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Review

Monoallelic Expression of *Pax5*: A Paradigm for the Haploinsufficiency of Mammalian *Pax* Genes?

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It is generally assumed that most mammalian genes are transcribed from both alleles. Hence, the diploid state of the genome offers the advantage that a loss-of-function mutation in one allele can be compensated for by the remaining wild-type allele of the same gene. Indeed, the vast majority of human disease syndromes and engineered mutations in the mouse genome are recessive, indicating that recessiveness is the 'default' state. However, a minority of genes are semi-dominant, as heterozygous loss-of-function mutation in these genes leads to phenotypic abnormalities. This condition, known as haploinsufficiency, has been described for five of the nine mammalian *Pax* genes, which are associated with mouse developmental mutants and human disease syndromes. Recently we have reported that the *Pax5* gene is subject to allele-specific regulation during B cell development. *Pax5* is predominantly transcribed from only one of its two alleles in early B-lymphoid progenitors and mature B cells, while it transiently switches to a biallelic mode of transcription in pre-B and immature B cells. As a consequence, B-lymphoid tissues are mosaic with regard to the transcribed allele, and heterozygous mutation of *Pax5* therefore results in deletion of B lymphocytes expressing only the mutant allele. The allele-specific regulation of *Pax5* raises the intriguing possibility that monoallelic expression may also be the mechanism causing the haploinsufficiency of other *Pax* genes. In this review, we discuss different models accounting for the haploinsufficiency of mammalian *Pax* genes, provide further evidence in support of the allele-specific regulation of *Pax5* and discuss the implication of these findings in the context of the recent literature describing the stochastic and monoallelic activation of other hematopoietic genes.

Key words: B cell development / Haploinsufficiency / Monoallelic expression / Pax5 / Single-cell RT-PCR.

Models to Account for Haploinsufficiency

The genetic term haploinsufficiency describes a situation where a null mutation in one allele of a gene results in a mutant phenotype despite the presence of the second, wild-type allele. A haploinsufficient gene therefore fulfills its normal function only in the presence of both wild-type alleles, and hence its activity is highly sensitive to gene dosage.

Haploinsufficiency is a relatively rare genetic phenomenon in mammals, as the majority of human disease syndromes and engineered mouse mutation are recessive (Fisher and Scambler, 1994). Nevertheless, a growing number of mammalian transcription factor genes have recently been reported to be haploinsufficient (Fisher and Scambler, 1994; Engelkamp and van Heyningen, 1996). While several explanations for haploinsufficiency have been suggested (Wilkie, 1994; Read, 1995), we will discuss below only the three most likely models.

The Protein-Protein Interaction Model

Transcription factors commonly depend on the interaction with partner proteins to fulfill their functions in gene activation and repression. Both positive and negative control by interacting proteins is frequently observed for transcription factors which bind DNA as heterodimeric complexes. This type of regulation is best illustrated by the family of transcription factors which interact with each other through the basic helix-loop-helix (bHLH) domain. As the function of these regulators is determined by the relative ratio of activating and inhibitory HLH proteins (Bain and Murre, 1998), it is conceivable that a two-fold change in the concentration of one partner protein could have a dramatic effect on the regulation of target genes within the cell. Indeed, several bHLH transcription factors including Sisterless-b (Sis-b) play an essential role in *Drosophila* sex determination where they cooperate to amplify a small quantitative (two-fold) difference in *sis* gene dose into a qualitative all-or-none effect on the transcription of the target gene *Sex-lethal* (Cline, 1993; Parkhurst and Meneely, 1994). A second example was revealed by targeted mutation of the murine *E2A* gene which demonstrated an essential role for this bHLH transcription factor in B cell development (Bain *et al*., 1994; Zhuang *et al*., 1994). The *E2A* mutation appears to be haploinsufficient, as 50% less B lymphocytes were detected in the fetal liver of heterozygotes compared to wild-type embryos (Zhuang *et al*., 1994). Moreover, increased expression of

the inhibitory HLH protein Id1 in the B-lymphoid lineage resulted in a phenotype resembling the *E2A* null mutation (Sun, 1994), indicating that B-lymphopoiesis is extremely sensitive to changes in the relative ratio of positively and negatively acting HLH proteins.

Binding Site Occupancy Model

This hypothesis is based on the assumption that the genome contains a large number of DNA-binding sites for a particular transcription factor which may itself be present only in limiting amounts within the cell. Under these conditions, only the binding sites of relatively high affinity will be occupied and hence potentially regulated by the transcription factor (Read, 1995). A small increase or decrease in protein concentration could consequently lead to a change in binding site occupancy and thus to an altered profile of target gene expression. Evidence in support of this model was obtained by analysis of the paired domain-containing transcription factor Pax6 (Schedl *et al*., 1996). *Pax6* is known to be haploinsufficient in mammals, as heterozygous mutations in this gene are responsible for the *Small eye* phenotype in mouse and for congenital eye abnormalitites known as aniridia and Peters' anomaly in humans (Hanson and van Heyningen, 1995). Surprisingly, a modest increase in *Pax6* expression due to the presence of additional copies of the wild-type *Pax6* locus generates a phenotype which is similar to that of the heterozygous *Small eye* mouse (Schedl *et al*., 1996). Hence, the transcription factor Pax6 is exquisitely dosage-sensitive, as it can control normal eye development only within a narrow concentration range.

The Monoallelic Expression Model

A third possible mechanism to account for haploinsufficiency is the monoallelic expression theory, which we will describe in detail in this review. According to this hypothesis, a given gene is transcribed from only one of its two alleles which are selected for expression on a purely stochastic basis. Consequently, the expressing tissue is mosaic with regard to transcription of this gene and can be subdivided into two cell populations, each transcribing either allele 1 or 2, respectively (Figure 1). Both cell populations can participate in normal development and differentiation, as long as the two alleles contain wild-type sequences. However, if one allele is mutated, one cell population lacks the function of the respective gene and consequently may not contribute to tissue formation, thus leading to developmental abnormalities (Figure 1).

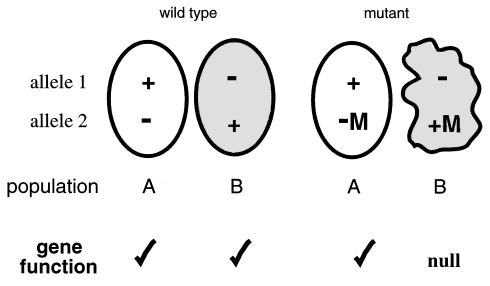


Fig. 1 Monoallelic Expression as a Possible Cause of Haploinsufficiency.
Transcriptionally active and silent wild-type alleles of a given cell are indicated by (+) and (–), respectively. M denotes an allele carrying a null mutation. The expressing tissue of a heterozygous individual is mosaic with regard to the transcription of a monoallelic gene, as it consists of a cell population A expressing the wild-type protein (√) and a population B lacking any functional protein (null).

Haploinsufficiency of Mammalian *Pax* Genes

The **pa**ired bo**x**-containing *(Pax)* genes constitute a highly conserved and evolutionary ancient family of transcriptional regulators (Noll, 1993). A hallmark of these transcription factors is the so-called paired domain (Bopp *et al*., 1986), which functions as a bipartite DNA-binding motif involved in target gene recognition (Czerny *et al*., 1993; Xu *et al*., 1995). The mammalian *Pax* family consists of nine different genes (Mansouri *et al*., 1996) and is of special interest to developmental biologists and clinical researchers alike, as natural mutations in five members cause developmental disorders in mouse and human (Table 1, and references therein). Although the spectrum of the observed genetic alterations is very broad, ranging from single base changes (resulting in amino acid substitutions and splice site mutations) to large deletions, loss of function is the common denominator of all these mutations. Hence, these developmental disorders arise as a result of haploinsufficiency (Strachan and Read, 1994; Hanson and van Heyningen, 1995). Heterozygous mutation of a given *Pax* gene is known to result in considerable phenotypic variability. These fluctuations do not correlate with specific mutations and are seen in the mouse even on a constant genetic background (Strachan and Read, 1994;

Table 1 Haploinsufficiency and Disease Association of Human and Mouse *Pax* Genes.

Gene	Human disease	Mouse mutant
Pax1	n.d.	*Undulated*
Pax2	Renal-coloboma syndrome	*Krd*
Pax3	Waardenburg syndrome	*Splotch*
Pax6	Aniridia Peters' anomaly	*Small eye*
Pax8	Hypothyroidism	n.d.

The indicated human disease syndromes and mouse developmental mutants are caused by heterozygous mutations in five different *Pax* genes (reviewed by Strachan and Read, 1994; Hanson and van Heyningen, 1995; Mansouri *et al*., 1996). References of the original publications: renal-coloboma syndrome (Sanyanusin *et al*., 1995), Waardenburg syndrome (Baldwin *et al*., 1992; Tassabehji *et al*., 1992), aniridia (Ton *et al*., 1991), Peters' anomaly (Hanson *et al*., 1994), congenital hypothyroidism (Macchia *et al*., 1998), *Undulated* (Wilm *et al*., 1998), *Krd* (**k**idney and **r**etinal **d**efects) (Keller *et al*., 1994), *Splotch* (Epstein *et al*., 1991), *Small eye* (Hill *et al*., 1991). n.d., not determined.

Hanson and van Heyningen, 1995). Interestingly, the haploinsufficient phenotype of a *Pax* gene is restricted to only a subset of its expression domains. For instance, heterozygous *Pax6* mutations cause malformations only in the eye, although this gene is widely expressed in the developing central nervous system, nose and pancreas in addition to the developing eye (Walther and Gruss, 1991; Callaerts *et al*., 1997). Hence, the haploinsufficient phenotypes are relatively mild compared to the homozygous null mutations which are lethal for most *Pax* genes analyzed in the mouse (Mansouri *et al*., 1996). Moreover, the function of Pax transcription factors appears to be highly dosage-sensitive as indicated by the fact that moderate overexpression of *Pax6* generates a similar phenotype as inactivation of one of the two *Pax6* alleles (Schedl *et al*., 1996). To date, the molecular mechanism responsible for this dosage sensitivity has not yet been elucidated. This phenomenon may be partly explained by the binding site occupancy model (see above; Schedl *et al*., 1996). However, no evidence for strongly interacting Pax partner proteins exists to date nor is dimerization required for DNA-binding of Pax proteins (Czerny *et al*., 1993). Hence, it seems unlikely that the function of Pax transcription factors is titrated within the cell by strong protein-protein interactions. Our recent data on the allele-specific regulation of *Pax5* (Nutt *et al*., 1999) raise, however, the possibility that monoallelic expression may be an important mechanism causing the haploinsufficiency of *Pax* genes.

The Heterozygous *Pax5* Mutant Mouse as a Model System to Test the Monoallelic Expression Theory

The *Pax5* gene coding for the B cell-specific activator protein (BSAP) is expressed throughout B cell development except in terminally differentiated plasma cells (Adams *et al*., 1992) (Figure 2A). Targeted inactivation of *Pax5* arrests B-lymphopoiesis at an early pro-B cell stage in the bone marrow of adult mice, while *Pax5* is already required for the generation of the earliest B-lineage committed precursor cells in the fetal liver (Urbánek *et al*., 1994; Nutt *et al*., 1997) (see Figure 2A). Pax5-deficient mice also display abnormal patterning of the midbrain and anterior cerebellum (Urbánek *et al*., 1994) consistent with expression of *Pax5* at the midbrain-hindbrain boundary during CNS development (Adams *et al*., 1992).

The identification of Pax5 target genes has been greatly facilitated by the fact that Pax5-deficient pro-B cells can be readily established as long-term *in vitro* cultures in the presence of stromal cells and interleukin-7 (Nutt *et al*., 1997). *CD19,* coding for a B cell surface protein, is a particularly interesting target gene, as its expression is entirely lost in Pax5-deficient pro-B cells (Nutt *et al*., 1997). *CD19* expression can, however, be fully reactivated in these cells by an estrogen-inducible version (BSAP-ER) of Pax5 (Nutt *et al*., 1998). Moreover, Pax5 is known to regulate the *CD19* gene by binding to a high-affinity site in the -30 promoter region (Kozmik *et al*., 1992). Hence, *CD19* was demonstrated by loss- and gain-of-function experiments to be a *bona fide* target gene whose expression is strictly dependent on Pax5 function.

A direct test of the monoallelic expression hypothesis depends on the possibility to follow the transcription of individual *Pax* gene alleles *in vivo*. Importantly, such a possibility was provided by the analysis of B lymphocytes from heterozygous *Pax5* (+/–) mice for the following reasons. First, as stated above, the expression of *CD19* is totally dependent on the presence of Pax5, indicating that CD19 is an ideal marker for Pax5 function (Nutt *et al*., 1997; 1998). Hence, only cells transcribing the wild-type *Pax5* allele express CD19 on the surface and can thus be detected as $CD19^+$ cells by anti-CD19 antibody staining and flow cytometric analysis (Figure 2B). Second, the inactivated *Pax5* allele contains an in-frame *lacZ* gene insertion, and hence its transcription leads to the synthesis of β-galactosidase, the activity of which can also be monitored by flow cytometric analysis using a fluorogenic substrate (Figure 2B). Analysis of the B cell compartments of heterozygous *Pax5* (+/–) fetal liver and bone marrow demonstrated that the majority of the pro-B cells express only the CD19 protein, but no β-galactosidase activity (Nutt *et al*., 1999) (Figure 2D). Hence, most heterozygous pro-B cells express exclusively the wild-type *Pax5* allele *in vivo*, indicating that *Pax5* is monoallelically transcribed at the onset of B lymphopoiesis. Monoallelic expression also predominates in mature B cells of the spleen and lymph node, while *Pax5* is transcribed from both alleles in pre-B and immature B cells (Nutt *et al*., 1999) (Figure 2C, D). The pattern of allelic *Pax5* transcription is therefore regulated during B cell development. This regulation is furthermore independent of the parental origin of the two *Pax5* alleles (Nutt *et al*., 1999), indicating that the monoallelic expression of *Pax5* is not caused by genomic imprinting which normally restricts expression either to the maternal or paternal allele (Barlow, 1995).

The phenomenon of monoallelic *Pax5* expression was further investigated by establishing pro-B cell lines from heterozygous mice *in vitro*. Single-cell cloning experiments and time course analyses demonstrated that individual cell colonies were able to switch expression between alleles within two weeks (Nutt *et al*., 1999). Moreover, replication timing analyses by fluorescence *in situ* hybridization (FISH) demonstrated that both *Pax5* alleles are synchronously replicated during the S-phase in B cells of heterozygous mice (Nutt *et al*., 1999). In summary, the allele-specific regulation of *Pax5* is stochastic, reversible, independent of parental origin and does not correlate with asynchronous replication in contrast to the monoallelic expression of genomically imprinted genes (Barlow, 1995; Watanabe and Barlow, 1996). As predicted by the model shown in Figure 1, the monoallelic expression of *Pax5* generates a haploinsufficient phenotype at the cellular level in heterozygous *Pax5* (+/–) mice. Cells which express only the mutant *(lacZ)* allele are absent in the B-lymphoid lineage past the developmental block of the *Pax5* mutation

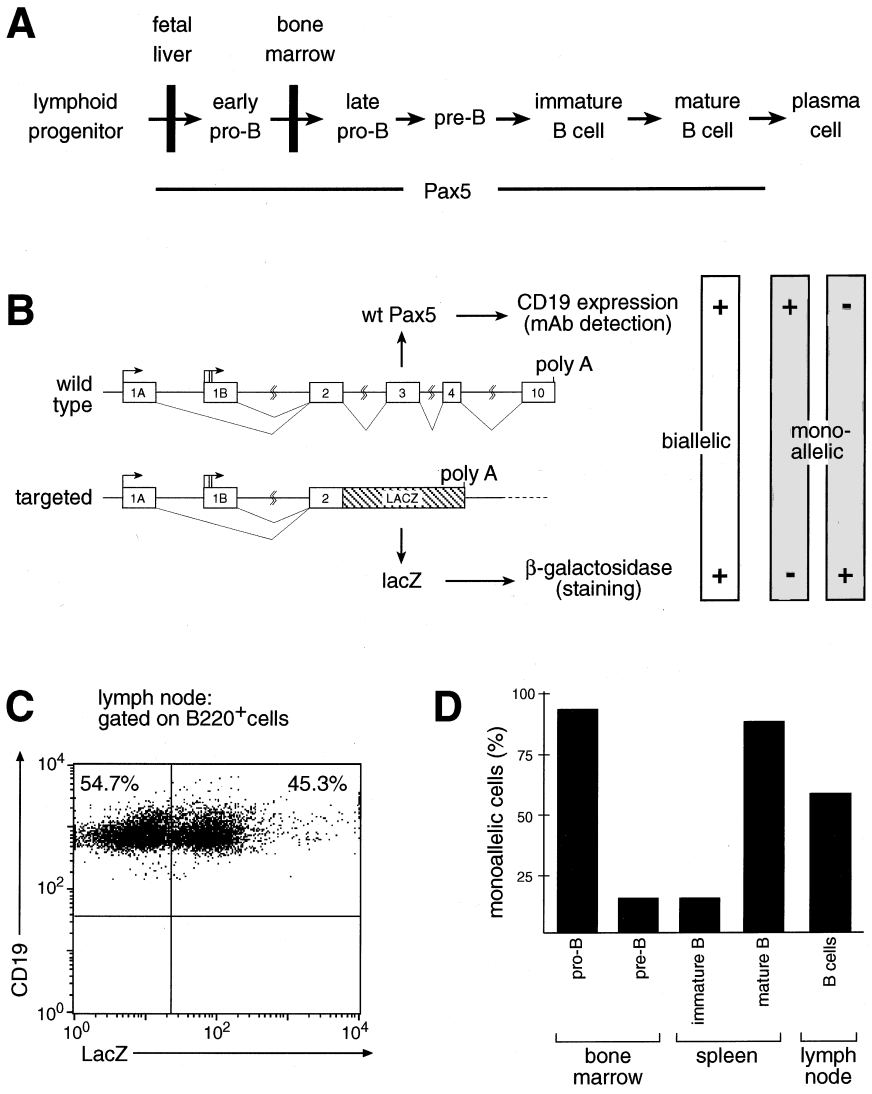


Fig. 2 Allele-Specific Regulation of *Pax5* in B Lymphocytes of Heterozygous *Pax5* (+/–) Mice.
(A) The different stages of B cell development are schematically shown together with the *Pax5* expression pattern and the early developmental block observed in fetal and adult B lymphopoiesis of *Pax5* (–/–) mice (Nutt *et al*., 1997). (B) Schematic diagram of the wild-type and disrupted *Pax5* alleles, explaining the experimental system used for demonstrating allele-specific regulation of *Pax5* in heterozygous mutant (+/–) mice. (C) Mono- and biallelically expressing B lymphocytes in the lymph node of *Pax5* (+/–) mice. Single-cell suspensions prepared from lymph nodes were analyzed by flow cytometry using anti-B220 and anti-CD19 antibodies in combination with the fluorogenic β-galactosidase substrate CMFDG as described (Nutt *et al*., 1999). $B220^+$ B-lymphocytes were gated, and the expression of CD19 and LacZ within this population is displayed. (D) The proportion of monoallelically expressing cells, as determined in the *Pax5* (+/–) mouse model (Nutt *et al*., 1999), is indicated for different stages of B cell development.

(Nutt *et al*., 1999) (Figure 2C). Interestingly, the *Pax5* (+/–) mouse has been ideal to prove the monoallelic expression theory, although it is phenotypically normal and does not provide a model for a human inherited disease. The main reasons for this apparent discrepancy are the functional redundancy of Pax2 and Pax5 in the developing midbrain (Urbánek *et al*., 1997; Schwarz *et al*., 1997) and the fact that a two-fold reduction in B cells is without phenotypic consequence. Hence, the human *PAX5* gene could not be linked to a primary immunodeficiency syndrome (Vorechovsky *et al*., 1995).

Testing the Monoallelic Expression Hypothesis in Genetically Unmanipulated B Cells ...

The evidence presented above is persuasive that the two *Pax5* alleles are independently regulated in B-lymphoid cells of heterozygous mutant mice. While the B lymphocytes of these mice all transcribe the wild-type *Pax5* allele, a variable number of B cells, depending on their developmental stage, also expresses the targeted allele (Figure 2D). These results can be interpreted in two ways. First, *Pax5* is regulated in an allele-specific manner during B cell development which is characterized by distinct phases of predominantly mono- or biallelic *Pax5* transcription (Nutt *et al*., 1999). Second, the transcriptional regulation of the

targeted *(lacZ)* allele has been adversely affected by insertion of the *lacZ/neomycin* expression cassette. In this case, the mosaic expression pattern of *Pax5* may simply reflect dysregulation of the targeted allele.

Evidence against the latter argument is provided by the expression analysis of *in vitro* cultured *Pax5* (+/–) pro-B cells (Nutt *et al*., 1999). We could clearly demonstrate that pro-B cells transcribing the wild-type *Pax5* allele *in vivo* could switch expression from the wild-type to the targeted allele upon *in vitro* culture, thus indicating that the targeted *lacZ* allele can be fully active in pro-B cells. Moreover, the wild-type *Pax5* allele lacking any genetic modification is transcriptionally silenced under these conditions, while this phenomenon was never observed with cultured pro-B cells from wild-type mice. Nevertheless, the insertion of a *neomycin* selection cassette upon gene targeting has been shown to affect the transcriptional control of several other genes in the mouse. An illustrative example is the disruption of the *myf6* gene, which codes for a bHLH transcription factor involved in myogenesis. Three different *myf6* null alleles have been reported which result in distinct phenotypes ranging from complete viability to lethality of the homozygotes (Olson *et al*., 1996). These phenotypic differences are caused by the specific orientation and position of the inserted *neomycin* gene which can interfere with *cis*-acting regulatory elements of the neighbouring myogenic gene *myf5* (Olson *et al*., 1996). Due to the large size of the *Pax5* locus (Busslinger *et al*., 1996), it is unlikely that the insertion of the *neomycin* gene affects the regulation of an adjacent gene which furthermore would have to be involved in the transcriptional control of *Pax5*. The *neomycin* gene insertion could, however, disrupt the regulation of the targeted *Pax5* locus itself. Indeed, different locus-specific effects were observed in lymphoid cells, whenever regulatory regions of the immunoglobulin *IgH* or *IgL* genes were inactivated either by insertion of a *neomycin* gene or by 'clean' deletion using the Cre-loxP system (Gorman *et al*., 1996).

To unequivocally prove the monoallelic expression hypothesis, it is therefore important to confirm the data obtained with the heterozygous mouse by analyzing *Pax5* expression in genetically unmanipulated B cells containing two equivalent wild-type *Pax5* alleles. Two techniques are currently available to detect the expression of wild-type alleles at the single-cell level. Below we will briefly describe the two methods and their results which provided further support for monallelic expression of *Pax5* in B lymphocytes.

… by RNA-FISH

The RNA-FISH method allows the detection of nascent primary transcripts at their site of synthesis in the nucleus. For this purpose, biotinylated DNA probes derived from introns of the gene of interest are hybridized to non-denatured nuclei, and the hybridization signal is subsequently amplified by incubation with fluorescently labelled avidin (Johnson *et al*., 1991; Panning and Jaenisch, 1996) (Figure 3A). As non-spliced pre-mRNA is primarily found at the site of RNA synthesis in the nucleus, each actively transcribing allele is revealed as a single signal (dot) by the use of intron-specific probes (Figure 3A). One dot per nucleus identifies a monoallelically transcribing cell, while two dots

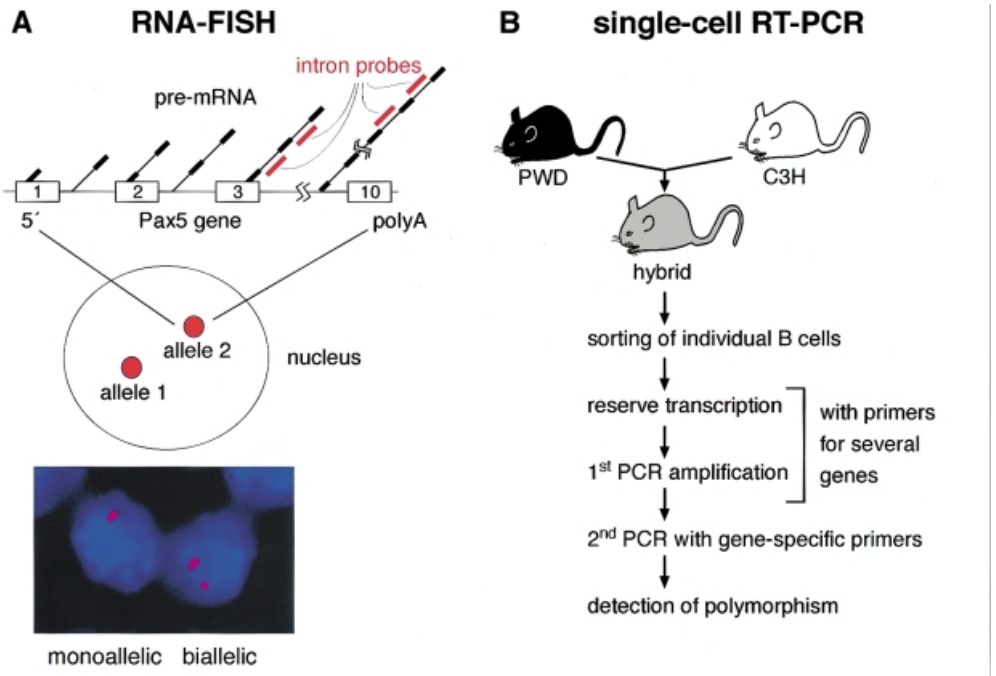


Fig. 3 Experimental Strategies for Demonstrating Monoallelic Expression in Single Cells.
(A) Schematic diagram of the RNA-FISH method with representative examples of quiescent splenocytes expressing *Pax5* mono- or biallelically. (B) Flow diagram of the single-cell RT-PCR technique used to demonstrate monoallelic expression of *Pax5* in wild-type B cells of the spleen.

are characteristic of biallelic transcription in a quiescent or G1 cell. This technique has been successfully used to visualize the transcriptional activity of globin genes in erythroid precursor cells (Wijgerde *et al*., 1995) and to follow the establishment of monoallelic transcription of the *Xist* gene during X chromosome inactivation (Panning and Jaenisch, 1996). We have recently adapted the RNA-FISH technique to study the transcription pattern of *Pax5* in B cells from the spleen of wild-type mice (Nutt *et al*., 1999).

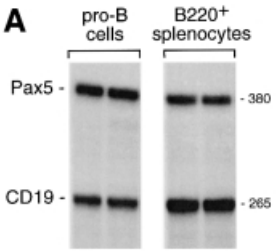
The *Pax5* mRNA is expressed at low abundance in splenic B cells (see below), and hence detection of primary *Pax5* transcripts by RNA-FISH is not trivial. Consequently, the RNA-FISH experiments had to be carefully controlled in order to avoid misinterpretation of the data. For instance, inefficient hybridization would inadvertently generate monoallelic expression data, as even potentially biallelic cells may appear monoallelic. To estimate the efficiency of hybridization, we determined the frequency of cells lacking any *Pax5* RNA signal, which ideally should be equivalent to the percentage of dead cells measured in the cell population at the onset of RNA analysis. The hybridization efficiency was thus assumed to be close to 100%, if every live cell gave rise to at least one RNA signal. Only the data of RNA-FISH experiments fulfilling this criterion were interpreted. To further assess the hybridization conditions, we performed the same RNA-FISH analysis with a second transcription factor gene, *Ikaros*, which is expressed in lymphocytes at a similarly low level as *Pax5* (Georgopoulos *et al*., 1992). Interestingly, the *Ikaros* probe detected 2-4 RNA signals at a high frequency (91%) in the nucleus, indicating that *Ikaros* is a biallelically transcribed gene. In contrast, monoallelic *Pax5* expression was observed in the majority (72%) of the same splenic B cells. A similar proportion (~75%) of monoallelically transcribing cells was determined by monitoring CD19 and lacZ expression in the heterozygous *Pax5* (+/–) spleen which normally contains fewer (~25%) immature B cells (biallelic) and more (~75%) mature B cells (monoallelic) (Nutt *et al*., 1999). In conclusion, the RNA-FISH experiments demonstrated that *Pax5* is subject to allele-specific regulation in genetically unmanipulated B cells.

... by Single-Cell RT-PCR Analysis

The expression of a given gene can also be studied at the single cell level by a modified two-step RT-PCR protocol (Hu *et al*., 1997) (Figure 3B). This technique can even be used to discriminate between the expression of individual polymorphic alleles in a single cell. Indeed, single-cell RT-PCR analyses have been successfully utilized to demonstrate monoallelic transcription of the odorant receptor genes in olfactory neurons (Chess *et al*., 1994) and of the *IL-2* and *IL-4* genes in T lymphocytes (Holländer *et al*., 1998; Bix and Locksley, 1998). We also employed this technique to investigate the allele-specific regulation of *Pax5* in wild-type B cells. Furthermore, we decided to analyze expression of *CD19* as a biallelic control gene in the same RT-PCR experiments. As shown by the RNase protection analysis in Figure 4A, the *Pax5* and *CD19* genes are expressed at similar levels in pro-B and mature B cells. Quantification of the RNase-protected signals revealed that a single splenic B cell contains as little as 30 and 80 molecules of *Pax5* and *CD19* mRNA, respectively (Figure 4B; see also legend). Hence it became apparent that the reverse transcriptase reaction had to be quite efficient to ensure the synthesis of several cDNA transcripts starting with so few mRNA templates in a single cell.

To be able to discriminate between transcribed alleles, we have searched for sequence polymorphisms in the *CD19* and *Pax5* mRNA of two related subspecies of the mouse, i.e. in the laboratory strain C3H and in the *Mus musculus* strain PWD (Forejt and Gregorová, 1992). cDNA cloning and sequence analysis revealed a cluster of changes in the 3′ non-coding region of *Pax5*, which were used to generate PWD- and C3H-specific oligonucleotide probes (Figure 5A). cDNA products amplified from B cells of the PWD and C3H strains could be readily distinguished by stringent hybridization with these probes (Figure 5B). The coding region of the *CD19* gene was furthermore shown to differ at position 665 by a C-to-T transition, which generated a *Bgl*II restriction site in the PWD sequence (Figure 5C). Hence, *Bgl*II digestion could be conveniently used to discriminate between the two allelic *CD19* transcripts that were amplified from B cells of F1 (C3H × PWD) hybrid mice (Figure 5D).

The two-step PCR protocol, which relies on the use of nested oligonucleotide primers (Figure 3B), is extremely sensitive, as it can detect a single cDNA molecule present in the initial RT reaction. Hence, a critical parameter of the single-cell RT-PCR experiment is the number of cDNA



B

	mRNA molecules / cell	
	Pax5	CD19
pro-B cell	30	30
splenic B cell	30	80

Fig. 4 Quantitation of the *Pax5* and *CD19* mRNA Molecules Present in a Single B Lymphocyte.

(A) RNase protection analysis. *CD19* and *Pax5* mRNA levels were quantitated by RNase protection assay in total RNA (10 μg) prepared from B220$^+$ sorted splenocytes and *in vitro* cultured pro-B cells. The size of the RNase-protected signal is indicated in nucleotides to the right. (B) Quantitation of the RNase protection data. The number of mRNA molecules per B lymphocyte was calculated from the known number of B cells required to obtain 10 μg of total RNA, the specific activity of each riboprobe and the radioactivity present in the RNase-protected signals (measured by phosphorimager analysis).

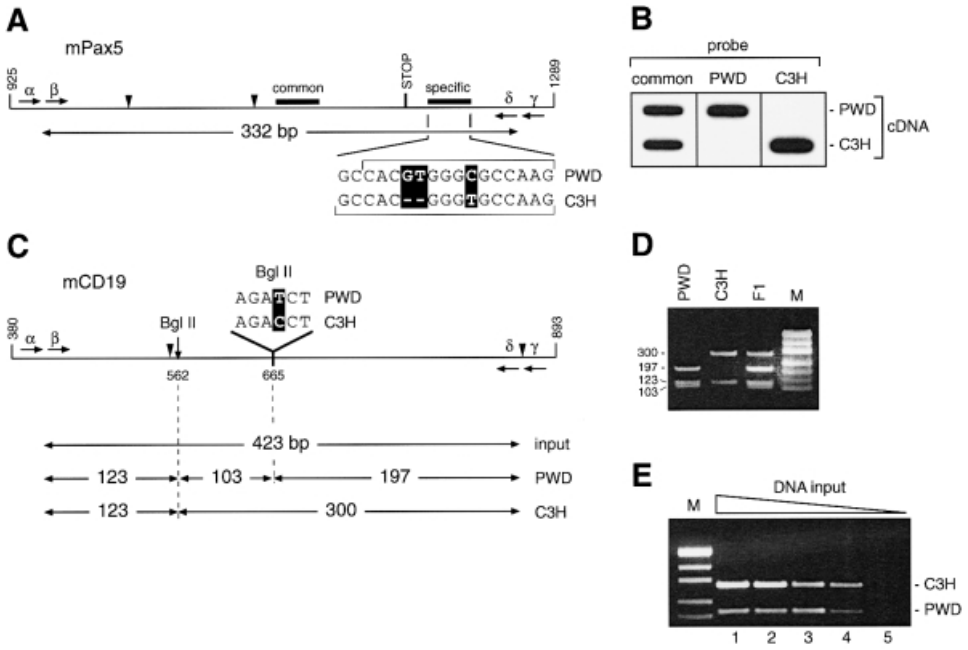


Fig. 5 Identification of Nucleotide Polymorphisms in *Pax5* and *CD19* mRNAs.
(A) Nucleotide polymorphisms were identified in the 3′ non-coding sequence of *Pax5* exon 10 by RT-PCR amplification and sequencing of a 332-bp cDNA fragment from the spleen of C3H and PWD mice. Nucleotide positions correspond to the published mouse *Pax5* cDNA sequence (Adams *et al*., 1992), and arrowheads denote the positions of introns. (B) The oligonucleotide probes shown by brackets in (A) were used for high-stringency slot-blot hybridization of *Pax5* PCR products which were amplified from B cells of PWD and C3H mice. (C) A polymorphic *Bgl*II restriction site in the coding region of *CD19* (Zhou *et al*., 1991) facilitates rapid determination of the allelic origin of *CD19* transcripts by *Bgl*II digestion. The sizes of the PCR product and its *Bgl*II restriction fragments are indicated in base pairs (bp). (D) *Bgl*II digestion of *CD19* PCR fragments amplified from the spleen of PWD, C3H and F1 (PWD × C3H) hybrid mice. (E) Unbiased PCR amplification of the polymorphic *CD19* transcripts. *CD19* cDNA (100 ng) derived from the PWD and C3H parental strains was mixed, serially diluted and then subjected to the 2-step PCR regimen described in the legend to Figure 6. PCR products were digested with *Bgl*II and analyzed on a 1.5% agarose gel.
Methods: F1 hybrid off-spring was obtained by intercrossing mice of the *Mus musculus* inbred strain PWD (Forejt and Gregorová, 1992) and the inbred laboratory strain C3H. The following oligonucleotides were used for single-cell RT-PCR analysis. mouse Pax5: (α) 5′-CCTACCCTATTGTCACAGGCC-3′; (β) 5′-GAGACTTGGCGAGCACAACC-3′; (γ) 5′-CCTCTGTCTGTCTCAGGGGGTT-3′; (δ) 5′-TCAGGGGTTGGGAGGCTGCC-3′. mouse CD19: (α) 5′-TGGAATGCTTCAGACGTCAGG-3′; (β) 5′-CCACAGGTCCACTTCTGGTTC-3′; (γ) 5′-AAGTCACCACTGGGACTATCC-3′; (δ) 5′-CTCAACAGCCAGAGCCACACT-3′. Discrimination of polymorphic *Pax5* and *CD19* transcripts: the allelic origin of the *CD19* PCR product was determined by *Bgl*II digestion followed by gel electrophoretic analysis. Approximately 100 ng of the *Pax5* PCR product was denatured by dilution in 0.4 M NaOH and heating to 100 °C for 10 min. The denatured DNA fragments were then slot-blotted onto nylon membranes and hybridized to end-labeled allele-specific or common *Pax5* oligonucleotides (see panel A). The specificity of allele detection was achieved by overnight hybridization in 10% formamide / 6× SSC at 50 °C, which corresponds to 2–5 °C below the predicted DNA melting temperature of the respective double-stranded oligonucleotides. Blots were washed in 1× SSC at 60 °C and exposed to X-ray film for ~ 1 h at –70 °C.

transcripts which are synthesized by reverse transcriptase prior to exponential amplification. Inefficient cDNA synthesis may generate only one cDNA transcript even from biallelically transcribing cells, thus creating an artifact, as every gene examined would appear to be monoallelically expressed. Using *CD19* mRNA as a template, we optimized several paramaters of the single-cell RT-PCR assay to achieve the desired goal of amplifying both allelic transcripts from a single B cell of an F1 hybrid mouse (for details see legend to Figure 6). In particular, two modifications proved to be crucial, as they increased the number of amplification-positive cells up to 95% and allowed the detection of both allelic *CD19* transcripts in > 90% of these cells (Figure 6A). First, the use of gene-specific oligonucleotides as opposed to random hexamer or oligo(dT) primers led to a significant increase in the number of cells from which *CD19* transcripts could be amplified. Second, RNase H digestion of the RNA-DNA hybrids of the RT reaction considerably improved the efficiency of PCR amplification. Importantly, the two *CD19* transcripts were amplified in an unbiased manner, as both alleles were detected with equal efficiency upon serial dilution of a corresponding cDNA mixture (Figure 5E).

The result of a typical single-cell RT-PCR experiment, which was performed under optimized conditions with individual $B220^+$ IgM^+ splenocytes, is shown in Figure 6. Both allelic *CD19* transcripts were detected in the majority of cells, as expected for a biallelically expressed gene. However, only 38% of the *CD19* biallelically transcribing cells expressed also both *Pax5* alleles (Figure 6B,C). Hence, the single-cell RT-PCR method revealed monoallelic *Pax5* expression in 62% of all splenic B cells which is in good agreement with the RNA-FISH data (72%) and flow cytometric analysis of the *Pax5* (+/–) mice (~ 75%)

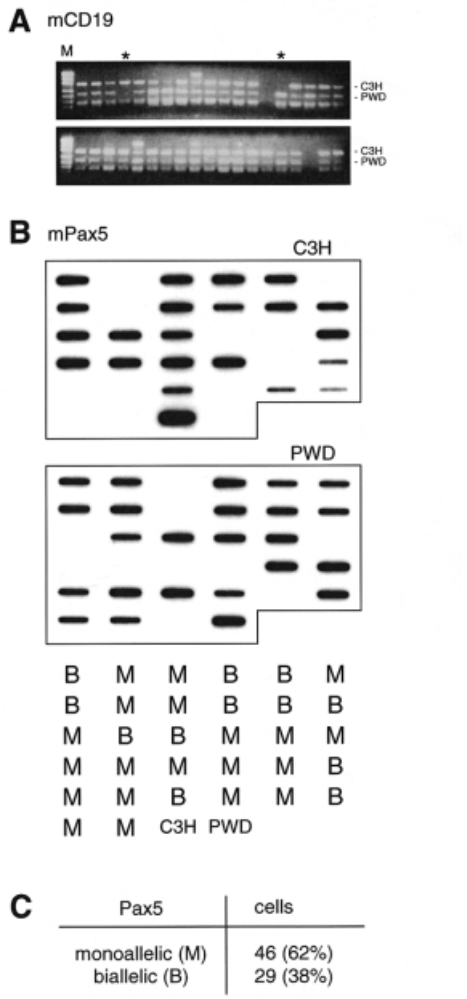


Fig. 6 Single-Cell RT-PCR Analysis of *Pax5* and *CD19* Expression in Splenic B Cells.

(A) Biallelic transcription of the polymorphic *CD19* alleles in individual B cells of F1 (PWD × C3H) hybrid mice. The PCR products of PWD and C3H origin were discriminated by *Bgl*II digestion. PCR reactions containing only one of the two allelic *CD19* transcripts (denoted by asterisks) were excluded from further analysis of *Pax5* expression. (B) Slot-blot analysis of the RT-PCR reactions of 32 individual B cells. The PCR reactions of only those cells which were determined to be biallelic for *CD19* transcription were analyzed by slot-blot hybridization with PWD- and C3H-specific probes (see legend to Figure 5). *Pax5* cDNA derived from the spleen of parental PWD and C3H mice was used as specificity control. M, monoallelic; B, biallelic. (C) Summary of *Pax5* transcription in 75 individual B cells which were all shown to biallelically express *CD19*.

Methods: Single-cell RT-PCR analysis: Single-cell suspensions prepared from the spleen of 4–6-week-old F1 hybrid mice were stained with phycoerythrin (PE)-conjugated anti-IgM mAb (Immunotech) and fluorescein isothiocyanate (FITC)-conjugated anti-B220 mAb (RA3-6B2, PharMingen). Individual $B220^+$ IgM^+ cells were sorted with a FACS Vantage TSO flow cytometer (Becton-Dickinson) into single wells of a 96-well PCR plate each containing 5 μl PBS. Plates were frozen at –70 °C until further use. Upon thawing, cells were lysed by heating to 70 °C for 2 minutes and then placed on ice. 10 μl of the reverse transcriptase (RT) reaction mixture [containing 1 mM dNTP, 10 mM DTT, 50 mM Tris-HCl pH 8.3, 7.5 mM KCl, 3 mM $MgCl_2$, 5 ng/μl RT-primers (*Pax5* and *CD19* oligonucleotides γ), 60 U SuperScript™ II (GibcoBRL) and 30 U RNasin (Promega)] were added to each well followed by incubation at 42 °C for 1 h. The RT reaction was stopped by heating the plate to 70 °C for 10 min followed by incubation with 0.5 U RNase H (Boehringer Mannheim) for 30 minutes at 37 °C and again heating to 70 °C for 10 minutes. 76 μl of the PCR mixture [1 × PCR buffer (Boehringer Mannheim) containing 0.8 mM dNTPs, 0.75 μg of the *Pax5* and *CD19* PCR primers α and γ, and 4 U *Taq* DNA polymerase (Boehringer Mannheim)] were then added to the RNase H-treated RT reaction followed by 25 cycles of PCR amplification. 2 μl of the first PCR reaction was then used for separate amplification of *Pax5* and *CD19* cDNA by utilizing the respective nested primer combinations β and δ for additional 25 cycles. The 332-bp *Pax5* and 423-bp *CD19* cDNA fragments could be readily visualized by analyzing 20 μl of the second PCR reaction on a 1.5% agarose gel.

(Nutt *et al*., 1999). In summary, the results obtained with three fundamentally different methods support the same conclusion that Pax5 is subject to allele-specific regulation in B lymphocytes.

Monoallelic Expression as a Result of Stochastic Activation of *Pax5* at Lineage Commitment

Pax5 is the first transcription factor gene which has been shown to be predominantly expressed from only one of its two alleles in a stochastic and reversible manner. This allele-specific regulation generates a haploinsufficient phenotype at the cellular level in B-lymphoid tissues of *Pax5* (+/–) mice (Nutt *et al*., 1999). However, for reasons discussed above, heterozygous mutation of the *Pax5* gene has no phenotypic consequences (Urbánek *et al*., 1994) and hence does not provide a model for a human disease syndrome (Vorechovsky *et al*., 1995). In contrast, heterozygous mutations of the closely related *PAX2* and *PAX8* genes of the same *Pax* subfamily are known to cause the renal-coloboma syndrome (Sanyanusin *et al*., 1995) and congenital hypothyroidism (Macchia *et al*., 1998), respectively. It is therefore tempting to speculate that allele-specific transcriptional regulation is responsible for the susceptibility of these and other mammalian *Pax* genes to single mutations. A model of stochastic allele-specific regulation of *Pax* genes could account for two aspects of *Pax*-related disease syndromes; i.e. their phenotypic variability and tissue preference. The severity of a heterozygous *Pax* mutation would be expected to depend on the stochastically determined ratio of cells activating either the wild-type or mutant allele. An increased frequency (> 50%) of cells expressing the wild-type allele may result in a milder phenotype, while a higher proportion (> 50%) of cells activating the mutant allele should cause a more severe phenotype. Moreover, the independent regulation of the two *Pax5* alleles during B cell development (Figure 2) suggests that other *Pax* genes may also be transcribed monoallelically in some tissues and biallelically in others, thus explaining why haploinsufficient phenotypes are observed only in a subset of the *Pax* expression domains

(Strachan and Read, 1994; Mansouri *et al*., 1996). Finally, it is worth noting that switching of expression between alleles may be deleterious in a heterozygous situation. For instance, a cell that differentiates along a particular lineage due to activation of the wild-type *Pax* allele, may lose its identity and possibly die upon switching to expression of the mutant allele.

Recently we have demonstrated that Pax5 is a key regulator of B-lineage commitment. Early pro-B cells lacking Pax5 function are able to differentiate along multiple hematopoietic lineages, but are entirely blocked in their ability to proceed along the B cell pathway (S.N., A. Rolink and M.B., unpublished data). Monoallelic transcription of *Pax5* at the onset of B-lymphopoiesis (Figure 2) is particularly interesting in view of these new findings. Multipotent progenitor cells are characterized by their ability to undergo self-renewal or development to several different lineages (Figure 7). High-level expression of a lineage-determination gene like *Pax5* might force such progenitor cells to differentiate along a single lineage and could thus deprive these cells of their various options. The multipotency of these cells could, however, be maintained by stochastically activating lineage-determination genes at a low frequency and efficiency, which may results in the transcription of only one of the two alleles (Figure 7). The monoallelic expression of *Pax5* in pro-B cells is therefore consistent with a stochastic model of B-lineage commitment. In this context it may be interesting to note that the transcription of several other lineage-specific genes is also initiated only at a low level in hematopoietic progenitor cells (Hu *et al*., 1997, and references therein).

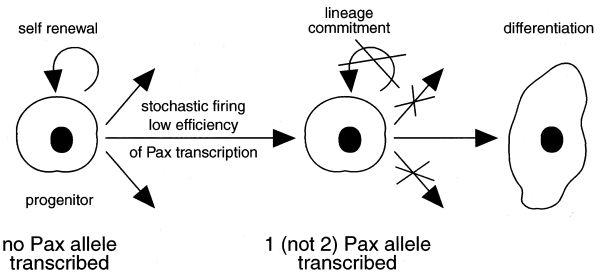


Fig. 7 Stochastic Activation of a Lineage Determination Gene during the Commitment Phase of Differentiation.
The commitment of progenitor cells to a particular cell lineage may be brought about by the stochastic activation of a lineage determination gene such as *Pax5* which could result in expression of only one of the two alleles due to low efficiency of transcription initiation.

Monoallelic Expression May be More Common in Mammals than Previously Anticipated

The majority of mutations in mouse and human genes are recessive, indicating that expression from both alleles is not essential for the function of most genes *in vivo*. A small proportion of mammalian genes (0.1% – 02%) is, however, monoallelically expressed as a result of genomic imprinting, while the extent of non-imprinted genes displaying monoallelic expression is not yet known (Watanabe and Barlow, 1996). Random monoallelic expression has been first described for the family of odorant receptor genes (Chess *et al*., 1994). In this case, monoallelic transcription is a mechanism to ensure that each individual sensory neuron of the olfactory system expresses only a single receptor on the surface. The lymphoid system, which is more amenable to analysis at the single cell level, has provided further insight into the regulation of monoallelic gene expression. The *Ly49* gene family codes for MHC class I-specific inhibitory receptors which are expressed on the surface of natural killer (NK) cells where they participate in the discrimination of self from nonself in innate immunity (Long and Wagtmann, 1997). Interestingly, individual alleles of these *Ly49* genes are stochastically chosen for stable expression in different NK cells. Monoallelic expression of the *Ly49* genes is therefore thought to be essential for generating a diverse repertoire of NK cell receptors (Held *et al*., 1995; Held and Raulet, 1997; Held and Kunz, 1998). More recently, the analysis of wild-type T cells and heterozygous mutant mice has revealed that individual alleles of the *IL-2* and *IL-4* genes are independently regulated by a stochastic process in T helper cells (Holländer *et al*., 1998; Bix and Locksley, 1998; Rivière *et al*., 1998). Allele-specific regulation may therefore establish a diverse repertoire of combinatorially assorted cytokine expression patterns in T lymphocytes (Bix and Locksley, 1998; Rivière *et al*., 1998).

Several human disease syndromes are caused by heterozygous mutations in transcription factor genes other than *PAX* genes *(MITF*, *HOXD13*, *EMX2*, *GLI3*, *THRB*, *HMGIC*, *SOX9*, *SOX10*, *CBP*, *EYA1*, *CBFA1)* (Engelkamp and van Heyningen, 1996, and references therein). It will be important to see whether monoallelic expression of some of these genes may be the underlying disease-causing mechanism. An interesting case in this repect is *CBFA1*, which upon mutation causes the cleidocranial dysplasia syndrome (Lee *et al*., 1997; Mundlos *et al*., 1997). Targeted gene inactivation in the mouse identified Cbfa1 as a key regulator of osteoblast differentiation (Otto *et al*., 1997; Komori *et al*., 1997), suggesting that this transcription factor may be essential for commitment to the osteoblast lineage similar to the role of Pax5 in B cell development.

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