoglobulin genes]

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The differentiation of B-lymphoid cells comprises two distinct phases (see Rolink and Melchers 1991). The first phase is antigen independent and includes a number of interdependent gene activation and rearrangement steps that assemble functional immunoglobulin heavy- and light-chain genes (see Alt et al. 1986). It ends with the generation of surface IgM-bearing virgin B cells. The second phase is antigen dependent and requires external, generally T-cell-derived signals to trigger a differentiation cascade that produces the mature immunoglobulin-secreting plasma cell. Control of the transcriptional activity of individual immunoglobulin gene loci is central to the regulation of this differentiation process and ultimately determines the functional capacity of the mature B cell.

The expression of immunoglobulin genes in B cells requires the interaction of many transcriptional regulators with their cognate binding sites in the promoters and enhancers of the immunoglobulin loci (see Calame 1989; Staudt and Lenardo 1991). One of these factors is Oct-2, a founding member of the POU family of transcription factors (Herr et al. 1988). Oct-2 binds to the octamer motif (consensus sequence, ATGCAAAT) by conserved POU-specific and POU homeo domains (Clerc et al. 1988; Ko et al. 1988; Scheidereit et al. 1988). Octamer motifs are strategically placed in both the heavy- and the κ light-chain immunoglobulin enhancers (Singh et al. 1986; Currie and Roeder 1989; Nelms and Van Ness 1990; Pettersson et al. 1990) and in all V-gene promoters (Falkner and Zachau 1984; Parslow et al. 1984), often adjacent to an additional octamer-binding motif, the heptamer (Eaton and Calame 1987).

An important role for Oct-2 in regulating immunoglobulin gene activity in B cells has been suggested but not proven (Dreyfus et al. 1987; Wirth et al. 1987; Muller et al. 1988; Junker et al. 1990; Jenuwein and Grosschedl 1991). Such a role was proposed partly because *oct-2* expression is predominantly B lineage specific (Staudt et al. 1986, 1988; Schlissel et al. 1991a). Also, Oct-2 is produced at all stages of the B lineage (Staudt et al. 1986, 1988; Schlissel et al. 1991a), giving it the ability to influence several developmental steps. However, it is not known at which developmental stage Oct-2 exerts its primary influence. Furthermore, it has not been established whether Oct-2 plays a role in B cells that clearly distinguishes it from other regulators of immunoglobulin gene expression that occupy adjacent binding sites or from the ubiquitously expressed octamer-binding protein, Oct-1.

The Oct-1 protein functions in cell cycle-specific regulation of histone H2B gene expression (LaBella et al. 1988) and activation of a number of other cellular and

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viral genes. Although Oct-1 and Oct-2 are products of different genes, they have structural similarities in several domains. The two proteins are most highly related in their DNA-binding (POU homeo) domains (Herr et al. 1988), and thus have almost identical DNA-binding specificity. Oct-1 and Oct-2 can cooperate in DNA-binding (Kemler et al. 1989), but the significance of this interaction, particularly in B cells where they are both expressed, is unknown (see Schaffner 1989; Ruvkun and Finney 1991). From in vitro experiments with octamer-containing promoter constructs, it has even been suggested that Oct-1 and Oct-2 are interchangeable (LeBowitz et al. 1989; Pierani et al. 1990; Kemler et al. 1991). Such experiments argue against the hypothesis that Oct-2 plays a unique role in B cells. Clearly, there are many unanswered questions regarding the relationship of Oct-2 to B-cell-specific gene expression.

The large ensemble of factors regulating immunoglobulin gene expression includes both B-cell-restricted and ubiquitous proteins (see Staudt and Lenardo 1991). Evidence for the function of these proteins comes primarily from experiments using cultured cells or cell-free systems. Although such approaches provide a valuable starting point, they cannot fully define the role an individual protein plays during the course of B-cell development in vivo. Isolated cells cannot represent the range of physiological states encountered in the body, and B-cell lines cannot perform many of the differentiation steps taken by B cells in vivo. A more definitive approach to establishing the function of a gene is through creation of a null mutant (Thomas and Capecchi 1987). We have used gene targeting in embryonic stem (ES) cells to generate ES cell lines that are heterozygous for a mutant *oct-2* gene. Unexpectedly, homozygous mutant mice derived from such a cell line die within hours of birth, although the cause of death has not been determined. B-cell differentiation in these young mutant animals appears to proceed normally to the stage of the virgin B cell, indicating that Oct-2 is dispensable during the antigen-independent phase of development. However, mutant B cells have difficulty developing past this stage, and stimulation of these cells in vitro reveals a severe functional defect. This implies that the Oct-2 transcription factor plays an essential role late in B-cell differentiation, when cells increase immunoglobulin expression in response to external stimuli.

Results

Targeting the oct-2 *gene*

Oct-2 binds to DNA through a bipartite-binding domain (Fig. 1A), comprising POU-specific and POU homeo domains; both are required for specific high-affinity binding to the octamer motif (Sturm and Herr 1988; Verrijzer et al. 1990). The *oct-2* targeting vector (see Materials and methods; Fig. 1B) was designed to introduce a nonsense mutation into the middle of the POU-specific domain of the protein (Fig. 1A). Any Oct-2 derivative expressed from the mutated gene would not be expected to bind to DNA, as it would lack the entire carboxy-terminal half of the protein, including the homeo box. After transfection of D3 ES cells with the targeting vector, G418-resistant (or G418 and gancyclovir doubly-resistant) clones were screened directly by Southern analysis. Clones bearing the desired mutation would contain a smaller *Xba*I fragment, by virtue of the *Xba*I linker carrying the nonsense mutation (Fig. 2A). Overall, ~1 in 14 G418-resistant clones resulted from a homologous event (Table 1). Several clones, both G418 resistant and doubly

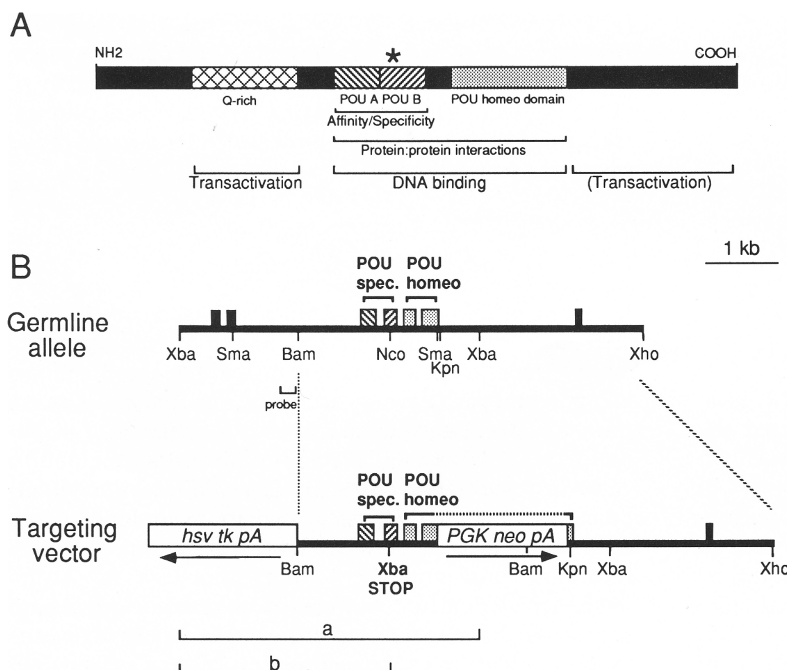

Figure 1. Oct-2 protein, gene, and targeting vector structures. (*A*) Diagrammatic representation of the Oct-2 protein, with functional domains highlighted. The asterisk (*) marks the position in the protein sequence where the nonsense mutation would occur in the mutated allele. (*B*) (*Top*) The structure of the wild-type murine *oct-2* gene in the region encoding the POU-specific and POU homeo domains (shading indicates corresponding regions in the gene and protein). Our unpublished data on the organization and sequence of the mouse gene agree with those published by Hatzopoulos et al. (1990). The targeting vector (*bottom*) contains the two selectable markers shown and an *Xba* linker containing multiple stop codons (*Xba* STOP) at the *oct-2* *Nco*I site. (Probe) The fragment used in Southerns blots shown in Fig. 2, a *Stu*I–*Bam*HI genomic fragment. a and b delineate fragments expected upon *Xba*I digestion of the germ line and targeted *oct-2* alleles, 4.6 and 3.4 kb, respectively. Arrows indicate transcriptional orientation of selectable marker genes.

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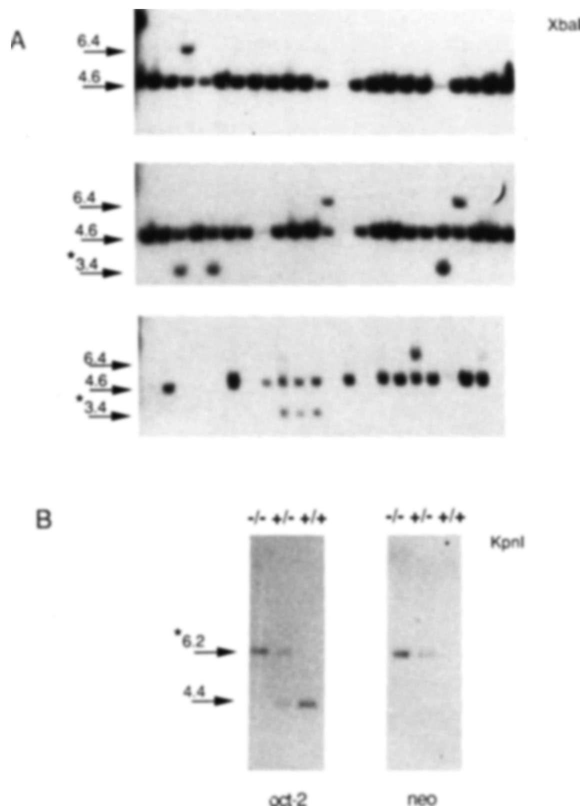

Figure 2. Southern analysis of selected ES cell clones to detect homologous recombination at the *oct-2* locus. (*A*) *Xba*I-digested DNA hybridized with the *oct-2* probe in Fig. 1B. Potential products of homologous recombination depend on the position of the 5′ crossover point with respect to the *Xba*I/nonsense linker. If recombination occurred 5′ to the *Xba*I linker, a 3.4-kb *Xba*I fragment would result. If it occurred 3′ to the linker, a 6.4-kb *Xba*I fragment would be generated. Approximately 1 in 20 clones contained both the *Xba*I/nonsense linker and *neo* (and thus yielded the 3.4-kb *Xba*I fragment), as the crossover had occurred in the 1.8-kb upstream of the *Xba*I linker mutation (see Fig. 1B). Those clones in which the crossover point fell between the *Xba*I linker and the *neo* gene were identified by virtue of the 6.4-kb *Xba*I fragment . The same fragment also hybridized to a *neo* probe (data not shown). This latter set of clones was excluded, as they had not incorporated the desired *oct-2* mutation. (*B*) Southern analysis of *Kpn*I-digested DNA from tails of wild-type, heterozygous, and *oct-2* mutant mice probed with the *oct-2* (Fig. 1B) or *neo* probes. The asterisk (*) marks the position of the mutated allele.

resistant, were chosen for injection into C57BL/6 blastocysts. Mice derived from one clone transmitted the *oct-2* mutation to their progeny; the mutation was carried by one-half of the offspring (38 −/+ and 41 +/+ of those analyzed). These F1 animals were healthy and subsequently proved to be fertile. Mice were subjected to further Southern analysis using *Kpn*I, to ensure that they carried a mutated *oct-2* allele with the correct structure, and only one copy of the *neo* gene. As *Kpn*I sites flank the *neo* insertion in the mutant allele, the larger *Kpn*I fragment detected by both the *oct-2* and *neo* probes corresponds to the mutant *oct-2* allele (Fig. 2B).

Oct-2 deficiency causes neonatal death

When animals heterozygous for the *oct-2* mutation were mated, genotypic analysis of all progeny in the first few litters revealed a ratio of *oct-2* genotypes close to the expected 1 : 2 : 1 (Table 2), indicating that the *oct-2* mutation was inherited in a Mendelian fashion and that most animals survived to birth, regardless of genotype. However, within a few hours of birth, some animals became lethargic, cyanosed and dehydrated, and finally died. Genotyping confirmed that the dying pups were *oct-2* nullizygotes (Table 2). No *oct-2*$^{-/-}$ mouse has survived for more than a few hours. Live-born *oct-2*$^{-/-}$ animals were distinguishable from their littermates only by the absence of milk in the stomach, because they do not suckle. However, starvation is unlikely to be the cause of death at such an early time, because normal mice delivered by Caesarian section in our animal facility have survived for 24 hr or more without feeding. Histologic examination did not reveal any indication of infection or of anatomical abnormality: The lungs were pink and inflated; the heart, spleen, liver and thymus were normal in size, color, and histologic appearance; and the kidneys were functioning, as indicated by full bladders. Because *oct-2* is expressed in the developing nervous system (Hatzopoulos et al. 1990; Stoykova et al. 1992), we paid particular attention to the brain and although we cannot rule out a physiological lesion, no structural defect was evident.

That neonatal lethality is the consequence of the *oct-2* mutation, and not some other undetected mutation, is supported both by the presence of a single copy of the targeting vector, integrated correctly at the *oct-2* locus (Fig. 2), and by the fact that lethality and homozygosity for the *oct-2* mutation have cosegregated without exception for more than six generations of breeding of many independent animals. Although the neonatal death of Oct-2-deficient animals remains to be explained, it attests to a late developmental role for Oct-2 outside of the immune system.

Early B-cell development is normal in oct-2$^{-/-}$ *mice*

Immunoglobulin genes are rearranged and transcribed Despite the neonatal lethality accompanying *oct-2*$^{-/-}$ deficiency, a significant part of the B-cell developmental pathway was accessible for analysis. During mid- to late gestation, the liver is a major site of hematopoiesis (Owen et al 1977). The immunoglobulin genes become transcriptionally active in fetal liver B-cell precursors before the onset of rearrangement and are then rearranged in a characteristic temporal sequence into functional antibody-coding units (see Alt et al. 1987; Schatz et al. 1992). These gene rearrangements are specific to lymphocytes, and the type of rearrangement present reflects the state of differentiation of a given cell.

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Table 1. *Frequency of homologous recombinants*

Experiment	Colonies screened		Number of homologous recombinants	Frequency	
	G418[R]	G418 + GANC[R]		G418[R]	G418 + GANC[R]
1	—	130	26 (18 + 8)[a]	—	1 in 5
2	95	—	5 (1 + 4)[b]	1 in 19	—

[a]Eighteen colonies had the desired double mutation, and 8 contained only the *neo* gene.
[b]One colony had the desired double mutation, and 4 contained only the *neo* gene (see text).

If Oct-2 were a regulator of germ-line immunoglobulin gene transcription through action at promoter or enhancer octamer sites and transcription were a prerequisite for gene rearrangement, as has been proposed (Alt et al. 1987), then mutant animals might show a defect in one or more of the rearrangement steps, potentially blocking differentiation. Immunoglobulin gene rearrangements in fetal liver cells were measured using a sensitive polymerase chain reaction (PCR) assay (Schlissel et al. 1991a). The assay detects the products of immunoglobulin gene rearrangements, with the size of each product reflecting the J_H element used in the rearrangement. The results in Figure 3 show that B-cell precursors of *oct-2* mutant mice were able to perform *D–J* and *V–DJ* rearrangements of the heavy-chain and *V–J* rearrangements at the κ locus as well as their normal littermates, and therefore indicate that fetal liver pre-B cells in mutant animals can progress normally through these early differentiation steps. The abundance of rearranged products varied somewhat among the animals but did not correlate closely with *oct-2* genotype. Therefore, Oct-2 binding is not a prerequisite for immunoglobulin gene rearrangement.

Fetal liver pre-B cells are susceptible to transformation by Abelson murine leukemia virus (A-MuLV) (Rosenberg et al. 1975). A-MuLV-transformed cell lines could be derived from day-16 fetal liver of both mutant and normal animals with similar efficiency (data not shown), indicating that A-MuLV targets occur in *oct-2*$^{-/-}$ animals at approximately normal frequency. These cell lines were used as a source of pure B-lineage cells to confirm that the *oct-2* mutation described in Figure1B was a true null mutation. Northern blot analysis was not sensitive enough to accurately measure *oct-2* mRNA levels in these early cells (data not shown). Other more sensitive methods of RNA detection, such as RNase protection or PCR, would only reveal transcription of a region of the gene and not whether stable, full-length RNA was present. Furthermore, the influence of the introduced phosphoglycerokinase (PGK) promoter and polyadenylation signals on *oct-2* transcription might make interpretation difficult. Therefore, Oct-2 protein levels were examined using a sensitive Western blotting assay. Nuclear proteins extracted from A-MuLV-transformed cells from wild-type and mutant animals were compared with similarly prepared (Schreiber et al. 1989) proteins from the mouse B-cell line, WEHI-231 (Gutman et al. 1981), using an anti-Oct-2 antiserum. This serum was raised against full-length, bacterially synthesized Oct-2 protein (Wirth et al. 1991). Whereas the cross-reactive Oct-1 protein was visible in extracts from both normal and mutant cells, Oct-2 was not detected in the mutant cells although it was readily detected in normal cells (Fig. 4). Furthermore, the antiserum did not detect any smaller

Table 2. *Genotypes of F2 animals*

Genotype	All animals[a] (%)	Neonatal deaths	Survivors
−/−	22 (20)[b]	16	0
−/+	63 (58)	1	42
+/+	24 (22)	0	18

[a](F2) Heterozygous females × heterozygous males, n = 109.
[b]Cannibalism of dead or dying pups by the mother reduced this frequency slightly.

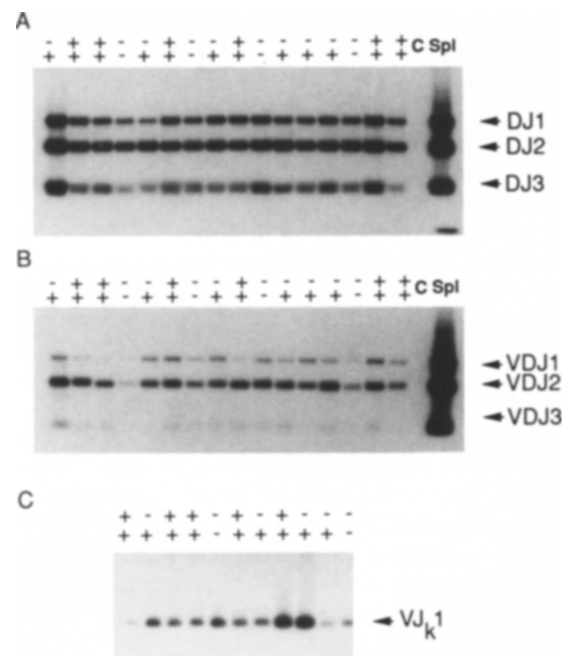

Figure 3. Immunoglobulin gene rearrangements in day 16 fetal liver cells. The reactions were performed and analyzed exactly as described previously (Schlissel and Baltimore 1989; Schlissel et al. 1991a) The *oct-2* genotype of each fetus is indicated. (*C*) Control reactions lacking DNA; (Spl) adult spleen control.

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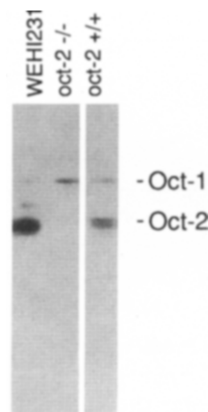

Figure 4. Oct-2 protein expression in mutant and control pre-B cell lines. Western blot of nuclear proteins from the mouse B-cell line WEHI-231 and from pools of A-MuLV-transformed fetal liver from Oct-2-deficient and wild-type animals. Coomassie staining indicated that approximately one-fifth as much protein was present in the WEHI-231 lane, compared with the A-MuLV lines (data not shown). The blot was reacted with rabbit serum (1 : 1000) raised against nearly full-length, bacterially synthesized Oct-2 (Wirth et al. 1991) and developed by enhanced chemiluminescence (ECL, Amersham). Both Oct-1 and Oct-2 are recognized by this antiserum.

Oct-2 derivatives in mutant cells, indicating that no stable protein is produced from the mutated allele.

To examine the effect of loss of Oct-2 on gene expression, transcription rates of genes characteristically expressed in pre-B cells were measured. These included genes for the immunoglobulin heavy and κ light-chains, for VpreB and λ5, the surrogate light-chains of pre-B cells, for the recombination-activating genes, RAG-1 and RAG-2, and for the immunoglobulin-associated chains, mb-1 and B29. Within this group are genes like B29 and the immunoglobulin genes, thought to be dependent on Oct-2 for their expression (Hermanson et al. 1989). Nuclear run-on assays measured transcription rates in cloned A-MuLV-transformed lines from control and mutant mice and also examined the effect of *Escherichia coli* lipopolysaccharide (LPS) treatment on the rate of transcription. Six individual pre-B clones (4 mutant and 2 wild-type) were examined, and typical results are shown in Figure 5. The genotypes of the mutant and wild-type cells were confirmed by Southern blotting to rule out contamination by wild-type cells (data not shown). Transcription rates of the genes examined were generally unaltered in the *oct-2* null clones. Clearly, Oct-2 is not required for the expression of immunoglobulin genes, or the other B-cell-specific genes tested, at the pre-B-cell stage of development.

Fetal pre-B- and B-cell numbers and surface phenotypes are not perturbed Maturing B-lineage cells in normal and mutant animals were quantitated. First, surface markers on cells from day 16/17 fetal liver were analyzed. The B220 molecule is present on very early B-cell precursors and persists through most of B-cell differentiation (Coffman and Weissman 1981). The μ chain appears later on the surface of B cells after productive rearrangement of a heavy-chain gene and association with a light chain. The results were the same for two independent litters analyzed; *oct-2* nullizygotes could not be distinguished from their littermates. All animals had similar numbers of $B220^+\mu^+$ (0.1–0.6%) and $B220^+\mu^-$ cells (1–3%; Fig. 6A and data not shown). Thus, flow cytometric analysis indicated that pre-B- and B-cell numbers were not affected by the mutation.

The number of clonable pre-B cells in normal and mutant animals was then compared. The cells were enumerated through their ability to proliferate when cultured on a feeder layer of IL-7-producing cells (Cumano et al. 1990; Rolink et al. 1991a; see Materials and methods). Limiting numbers of live cells from newborn liver and spleen were plated on 3T3 cells stably expressing IL-7 (Rolink et al. 1991a). After 10 days, proliferation of cells resembling lymphocytes was scored by microscopic examination. Analysis of surface markers on cells at the end of the incubation period indicated that >98% of cells were $B220^+$ and $PB76^+$ (data not shown), as expected for pre-B cells (Strasser 1988; see Rolink and Melchers 1991). Regression analysis of the proliferation data yielded the frequencies shown in Figure 6B. Although some variation in early pre-B-cell frequencies was observed among the individuals, there was no significant difference between mutant and heterozygous or

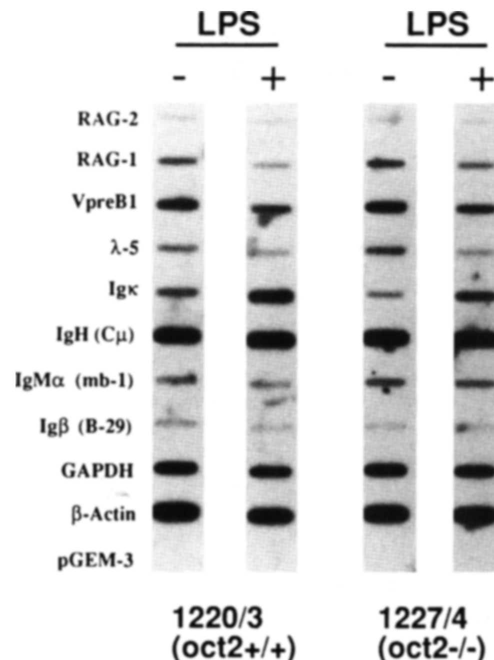

Figure 5. Transcriptional activity of genes in mutant and normal pre-B-cell lines. A-MuLV-transformed pre-B cells from an *oct-2* null animal (1227/4) and a control littermate (1220/3) were analyzed by nuclear run-on assay. Transcription rates of B-lineage-specific genes were measured in the absence (−) and presence (+) of LPS. pGEM-3 is a vector control.

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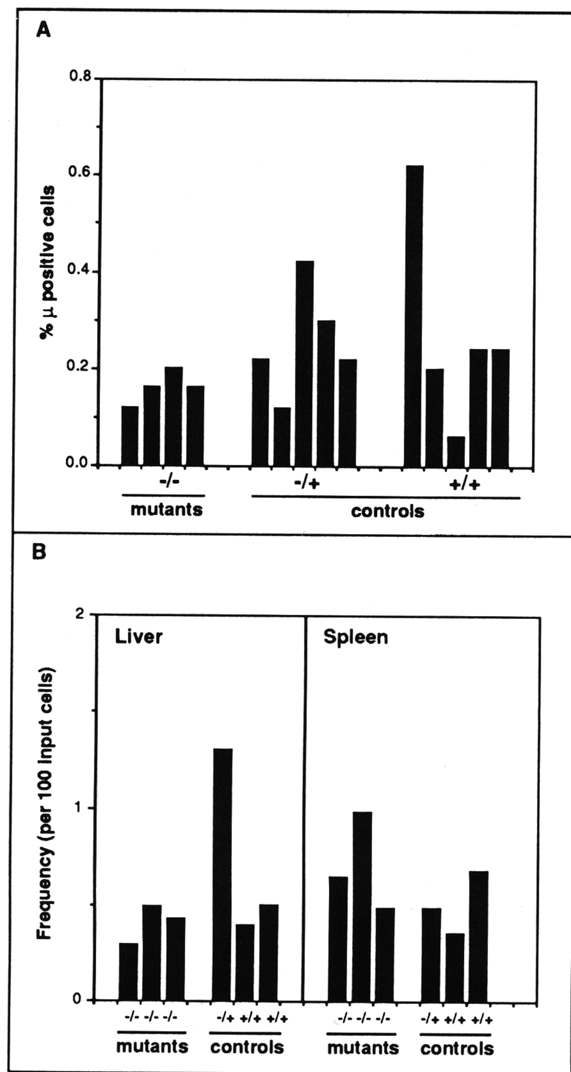

Figure 6. Enumeration of early B cells in fetal and newborn tissues. (*A*) B cells in fetal liver of mutants and controls. Values are percentages of B220$^+$, μ^+ cells in day 16 fetal liver. Each value is from an individual fetus; two litters were analyzed. Genotypes of controls are indicated. (*B*) Frequency of clonable pre-B cells from control and mutant mice. Values are frequencies of clonable pre-B cells/100 unfractionated newborn liver or spleen cells. Each value represents an individual animal, and all are littermates. Genotypes of control animals are indicated.

wild-type animals. Both liver and spleen showed a mean pre-B-cell frequency of ~1 in 170. Proliferative capacity of pre-B cells, as judged by the size of visible colonies, seemed unaffected by the *oct-2* mutation. The conclusion from this series of experiments is that Oct-2 is not required for the generation or proliferation of B-cell precursors or for the productive rearrangement or expression of immunoglobulin genes in developing B cells.

Mature B-cell numbers are marginally depressed in oct-2*-deficient neonates*

From mid-gestation to birth, the liver is the primary site of B-cell production, whereas the spleen is the site to which preformed B cells migrate for further development in response to antigenic and other signals. Because *oct-2* nullizygotes died at birth, the most mature B cells available were those in the neonatal liver and spleen. These tissues were taken from newborn mice shortly after delivery, when both mutant and control animals were viable and quite mobile. Flow cytometric analysis was performed to determine the proportion of B cells (B220$^+$, μ^+) present. Several additional strategies were included to ensure that the cells detected represented the true B-cell population in the organ examined (Materials and methods), because previous work had demonstrated that these cells represent a small minority of cells in such young animals (Nossal and Pike 1973).

B-cell numbers were compared for 22 animals from four litters, including 9 homozygous mutants, and representative data are shown in Figure 7. The flow cytometric data in Figure 7 show only the B220 and μ profiles; the μ^+ cells from both mutant and control livers

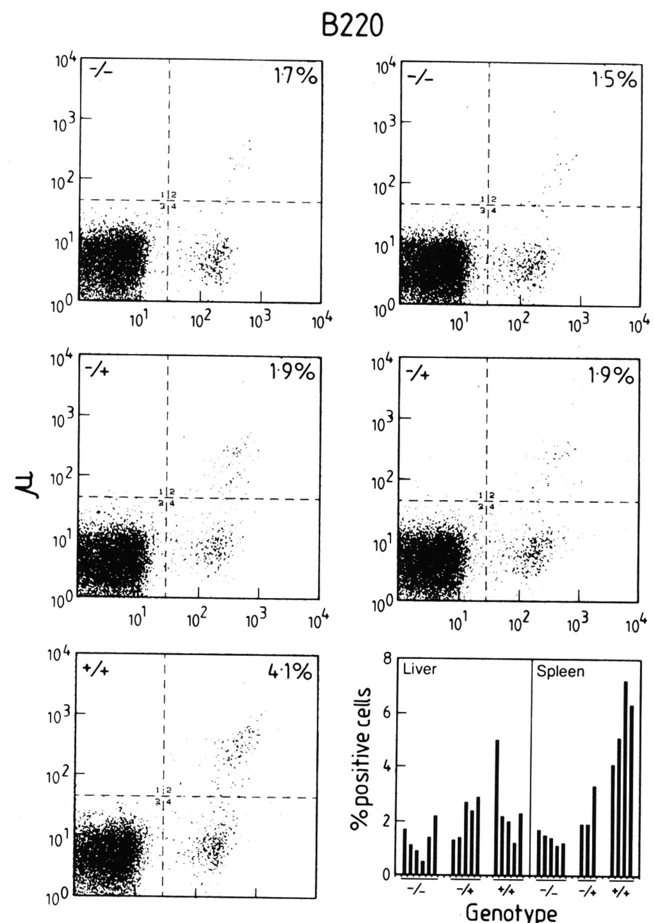

Figure 7. B220$^+$, μ^+ B cells in newborn liver and spleen. The percentage of B220$^+$, μ^+ cells is shown in each dot plot, along with the genotype of the animal. The flow cytometry plots are only shown for spleen, but the summary (*bottom right*) includes data from liver. Each value in the summary graph represents a separate animal.

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were also predominantly κ^+, BP-1$^+$, PB76$^+$, IgD$^-$, CD23$^-$, and class II$^-$ (data not shown). This surface phenotype classifies these cells as virgin or immature (IgM$^+$, D$^-$) B cells (see Rolink and Melchers 1991). By the time of birth, the numbers of B cells had risen significantly from their day 16/17 levels (in Fig. 6A). Whereas total nucleated cell numbers in neonatal liver and spleen were found to be equivalent for mutant and control animals (data not shown), hepatic B cells were, on average, slightly more abundant in +/+ animals than in mutants, with heterozygotes having an intermediate level. There was, however, considerable overlap in the range of B-cell numbers measured in liver for mice of each genotype (Fig. 7, bottom), and some litters showed little difference between mutants and controls. In contrast, there was a clear effect on the proportion of B cells in newborn spleen; wild-type animals averaged four times more IgM$^+$ cells than mutants. This first evidence of the phenotypic consequence of Oct-2 deficiency implies a differentiation or proliferation defect in the virgin B cell. The difference between liver and spleen may reflect the primary and secondary nature, respectively, of these two lymphoid organs.

A closer look at the B-lineage cells in mutant and normal spleens revealed not only that IgM$^+$ cells were more abundant in wild-type animals, but the ratio of pre-B to B cells was slightly altered by the *oct-2* mutation. The BP-1 molecule, present on pre-B and immature B cells (Cooper et al. 1986), is lost with further B-cell differentiation (Rolink and Melchers 1991). In spleens from control animals, mature B cells (μ^+, BP-1$^-$) predominated over pre-B cells (μ^-, BP-1$^+$) (Fig. 8). However, in *oct-2$^{-/-}$* animals, the balance was skewed toward the less mature (μ^-, BP-1$^+$) cells, indicating either a block or a delay in the differentiation of mutant B cells at this late stage. This may account, in part, for the depression of IgM$^+$ B-cell numbers in spleens of *oct-2* nullizygotes. The distribution of PB-76, CD23, and class II markers on IgM$^+$ cells was not affected by the mutation (data not shown).

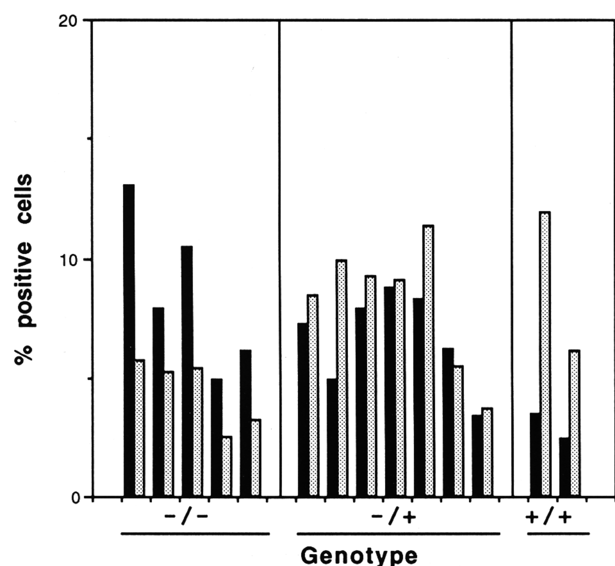

Figure 8. Pre-B and B cells in newborn spleen. The relative proportions of pre-B (BP-1$^+$, μ^-; solid bars) and B (BP-1$^-$, μ^+; strippled bars) cells in spleens of individuals from two independent litters are shown, with corresponding *oct-2* genotype.

Oct-2 mutation disrupts activation and antibody secretion by mature B cells

When B cells are stimulated in vitro by the mitogen LPS, in the presence of 3T3 filler cells and optimal concentrations of IL-2, IL-4, and IL-5, clones of antibody-forming cells are induced that first secrete IgM and then later frequently switch to secretion of IgG1 and/or IgE (McHeyzer-Williams 1989). Cells from lymphoid organs of newborn *oct-2$^{-/-}$* animals and normal littermates were stimulated under such culture conditions (Materials and methods) to measure their capacity to differentiate to activated IgM-secreting cells and to switch to IgG1 secretion. A dilution series of viable cells from individual livers and spleens was plated and at the end of the 7- to 8-day culture period microscopic inspection indicated that all samples had a similar proportion of wells containing typical clusters of lymphocytes. IgM and IgG1 levels were measured in all culture supernatants at the end of the culture period, and the frequency of antibody-forming clones (AFC) was determined. A comparison of these frequencies (Fig. 9A,B) revealed a dramatic effect of Oct-2 deficiency; the livers of mutant animals contained 20-fold fewer cells capable of secreting antibody of either the IgM or IgG1 isotype, compared with homozygous wild-type animals. In the spleen, the average deficit was 100-fold for IgG1 and nearly 200-fold for IgM producers, respectively. Once again, heterozygous animals tended to have intermediate values. Furthermore, the clones from mutant animals that could secrete immunoglobulin did so to a much lesser extent. Figure 9C summarizes data obtained by measuring the amount of immunoglobulin secreted by individual clones (Materials and methods). Liver-derived clones from *oct-2* mutants secreted, on average, 16- and 10-fold less IgM and IgG1, respectively, than wild type, whereas B cells from mutant spleen were 55- and 70-fold less productive (for IgM and IgG1, respectively) than +/+ controls. Immunoglobulin production by heterozygotes fell between the two extremes. If the antibody-secreting capacity of the whole organ is considered, then the compound effects of the paucity of antibody-secreting cells in mutant animals and the poor performance of those few functional cells present would translate into a significant immunodeficiency. These results predict that de novo immunoglobulin production by the two major B-lymphoid organs of the *oct-2$^{-/-}$* newborn would be severely depressed (200- to 320-fold for liver and 7000- to 10,000-fold for the spleen).

Discussion

Oct-2-deficient mice provide the first opportunity to de-

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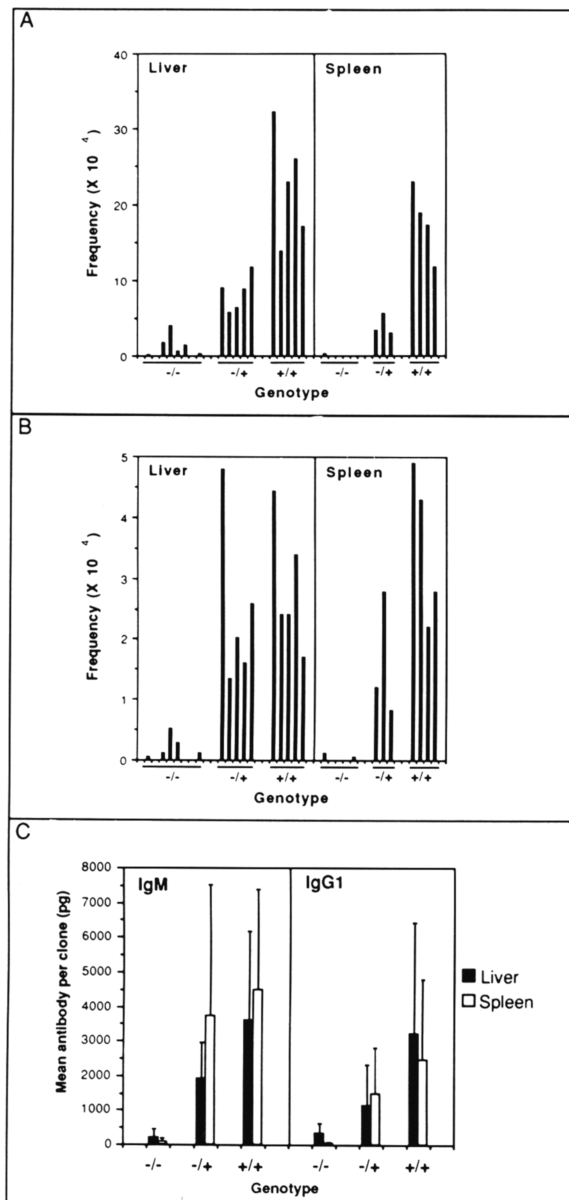

Figure 9. Immunoglobulin secretion by LPS-stimulated B cells. (*A*) Frequency of IgM-secreting clones induced by LPS treatment of unfractionated cells from neonatal liver and spleen. For liver, six mutant, five heterozygous, and five wild-type animals were analyzed. The spleens from a subset of these animals were also analyzed (5 −/−, 3 −/+, and 4 +/+. (*B*) Frequency of IgG1 secretion by the same cells analyzed in *A* above. (*C*) The amount of antibody secreted by antibody-secreting clones, pooled according to *oct-2* genotype (Materials and methods). (Solid bar) Liver; (open bar) spleen. The large error bars (standard deviation) reflect the small number of pooled samples and the fact that immunoglobulin secretion varied dramatically among pools. However, the *oct-2*$^{-/-}$ cells always had the lowest average level of antibody production.

termine the role of Oct-2 in the development of the B cell. Previous studies with cell lines have failed to establish a definitive role for Oct-2 in immunoglobulin gene expression. On the basis of such experiments, it could be argued that Oct-2 is required for the expression of any immunoglobulin gene or that it makes only a modest contribution to that expression. Furthermore, the early notion of a specialized function for Oct-2 in B cells had been challenged by evidence for functional overlap between Oct-1 and Oct-2. The experiments reported here show that although the *oct-2* gene is expressed early in the B-cell lineage, it has no recognizable function in the early fetal B lymphocyte but is clearly needed for the later functional development of the B cell. The *oct-2* gene product is also critical for viability of the newborn mouse, but the organ system or cell type in which it is required could not be identified.

Oct-2 is dispensable during early B-cell development

Despite the fact that *oct-2* is expressed at very early stages of the B-cell lineage (Schlissel et al. 1991a), this work indicates that the protein is not required for the early transcription of immunoglobulin genes (Fig. 5) or for immunoglobulin gene rearrangements in pre-B cells (Fig. 3). Furthermore, A.L. Feldhaus, C.A. King, K.L. Arvin, and H. Singh (pers. comm.) have largely eliminated expression of the *oct-2* gene by homologous recombination in WEHI-231 B-lymphoma cells, and have observed no diminution of immunoglobulin gene transcription in such cells, in agreement with our observation that lymphoid cells can mature to the B-cell stage without Oct-2. Therefore, other transcriptional activators that act at sites in the immunoglobulin promoters and enhancers, such as the helix–loop–helix proteins that bind to the so-called E boxes (Eaton and Calame 1987) or the factors that bind to the μB or π boxes (Libermann et al. 1990; Nelsen et al. 1990), must provide a level of transcription that is adequate to achieve early steps of B-cell differentiation. Overexpression of the E47 protein in T cells activates germ-line transcription and *D*-to-*J* rearrangement of the immunoglobulin heavy chain (Schlissel et al. 1991b). Another obvious candidate for a factor that might complement an Oct-2 deficiency in early B cells is the ubiquitously expressed Oct-1. In developing B cells that lack Oct-2, Oct-1 might participate in immunoglobulin expression through action at the octamer motif, as has been demonstrated in vitro (Pierani et al. 1990). However, the work described here shows clearly that Oct-1 cannot adequately complement an Oct-2 deficiency in mature B cells nor in the system that depends on Oct-2 for animal survival, indicating unique biochemical roles for Oct-2.

Maturation defect in oct-2*-deficient B cells*

The first evidence of a B-cell developmental defect in Oct-2-deficient mice was a deficit in splenic B cells at the time of birth, due in part to the slightly impaired maturation capacity of these cells. Because the animals died postnatally, other events in B-cell differentiation had to be followed in vitro. These studies uncovered a significant developmental arrest. Although LPS could in-

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duce normal B cells to develop into immunoglobulin-secreting cells, the *oct-2*$^{-/-}$ cells responded poorly. Few were induced to secrete immunoglobulin, and these secreted very little. Surprisingly, even heterozygotes showed a deficiency in development to immunoglobulin secretors, implying that Oct-2 is a limiting factor in this maturation step. In the few antibody-producing clones derived from mutant cells, the switch to IgG1 synthesis occurred with normal efficiency, despite the presence of several potential Oct-2-binding sites in the γ1 switch region (Schultz et al. 1991).

LPS induces a pleiotropic response in B-lineage cells, involving functional activation, proliferation, and differentiation (e.g., see Anderson et al. 1977). Whereas antibody levels in culture supernatants could readily be measured in the in vitro activation experiment, the small numbers of cells involved and their failure to survive the assay have not allowed us to characterize the cells after LPS treatment. It therefore remains a possibility that Oct-2 affects the LPS-induced proliferation of B cells, rather than the rate of antibody secretion, and detailed frequency analysis of clonal proliferation is under study. It should be noted that whereas blastogenesis, clonal proliferation, and differentiation to antibody-secreting status are usually coupled events in B-cell physiology, little is known about the regulation of continued division versus terminal differentiation. There are even some instances where B cells can be induced to secrete antibody without prior cell division (Melchers et al. 1980).

LPS treatment induces dramatic changes in immunoglobulin gene expression (Yancopoulos and Alt 1985) and increases *oct-2* transcription (Staudt et al. 1986, 1988; Miller et al. 1991). LPS may therefore influence immunoglobulin gene activity, in part, through induction of *oct-2*, and *oct-2*$^{-/-}$ B cells may fail to secrete immunoglobulin in response to LPS because they cannot up-regulate Oct-2 production. In this scenario, binding sites in the V-gene promoter and the 3' IgH enhancer might be the critical regulatory elements at which Oct-2 would act, as they have been shown to contribute strongly to immunoglobulin gene expression in mature B cells in vivo (Jenuwein and Grosschedl 1991) and plasma cells in vitro (Dariavach et al. 1991).

Although the defect in immunoglobulin secretion may be the primary lesion in *oct-2*$^{-/-}$ B cells, maturation involves the action of many genes, any one of which may be affected by the mutation. For example, Oct-2 may, in an undefined way, participate in a late maturation step that makes an IgM^{+} B cell LPS responsive. Alternatively, the protein may influence signal transduction at the B-cell surface, through levels of the B29 molecule. B29 is an immunoglobulin-associated protein, analogous to a component of the CD3 signal-transducing complex of the T-cell receptor (Cambier and Campbell 1990), which is phosphorylated in response to surface immunoglobulin cross-linking (Gold et al. 1991). Transcription of the B29 gene is potentially regulated by Oct-2, through a perfect octamer motif in the B29 gene promoter (Hermanson et al. 1989).

Finally, it is important to remember, when considering the general role of Oct-2 in B-cell differentiation, that the B-cell populations of newborn mice and adults differ. Ly-1 B cells, which arise early in development, are regarded as a distinct lineage, with surface markers, antibody repertoires, and regeneration characteristics that distinguish them from the conventional B cells that predominate in the adult (Hardy and Hayakawa 1986; Herzenberg et al. 1986). The important effects of Oct-2 deficiency on B-cell maturation in newborns described here may differ from effects on adult B cells.

Effects on other hematopoietic cell lineages

It has been reported that B cell precursors can give rise to B cells or myeloid cells, depending on the lymphokines present (Cumano et al. 1992), and that Oct-2 is expressed in some myeloid cell lines (Staudt et al. 1988; Cockerill and Klinken 1990). Nevertheless, myeloid development was not disturbed in *oct-2* mutant animals; the number and morphology of colonies observed in agar culture of day 13 fetal liver were normal, and Oct-2 null macrophages generated in the presence of M-CSF were highly phagocytic (L.C. Metcalf and D. Metcalf, unpubl.).

Oct-2 is also expressed in some T-cell lines (Staudt et al. 1988; Cockerill and Klinken 1990; Schlissel et al. 1991a), and in primary T cells, where it is induced by antigenic stimulation (Kang et al. 1992). In preliminary experiments, newborn thymocytes from Oct-2-deficient animals appeared in normal numbers and with normal surface phenotypes. However, very few mature cells are present in the thymus at this age, so the behavior of mature T cells could not be assessed.

This study of B-cell development in the absence of a functional *oct-2* gene provides information about Oct-2 function at late stages of B-cell development and therefore distinguishes the actions of Oct-2 from the other transcription factors influencing immunoglobulin gene expression. By removing Oct-2 from consideration, essential elements controlling immunoglobulin gene expression in immature B cells should now be defined more easily. Oct-2-deficient B cells may also help to define the molecular events that occur when virgin B cells respond to environmental signals and differentiate to immunoglobulin-secreting cells. Finally, SCID (severe combined immunodeficiency) mice, which fail to make their own B or T lymphocytes, can be repopulated with Oct-2-deficient B cells. The phenotypically mature B cells in such repopulated animals display similar functional defects to their neonatal counterparts, both in vivo and upon activation in vitro. These animals may represent a new model of immunodeficiency, with the *oct-2* mutation imparting a kind of nonspecific anergy to mature B cells, in contrast to the early defects that derail B-cell development in mice lacking functional λ5, μM, RAG-1, or RAG-2 genes (Kitamura et al. 1991, 1992; Mombaerts et al. 1992; Shinkai et al. 1992).

Materials and methods

oct-2 *targeting vector*

The mouse *oct-2* gene was isolated from a genomic library from

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T-cell hybridoma 1.9.2 [a hybrid between cells from B10.A and AKR inbred mouse strains (Winoto et al. 1985)], using a human *oct-2* cDNA probe (Staudt et al. 1988; Clerc et al. 1988). In constructing the substrate for gene targeting, three changes were made to the *oct-2* gene (Fig. 1C). All were insertions; no *oct-2* sequences were replaced or deleted during the construction. The first was a nonsense mutation, in the form of an *Xba*I linker with stop codons in all frames, introduced into the *Nco*I site in the POU-specific domain. A cassette containing the *neo* gene (Mansour et al. 1988) was then inserted at a site just downstream of the homeo domain (Fig. 1C). As the *oct-2* gene is not appreciably transcribed in ES cells (RNase protection results not shown), we supplied the *neo* gene with the strong PGK promoter and polyadenylation signal (Tybulewicz et al. 1991). A thymidine kinase cassette was inserted at the 5′ end of the targeting substrate for enrichment by selection against random integration of DNA (Mansour et al. 1988). The resulting targeting vectors had 2.9 kb of *oct-2* sequences, 5′ of the *neo* insertion, and 3.0 kb of homology, 3′. The distance between the *Xba*I linker and the *neo* gene was 1.1 kb.

Transfection, selection, and screening for homologous recombinants

D3 ES cells (Gossler et al. 1986) were propagated on γ-irradiated (20 Gy) STO *neo* fibroblasts as described (Zijlstra et al. 1989). ES cells (8×10^6) were electroporated (400 V, 25 μF) in the presence of 20 μg of linearized targeting vector, and G418 (300 μg/ml), with or without gancyclovir (2 μM) selection was initiated 24–48 hr later. Individual clones were picked after 6–8 days of selection, and expanded, and separate aliquots were frozen and were processed for Southern analysis.

Nuclear run-on assay

Nuclear run-on assays were performed essentially as described (Linial et al. 1985; Spanopoulou et al. 1988). Briefly, cells were grown to a density of 5×10^5/ml in duplicate flasks. To one flask LPS was added to a final concentration of 10 μg/ml. Cells were harvested 24 hr later. Nuclei were isolated from $1–2 \times 10^8$ cells by lysing cells in buffer containing the nonionic detergent NP-40 and immediately frozen in liquid nitrogen. Thawed nuclei were incubated in the presence of [α-^{32}P]UTP, and RNA was purified. Equal amounts of radiolabeled RNA were hybridized to nitrocellulose filters to which various cDNAs had been blotted.

In vitro culture of pre-B cells

A dilution series of unfractionated, viable cells from newborn livers and spleens (from mutant, heterozygous or wild-type littermates) was plated on 96-well plates, in the presence of 2×10^4 3T3 cells stably expressing IL-7 (Rolink et al. 1991a). Thirty-two wells were plated for each dilution (1000, 100, and 10 cells). Cells were cultured in RPMI-1640 + 10% fetal calf serum supplemented with 50 μM 2-mercaptoethanol. After 10 days, wells were examined microscopically and scored for the presence of proliferating lymphoctyes.

Activation of mature B cells

A threefold dilution series of 5000–150 viable cells from newborn liver or spleen was plated on 96-well dishes (24 wells for each dilution) and cultured for 7–8 days in the presence of LPS, IL-2, IL-4, and IL-5, and 3T3 cells (5000 cells per well) as described (McHeyzer-Williams 1989). The lymphokines IL-2, IL-4, and IL-5, act as B-cell growth and differentiation factors, and IL-4 can promote maturation and heavy-chain class switching to the γ1 gene (Arai et al. 1990). 3T3 feeder cells provide additional support, potentially via IL-6 and steel factor (see Arai et al. 1990; Rolink et al. 1991b). At the end of this period, 25 μl of each culture supernatant was assayed for the presence of IgM or IgG1 by standard ELISA (Karvelas and Nossal 1992). Clones with an OD. > 0.1 were scored as positive. Levels of antibody production by individual clones were estimated by measuring antibody titers in pools of supernatants from positive clones of each *oct-2* genotype, from wells that were statistically unlikely to contain more than one clone of antibody-secreting cells.

Flow cytometry

Monoclonal antibodies RA3-6B2 [anti-CD45R(B220), R-phycoerythrin conjugated], and R6-60.2 (anti-IgM, fluorescein conjugated) were obtained from Pharmingen, and G10 (anti-BP-1, biotin conjugated) was a kind gift from Dr. Andeas Strasser (The Walter and Eliza Hall Institute, Melbourne, Australia). Cytometric analysis was performed with a FACScan (Becton Dickinson). Controls and parallel experiments were performed to ensure the specificity of labeling B-lineage cells, rather than cells capable of the passive acquisition of immunoglobulin. The controls included incorporation of unlabeled anti-Fcγ receptor monoclonal antibody 2.4G2 in staining steps and staining of equivalent samples with irrelevant, but species- and isotype-matched monoclonal antibodies. Finally, cells stained with FITC-labeled sheep antimouse antibody were incubated at 37°C for 30 min and examined by fluorescence microscopy. The stained cells had "capped" the fluorescent antibody, a behavior characteristic of B cells. The proportion of labeled, capped cells agreed well with the flow cytometric data (Fig. 7) on the same cell suspensions.

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Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival.

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