

The Epigenetic Stability of the Locus Control Region-Deficient IgH Locus in Mouse Hybridoma Cells Is a Clonally Varying, Heritable Feature

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ABSTRACT

Cis-acting elements such as enhancers and locus control regions (LCRs) prevent silencing of gene expression. We have shown previously that targeted deletion of an LCR in the immunoglobulin heavy-chain (IgH) locus creates conditions in which the immunoglobulin μ heavy chain gene can exist in either of two epigenetically inherited states, one in which μ expression is positive and one in which μ expression is negative, and that the positive and negative states are maintained by a *cis*-acting mechanism. As described here, the *stability* of these states, *i.e.*, the propensity of a cell to switch from one state to the other, varied among subclones and was an inherited, clonal feature. A similar variation in stability was seen for IgH loci that both lacked and retained the matrix attachment regions associated with the LCR. Our analysis of cell hybrids formed by fusing cells in which the μ expression had different stabilities indicated that stability was also determined by a *cis*-acting feature of the IgH locus. Our results thus show that a single-copy gene in the same chromosomal location and in the presence of the same transcription factors can exist in many different states of expression.

PHYSIOLOGY and evolution are finely balanced between stability and variability. While biochemical and genetic stability ensure robust metabolic pathways and their faithful transmission to offspring, variability allows adaptation to changing environmental conditions and acquisition of novel phenotypic traits. Several mechanisms exist to ensure stability and to prevent variation: recombination and DNA repair prevent mutation; the effects of mutation on protein structure are minimized by codon redundancy; and gene expression and enzyme activity are often subject to homeostatic mechanisms, such as feedback regulation.

Tissue differentiation in complex organisms also depends on stability and variability in the sense that clonal variability in gene expression is necessary to generate cells of different types, while clonal stability is necessary for organogenesis. However, the same gene is sometimes differentially expressed in seemingly equivalent cells. The classic example is position-effect variegation (PEV), in which a gene that has been translocated to a heterochromatin-proximal location is silenced in some cells, but not in others. On the one hand, PEV illustrates clonal variation, as the translocated gene is differentially expressed in otherwise identical cells. On the other hand, the expressed and silent states of the variegating gene are heritable, and PEV is therefore also an example of clonal stability.

Clonal variegation also occurs for normal genes.

Thus, independent T-cell clones differ in the fraction of cells that express the interleukin 4 (IL4) gene after stimulation, and this fraction is a clonally inherited feature (HU-LI *et al.* 2001; GUO *et al.* 2002). IL4 is toxic at high concentration, and clonal variegation might be a mechanism by which IL4 can sometimes be produced locally at a high rate without systemic toxicity. Variegation might thus allow IL4 to function sometimes as an autocrine and sometimes as a paracrine signal or allow different T-cell clones to activate cells that have greatly differing sensitivities to IL4. In another example, early erythroid cells express different combinations of the α - and β -globin genes, and these patterns are clonally inherited through several cell divisions (DE KROM *et al.* 2002). In this case, the physiological function, if any, for clonal variation is obscure.

Other molecular features, in addition to heterochromatin proximity, have been found to affect variegation. Thus, variegation can be reversed by higher concentrations of specific transcription factors (APARICIO and GOTTSCHLING 1994; BECSKEI and SERRANO 2000; LUNDGREN *et al.* 2000; McMORROW *et al.* 2000) and induced by removing specific *cis*-acting elements, such as enhancers and locus control regions (LCRs), as can be seen for transgenes and endogenous genes both in cell lines and in animals (RONAI *et al.* 1999; FIERING *et al.* 2000; ELLMEIER *et al.* 2002; GAREFALAKI *et al.* 2002). In the particular case of the immunoglobulin heavy chain (IgH) locus, targeted deletion of an intronic LCR alters the locus such that the μ gene can exist in either of two states: positive (P), in which μ expression is highly expressed, and negative (N), in which μ expression is

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nearly extinguished (RONAI *et al.* 1999). Each of these states is heritable, in that cells in a particular state usually yield progeny cells in the same state. Cells can switch between the P and N states, and the rates of switching are much higher than mutation rates, suggesting that the P and N states are inherited by an epigenetic rather than a genetic mechanism (RONAI *et al.* 1999).

In the case of variegating transgenes, the stability of the expressed state, *i.e.*, the rate at which the expressed gene is silenced, varies among independent transfectants (MAGIS *et al.* 1996; FRANCASTEL *et al.* 1999). The variation in the stability of transgene expression might occur because different, but undefined, regulatory elements adjoin some insertion sites. Alternatively, stability might be a variegating property, as suggested from the analysis of IL4 (HU-LI *et al.* 2001; GUO *et al.* 2002). To test whether stability is subject to clonal variation, we have used the LCR-deficient IgH locus and measured the extent of switching between the P and N states of many subclones. As reported here, we found that the stability of expression, *i.e.*, the propensity to switch from one state of expression to another, was a clonal, epigenetically inherited property. That is, in the absence of the intronic LCR, the endogenous, recombinant IgH locus could exist in many different states of stability, and cells of a particular stability usually yielded progeny cells with the same stability. We also show that stability is determined by a mechanism that acts *in cis* with the IgH locus.

MATERIALS AND METHODS

Construction of hybridoma recombinants and derivation of subclones: We have previously described construction of the LCR-deficient recombinant E⁻M⁻S⁻44 (WIERSMA *et al.* 1999) and of the analogous recombinant in which the BssSI site in the C μ 2 exon was changed to a HindIII site (RONAI *et al.* 2002). These recombinants are also denoted here as the B and H cell lines, according to whether the C μ 2 exon bears the BssSI or HindIII site, respectively. E⁻M⁻S⁻44 was transfected with a transgene encoding resistance to puromycin, and a predominantly negative, puromycin-resistant colony was isolated. To obtain isogenic positive subclones, cells from this puromycin-resistant colony were surface labeled with FITC-coupled anti-IgM antibodies (Jackson) from which a fraction enriched in positive cells was isolated by cell sorting (RONAI *et al.* 2002), and the stable and unstable positive subclones were isolated from this fraction. In some cases, rare μ -positive cells were obtained by plucking cells from plaques, as described by BAAR and SHULMAN (1995). Other subclones were obtained simply by plating cells at limiting dilution.

Hybrids were constructed by fusing the B and H cell lines as described (RONAI *et al.* 2002). The B cell line but not the H cell line was deficient in thymidine kinase and therefore unable to grow in hypoxanthine, aminopterin, and thymidine (HAT) medium; the former cell line was rendered resistant to puromycin by transfection (RONAI *et al.* 2002). A total of 10⁷ cells of each parent were washed in serum-free medium and resuspended in 1 ml polyethylene glycol-4000 (GIBCO, Gaithersburg, MD) at 37°. Cells were then slowly diluted in serum-free medium at room temperature, washed once, and resuspended in double-selection medium, *i.e.*, medium supplemented with puromycin (Sigma, St. Louis) and HAT

(GIBCO). Puromycin-resistant, HAT-resistant transfectants were subcloned at limiting dilution to ensure that the hybrids analyzed were derived from single cells.

Single-cell analysis of IgM production: Flow cytometry of para-formaldehyde-fixed cells stained for intracellular IgM with FITC-labeled anti-IgM antibodies (Jackson) and the plaque-forming cell (PFC) assay were described previously (RONAI *et al.* 2002).

The Elispot assay (CZERKINSKY *et al.* 1983) was used as an alternative to the PFC assay to estimate the fraction of positive cells in mostly negative subclones. A total of 6 × 10³ to 6 × 10⁴ cells/well were distributed to six wells of enzyme-linked immunosorbent assay plates (Nunc) coated with goat anti- μ -antibody (Jackson) and incubated for 2 hr at 37°. Wells were washed and incubated with biotinylated goat anti- μ -antibody coupled with alkaline phosphatase (Jackson) and then incubated with streptavidin-coupled alkaline phosphatase. Finally, 5-bromo-4-chloro-3-indolyl phosphate dipotassium salt (GIBCO) was used to develop the spots generated by IgM-producing cells. Spots were enumerated under a dissecting microscope and reported as the total for the six wells.

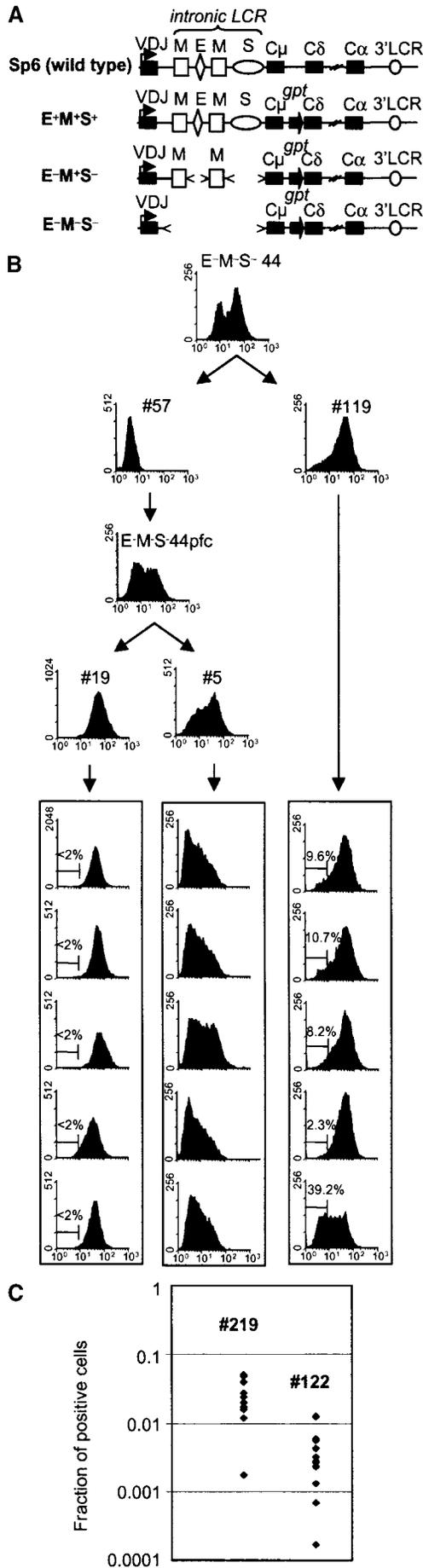
Analysis of RNA: Total RNA was prepared with Trizol reagent (GIBCO) according to the manufacturer's specifications. Northern blots were done according to standard protocols. Probes were amplified by PCR and labeled with ³²P by random priming. To probe RNA derived from the μ region, we used the HindIII fragment corresponding to C μ 3 and C μ 4. The β -actin probe was made by RT-PCR using actin-specific primers purchased from CLONTECH (Palo Alto, CA).

PCR and RT-PCR assays for specific alleles: For PCR and RT-PCR, the following primers were used: the sense primer 5'-ATG TCT TCC CCC TCG TCT CCT-3' and the antisense primer 5'-TAC ACA TTC AGG TTC AGC CAG TC-3'. cDNA was synthesized using the One-Step RT-PCR system (Invitrogen, San Diego), according to the manufacturer's specifications. PCR products were purified using a PCR purification kit (QIAGEN, Chatsworth, CA) and digested with restriction enzymes.

Treatment with azacytidine and trichostatin: A total of 10⁵ cells were treated with 4 μ M 5-azacytidine (5-azaC; Sigma) for 48 hr or with 5 nM trichostatin A (TSA; ICN) for 24 hr. Cells were also treated with a combination of the two drugs. In this case, cells were incubated with 5 μ M trichostatin A and 4 μ M 5-azacytidine together for 24 hr, after which the cells were washed and then incubated with 5-azacytidine alone for an additional 24 hr.

RESULTS

Expression of genes in the IgH locus is controlled in part by elements in the J_H-C μ intron that are components of the intronic LCR: the core enhancer (E μ), the matrix attachment regions (MARs), and the switch region (S μ ; Figure 1A; GRAM *et al.* 1992; FORRESTER *et al.* 1994; OANCEA *et al.* 1995; ARULAMPALAM *et al.* 1997). We previously described a cell culture system to study the role of these elements in the endogenous IgH locus. Using this system, we have examined expression of immunoglobulin μ heavy chain gene in the mouse B cell hybridoma Sp6, in which the IgH locus has been modified by targeted recombination and so lacks one or more of the components of the intronic LCR (Figure 1A; RONA *et al.* 1999). To test whether μ expression was uniformly positive or variegated in the recombinants, intracellular μ protein was stained with fluorescent μ -specific antibodies, after which μ expression in individual cells was assessed by flow cytometry. As summarized in the



Introduction, deletion of the intronic LCR (E⁻M⁻S⁻ recombinant in Figure 1A) by targeted recombination in Sp6 resulted in variegated (bimodal) expression of IgH; *i.e.*, in the LCR-deficient recombinant cells, expression of the μ gene either was at a high level, similar to wild-type cells (positive, P), or was nil (negative, N). In contrast to these LCR-deficient recombinants, the expression of the μ gene in wild-type cells and recombinants that retained at least the core enhancer was uniformly positive. Expression of the μ gene as assayed by flow cytometry correlated with expression as measured by Northern blot (RONAI *et al.* 1999; data not shown).

In this previous analysis we estimated the rate of switching by measuring the fraction of cells that had switched from one state to the other during a defined time period. These measurements were made for several independent recombinants and suggested that switching occurred at a single characteristic rate. To test more extensively whether switching occurred at only a single rate, we measured the fraction of switched cells in a large number of subclones, as described below.

FIGURE 1.—Variegated μ expression in LCR-deficient colonies. (A) Structure of wild-type and recombinant IgH loci in hybridoma cells. As described in the text, targeted recombination of the wild-type cell line, Sp6, was used to modify the μ heavy chain gene in the IgH locus. The heavy chain variable domain of Sp6 is encoded by the rearranged segments, VDJ, and the transcription initiation site is indicated by the arrow. Solid rectangles, the multiple exons encoding the variable and constant region domains; open symbols, *cis*-acting, regulatory elements. As indicated, the intronic LCR contains matrix attachment regions (M), the core Eμ enhancer (E), and the switch region (S). Another LCR is located 3' of Cα (3' LCR). The structure of the endogenous IgH locus is also shown for the recombinant hybridomas in which these components of the intronic LCR were either present (E⁺M⁺S⁺) or deleted (E⁻M⁻S⁻ and E⁻M⁺S⁻). < > indicates the absence of specific elements. As described in the text and in WIERSMA *et al.* (1999), the *gpt* cassette was used as the selectable marker in constructing these recombinants. (B) Flow cytometry of LCR-deficient colonies. The E⁻M⁻S⁻ 44 recombinant was subcloned, as described in the text, and these subclones were analyzed by flow cytometry after staining the cells for intracellular μ chains. In these histograms the relative fluorescence intensity is shown on the horizontal axis, and the cell number on the vertical axis. All experiments were calibrated such that the IgM-negative cell line lacking the μ gene (X10) stained in the first decade. The fraction of IgM-negative cells, *i.e.*, cells that stained in the range marked by |—|, is indicated. The arrows indicate the origin of the subclones, which were generally obtained by limiting dilution. However, to obtain the subclone denoted E⁻M⁻S⁻ 44pfc, colony 57 was plated for plaque-forming cells, and a rare μ-positive cell was plucked from a plaque and then expanded in culture (BAAR and SHULMAN 1995). (Bottom) μ expression was measured for five subclones each of colonies 19, 5, and 119. (C) Measurement of positive cells in predominantly negative colonies. Two colonies, 219 and 122, derived as subclones of the E⁻M⁻S⁻ 44 recombinant, were further subcloned. The subclones were then assayed for positive cells using the Elispot assay (MATERIALS AND METHODS).

TABLE 1

Frequency of positive cells in subclones of parental colonies used to create somatic cell hybrids

Subclone	Frequency of positive cells in subclones (\pm SD)	
	At the time of fusion (mean \pm SD)	2 mo after fusion (mean \pm SD)
B(P ^s)	>0.98	>0.98
H(P ^s)	>0.98	0.86 (\pm 0.27)
B(P ^u)	0.41 (\pm 0.15)	0.15 (\pm 0.13)
H(P ^u)	0.37 (\pm 0.48)	0.19 (\pm 0.14)
B(N)	3.0 (\pm 1.8) $\times 10^{-3}$	ND
H(N)	1.9 (\pm 1.3) $\times 10^{-2}$	ND

To distinguish stable and unstable positive colonies [B(P^s), H(P^s), B(P^u), and H(P^u), Figure 2B], predominantly positive colonies were subcloned and the frequency of positive cells in each of 10 subclones was measured by flow cytometry. The predominantly negative colonies [B(N) and H(N)] were subcloned and the frequency of positive cells in each of 10 subclones was measured with the Elispot assay (MATERIALS AND METHODS).

Detection of heritable positive and negative states of different stabilities: As illustrated in Figure 1B, expression of the immunoglobulin μ heavy chain gene in the LCR-deficient recombinant E⁻M⁻S⁻44 is bimodal, and the fraction of positive and negative cells typically differs greatly among subclones, as shown for colonies 57 and 119. As the starting point for comparing the behavior of positive cells, we isolated a rare positive (plaque-forming) cell from colony 57 and expanded this cell to generate a colony, E⁻M⁻S⁻44pfc, which was subcloned by limiting dilution. The resulting colonies were examined by flow cytometry, and those with markedly different levels of negative cells were resubcloned; this protocol was then repeated several times. Figure 1B presents two extreme examples: colony 19, which contained no detectable negative cells, and colony 5, which contained a large fraction of negative cells. To test whether the fraction of negative cells was a heritable feature of each colony, colonies 19 and 5, as well as 119 (Figure 1B), were subcloned, and the fraction of negative cells in these subclones was measured. As illustrated, colony 19 yielded subclones with no detectable (<2%) negative cells. By contrast, subclones of colonies 5 and 119 contained a measurable, but significantly different fraction of negative cells (mean \pm SD = 0.60 \pm 0.07 and 0.14 \pm 0.14, respectively) and were thus similar to their respective parental colonies. This analysis can thus distinguish at least three types of colonies.

The colonies 19 and 119 contained predominantly positive cells, and their subclones therefore arose from positive cells in all or almost all cases. The fraction of negative cells thus measures the propensity of the positive cells in these subclones to switch to the negative state. We use the term *stability* to denote this propensity of positive cells to switch their state of expression, as

measured by the fraction of negative cells that arose in subclones after a defined period of time, usually a few weeks. We refer to the cells of colony 19 as *stable positive* cells, because they did not yield detectable negative cells, and the cells of colony 119 as *unstable positive* cells, because of their detectable propensity to switch to the negative state. In the case of colony 5, each subclone contained a large fraction of both negative and positive cells. The flow cytometry patterns suggest that both the positive and the negative cells in these subclones were unstable, to the point that it was not possible to infer whether the starting cells for these subclones were in the positive or negative state. Our finding that subclones of one colony usually resembled each other in this assay more closely than they resembled the subclones of another colony indicates that the cells that were used to generate the colonies differed in a heritable feature that determined their stability. Inasmuch as most cells in a colony had similar stability, we use this property to define colonies as stable or unstable.

We also tested whether the *negative* state could occur with different degrees of stability. For this purpose we measured the fraction of positive cells in subclones that arose from negative cells after a defined period of time, again usually a few weeks (Table 1). Figure 1C illustrates two such examples, the mostly negative colonies 122 and 219, which were themselves subclones of the E⁻M⁻S⁻44 recombinant. These colonies were subcloned, and the fraction of cells that had switched to a positive state was measured. Because the frequency of switched cells was too low to detect by flow cytometry, we used the Elispot assay, in which individual μ -secreting cells yield a visible spot in microtiter wells (see MATERIALS AND METHODS). Using the same subcloning strategy as for positive colonies, we found mostly negative colonies whose subclones generated significantly different numbers of positive cells (Figure 1C). Thus, in subclones derived from colony 219, 2.5 (\pm 1.4) $\times 10^{-2}$ of the cells were positive for μ expression, while in subclones of colony 122 only 0.38 (\pm 0.34) $\times 10^{-2}$ of the cells were positive. This difference between the subclones is statistically significant according to Student's *t*-test ($P < 0.0001$). Therefore, cells could differ in the stability of the negative state of expression.

We conclude that positive and negative states of μ expression in the LCR-deficient recombinants could each occur with different stabilities and that stability was a heritable trait. Because we obtained these subclones after extensive selection and elimination of candidate subclones, we cannot estimate the frequency at which subclones of different stabilities arose. Nevertheless, the fact that this simple protocol was sufficient to yield subclones with different stabilities indicates that the rate at which stability changed was much higher than traditional mutation rates. The high frequency at which cells can give rise to cells of different stability argues that the differ-

ences in stability were an epigenetically rather than a genetically encoded feature.

Analysis of MAR-containing recombinants: The μ gene includes two MARs, which can affect gene expression. For example, MARs facilitate demethylation and extend the domain of accessibility and histone acetylation created by $E\mu$ (KIRILLOV *et al.* 1996; FORRESTER *et al.* 1999; FERNANDEZ *et al.* 2001). We previously reported that

$E^-M^+S^-$ recombinants (Figure 1A), which bore only the MARs in the J_H - $C\mu$ intron, expressed the immunoglobulin μ heavy chain gene at the normal high level (WIERSMA *et al.* 1999). We have examined these recombinants using subcloning assays similar to those described above for the MAR-deficient recombinants. This analysis indicated that the MAR-containing recombinants, like the MAR-deficient recombinants, can occur as both positive and negative cells and that the stability of these states is also a clonal feature (see online supplemental Table 1 at <http://www.genetics.org/supplemental/>). The variation that we detected among the MAR-containing ($E^-M^+S^-$) recombinants was similar to the variation that we found for the MAR-deficient ($E^-M^-S^-$) recombinants. We conclude that no difference between the MAR-containing and MAR-deficient recombinants was evident in this hybridoma system.

The stability of the positive state is maintained by a cis-acting mechanism: Many proteins, both tissue specific and nonspecific, are required to maintain transcription of the μ gene. In principle, cells in different epigenetic states—positive, negative, stable, unstable—might differ in their content of transcription or chromatin-modifying factors. Alternatively, the differences might be confined to the IgH locus itself and act *in cis*. A general method of distinguishing between *trans*-acting and *cis*-acting mechanisms is to fuse cells that are in different states and then test whether the two genes in the hybrid cell have assumed the same state of expression or persist in their distinct states (Figure 2A).

We previously used this type of analysis to test whether the positive and negative states are maintained by a *cis*-acting mechanism (RONAI *et al.* 2002). As parental cell lines, we used the original targeted recombinant, $E^-M^-S^-44$, and a related targeted recombinant that was constructed in the same way as $E^-M^-S^-44$, but with a distinguishing silent mutation, a single nucleotide substitution in the exon for $C\mu 2$, that changed a *Bss*SI site to

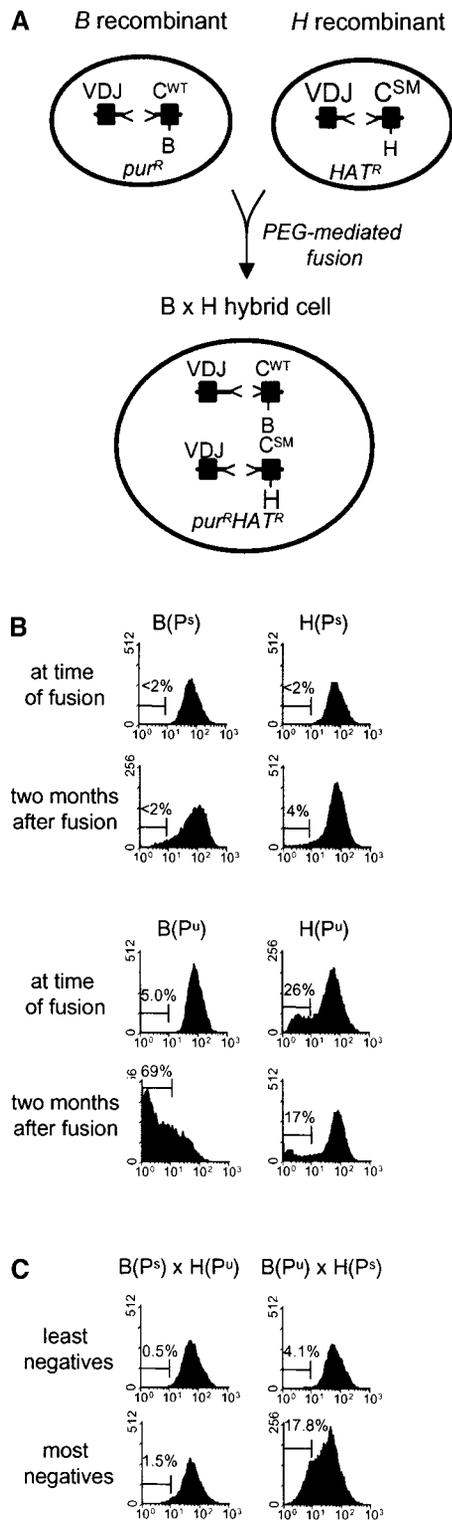


FIGURE 2.—Generation of somatic cell hybrids. (A) Strategy for generating hybrids. The parental lines were $E^-M^-S^-$ subclones with wild-type $C\mu$ (*B* recombinant) or $C\mu$ with a 1-bp substitution that created a silent mutation (*H* recombinant), which converted the normal *Bss*SI site to a *Hind*III site. The cell line with the *Bss*SI site was rendered puromycin resistant and HAT sensitive, as described in MATERIALS AND METHODS. (B) Flow cytometry of parental cell lines. The parental *stable* positive colonies, B(P^s) and H(P^s), and the parental *unstable* positive subclones, B(P^u) and H(P^u), were analyzed for intracellular IgM by flow cytometry. The histograms show the state of the parental cells at the time of fusion and 2 months after fusion, when hybrids had been cloned and analyzed. (C) Flow cytometry of selected hybrids from the P^s × P^u fusions. The hybrids obtained from the reciprocal fusions, B(P^s) × H(P^u) and B(P^u) × H(P^s), were analyzed by flow cytometry. In each case, the large majority of the cells were μ positive. To illustrate the range of results that were obtained, the hybrid with the lowest and the hybrid with the highest fraction of negative cells are shown here for each of the reciprocal fusions.

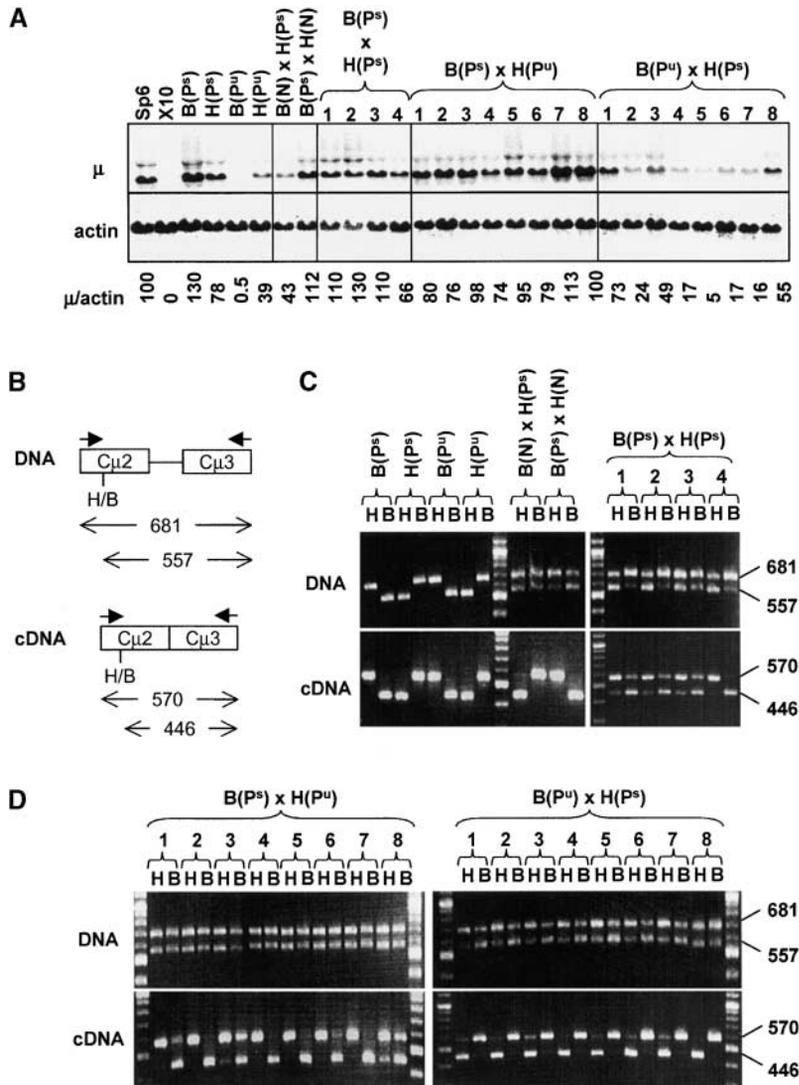


FIGURE 3.— μ RNA content of hybrid cells. Cells from the indicated colonies, which had been determined to have predominantly negative cells (N) or stable or unstable positive cells (P^s or P^u), were fused, and hybrid cells were selected by plating cells at limiting dilution in HAT/puromycin medium. The resulting hybrid colonies were subcloned to ensure that hybrids were derived from a single fused cell and then tested for the presence of both alleles. The parental cell lines were grown in parallel with the hybrids so that the alleles could be compared for their stability over a similar time period. The hybrids and parental cells were analyzed ~ 2 months after the time of fusion. (A) Total μ RNA. RNA was prepared from the indicated parental subclones and hybrid cells, and 10 μ g was analyzed by Northern blot, probing first for μ and then for actin. The RNA was isolated from cells ~ 2 months after fusion. μ RNA in these cell lines was made as both the secretory (bottom band) and the membrane (top band) forms. The intensity of the secretory μ band and the actin band was measured, and the ratio of secretory μ to actin is indicated below each lane. (B) PCR and RT-PCR assays for μ alleles and allele-specific μ RNA. This diagram shows the products from DNA or RNA that were isolated from cells and then subjected to PCR or RT-PCR using primers in the C _{μ} 2 and C _{μ} 3 exons. Digestion of the products with the allele-specific enzymes, *Bss*SI and *Hind*III, then served to separate the products arising from each allele. Numbers indicate the expected size of the restriction fragments in base pairs. (C) Allele-specific μ RNA in parental and control hybrid cells. As depicted in B, DNA and cDNA were prepared from the indicated parental colonies, the N \times P^s hybrids, and four $P^s \times P^s$ hybrid cells (1–4). These DNAs were then amplified by PCR as described in MATERIALS AND METHODS, and the PCR products were digested with either *Hind*III (H) or *Bss*SI (B) and electrophoresed in agarose. The size (in base pairs) of the fragments is indicated to the right. (D) Allele-specific μ RNA in $P^s \times P^u$ hybrids. As indicated in B, DNA and cDNA were prepared from eight independent hybrids from each $P^s \times P^u$ fusion, amplified by PCR, and tested for cutting by *Hind*III and *Bss*SI.

a *Hind*III site without altering the amino acid sequence of the heavy chain (see legend to Figure 2 and RONAI *et al.* 2002). For simplicity, we denote recombinant cells and alleles that bear the wild-type allele or silent mutation with the letters *B* or *H*, respectively, according to whether the C _{μ} 2 exon bears the *Bss*SI or *Hind*III site. As described in MATERIALS AND METHODS, the *B* cell line was rendered deficient in thymidine kinase and resistant to puromycin, so that *B* \times *H* hybrid cells could be selected in HAT medium containing puromycin. The PCR and RT-PCR assays used to distinguish the two μ alleles and their transcripts are illustrated in Figure 3B.

As described above, we examined numerous subclones of the *B* and *H* cell lines to obtain predominantly positive colonies that were stable and unstable (P^s , P^u , respectively) and to obtain predominantly negative (N)

subclones. Thus, we determined that the colonies *B*(P^s) and *H*(P^s) were composed of stable positive cells, because very few, if any, negative cells were detected by flow cytometry in any of 10 subclones after a few weeks of growth in culture [$<2\%$ negative cells in *B*(P^s) and $\sim 2\%$ in *H*(P^s); data not shown]. We also determined that the colonies *B*(P^u) and *H*(P^u) were composed of unstable positive cells, since 10 subclones of these colonies each contained a substantial number of negative cells (data not shown). Figure 2B shows the flow cytometry profiles of the fusion partners at the time of fusion and 2 months thereafter, when subclones of the hybrids were analyzed for RNA production (see below). Both at the time of fusion and 2 months later, the stable colonies, *B*(P^s) and *H*(P^s), were nearly 100% positive for μ expression. The unstable colonies, *B*(P^u) and *H*(P^u),

were 95 and 74% positive, respectively, at the time of fusion and ~ 31 and 83% positive 2 months later. These results imply that when the hybrids were initially formed, both μ genes should have been expressed in 95 and 74% of the hybrids in the respective fusions. As noted above, if the difference between the stable and unstable states were due to the presence or absence of a stabilizing or destabilizing factor, then both μ genes should be equally stable or unstable in the hybrids. Conversely, if the difference in stability were due to a *cis*-acting feature of the μ gene, then the stable allele should be expressed significantly more often than the unstable allele.

As described below, we fused cells in various combinations. Following outgrowth of the hybrid cells, we tested whether hybrids retained both μ alleles by amplifying μ DNA by PCR and then digesting the PCR product with the allele-specific enzymes, *BssSI* and *HindIII* (Figure 2A). Hybrids bearing both μ alleles were subcloned and then analyzed for μ expression by flow cytometry. Figure 2C illustrates the range of these results and shows the hybrids with the least and the most negative cells. Flow cytometry of these hybrids indicated that μ was expressed from at least one of the two alleles in all hybrids analyzed. We confirmed this observation by performing Northern blot analysis (Figure 3A) and then determined which allele was μ expressed by competitive, semiquantitative RT-PCR (Figure 3, C and D).

Northern blots were probed for both μ and actin (Figure 3A). μ RNA in these cell lines is made in two forms, which correspond to the secretory [bottom (major) band] and the membrane [top (minor) band] forms of the RNA and protein (Figure 3A). The μ /actin ratio was calculated using the secretory μ band and is listed below each lane. This assay indicated that the stable parents contained substantially more μ RNA than the unstable parents, in keeping with the flow cytometry results (Figure 2B). Also, the B(P^s) parent contained ~ 1.7 -fold as much μ RNA as did the H(P^s) parent; the basis for this difference is under investigation. As expected, in the RT-PCR assay for allele-specific μ RNA, the B and H parental cell lines yielded products that were digested only with *BssSI* and *HindIII*, respectively.

Some control fusions that were done in parallel are included here. The fusion of two stable positive cells, B(P^s) \times H(P^s), served to confirm that hybrids expressing two μ alleles were viable. As illustrated in Figure 3A, the μ /actin RNA ratio of these hybrids was approximately the average of the two parents. The RT-PCR analysis of the RNA of these hybrids indicated that both μ alleles were substantially expressed but that somewhat more μ RNA was derived from the B allele than from the H allele, similar to the difference between the B(P^s) and H(P^s) parents noted above. The significance of hybrid 4 in which expression of the H allele is greatly reduced is considered in the DISCUSSION. The reciprocal fusions of positive and negative cells, B(P^s) \times H(N) and

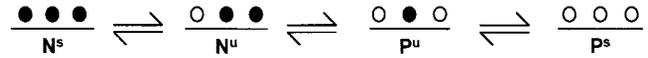


FIGURE 4.—Model for determination of multiple states of expression and stability. This model depicts a segment of the μ gene in which a *cis*-acting, self-templating feature can exist in either of two conditions (for example, cytidine, which is methylated or unmethylated). In this example, there are three sites (circles) for this feature that can be solid or open, and four states of the μ gene are illustrated. The positive or negative state is determined by which condition (solid or open) is in excess, and stability is determined by how many sites must change from solid to open to switch the majority condition.

B(N) \times H(P^s) served to confirm that the positive and negative states were determined *in cis*, as reported previously (RONAI *et al.* 2002). That is, the μ /actin RNA ratio of these hybrids was approximately the average of the two parents (Figure 3C), but in these hybrids the μ RNA was derived almost exclusively from the allele donated by the positive parent (Figure 3B). Thus, both positive and negative states of μ expression could exist in the presence of the same set of transcription factors and were therefore determined by a *cis*-acting mechanism.

To test whether stability was determined by a *cis*-acting or *trans*-acting mechanism, we constructed the hybrids from the reciprocal fusions, B(P^s) \times H(P^u) and B(P^u) \times H(P^s). Total μ RNA in these hybrids is shown in Figure 3A and again corresponds to the average of the two parents. As indicated above, flow cytometry of these hybrids indicated that all [B(P^s) \times H(P^u)] and most [B(P^u) \times H(P^s)] cells expressed the μ gene (Figure 2C). However, the RT-PCR results indicate that in all cases the allele donated by the stable parent was expressed at much higher levels than the allele from the unstable parent (Figure 3D, bottom). Considering that $\sim 70\%$ of the H parental cells expressed μ at a high level at the time of fusion in B(P^s) \times H(P^u) hybrids, it is likely that in most cases expression of the H allele was decreased after fusion. Similar relationships are seen in the reciprocal fusion. That is, in H(P^s) \times B(P^u) hybrids, although $\sim 95\%$ of the B(P^u) cells expressed the μ gene at a high level at the time of fusion, the H allele was expressed at much higher levels than the B allele. In summary, the flow cytometry results indicate that nearly all hybrids should have been derived from cells in which μ was expressed. Our finding that in almost all hybrids the μ allele donated by the stable parent was expressed much more than the μ allele donated by the unstable parent indicates that the difference in stability persisted even when the two μ alleles were in the same cell. We conclude therefore that stability of the positive state was maintained by a mechanism that acted *in cis* with the μ locus.

Molecular basis of variegation: Figure 4 depicts a simple model to illustrate how a *cis*-acting, self-templating, graded feature could account for the epigenetic con-

tol of stability and the positive and negative states. In this model the positive or negative state of μ expression is determined by whether the relevant feature is above or below a threshold, and stability is determined by how many changes are required to cross the threshold. Chromatin in mammalian cells can vary in several features: accessibility, cytosine methylation, histone acetylation and methylation, proximity to a centromere, and subnuclear localization. These features are expected to exert their effects on expression, if any, *in cis*. In the case of CpG dinucleotides, cytidine methylation is self-templating, due to the strong preference of the maintenance methylase for hemi-methylated DNA. It is unknown whether any of the other chromatin-associated features are self-templating.

Considering that cytidine methylation is a *cis*-acting, self-templating feature of chromatin, we undertook to test whether graded methylation determined the stability of the positive and negative states, as in the model of Figure 4. In principle, methylation might inactivate or activate μ expression. Thus, cytidine methylation can extinguish gene expression by preventing binding of some transcription factors to the regulatory regions of genes (YEIVIN and RAZIN 1993; EDEN and CEDAR 1994) or by causing heterochromatinization (NAN *et al.* 1998; WADE *et al.* 1999; ROBERTSON *et al.* 2000). Methylation can also inhibit binding of CTCF, a factor that functions in insulating a promoter from an enhancer, and in such cases methylation can activate expression (BELL and FELSENFELD 2000; HARK *et al.* 2000). We previously reported that treatment of negative cells with the methylation inhibitor (5-azaC), a compound that causes demethylation, increased the frequency of positive cells and that treatment with the histone deacetylation inhibitor trichostatin A had no effect, either alone or in conjunction with azaC (RONAI *et al.* 2002). These results suggested that methylation of the μ gene inactivates expression. Accordingly, in the model in Figure 4, methylation of CpG's in the μ gene would be farther from the threshold in stable negative cells, N^s , than in unstable negative cells, N^u . Less demethylation should therefore be needed to bring unstable negative cells across the threshold than stable negative cells. Consequently, treatment of the cells with azaC should activate μ expression in more N^u cells than N^s cells. To test this prediction, two colonies, 13 and 16, which had been obtained from the MAR-containing recombinant and differed significantly in their stability ($P = 0.0055$), were treated with azaC. These colonies were then subcloned, and the number of positive cells was then measured by the Elispot assay in each of 10 subclones, treated and untreated with azaC. AzaC treatment increased the number of positive cells in each subclone of both the stable and unstable colonies, but approximately sevenfold as many cells were activated in the subclones of the unstable colony as in the subclones of the stable colony. That

is, in the untreated subclones of the unstable colony, 16, the frequency of positive cells was $9 (\pm 7) \times 10^{-5}$ without azaC treatment and $2 (\pm 1) \times 10^{-2}$ with treatment; in the untreated subclones of the stable colony, 13, the frequency of positive cells in the untreated cultures was $1.2 (\pm 0.7) \times 10^{-5}$, which was increased to $0.3 (\pm 0.2) \times 10^{-2}$ with azaC treatment. The larger azaC-induced increase in the subclones of the unstable colony is consistent with the model of Figure 4.

Other experiments did not support the hypothesis that stability is determined by cytidine methylation in the manner indicated in Figure 4. Thus, this model predicts that treatment with azaC should convert unstable positive cells to stable positive cells. However, analysis of 24 subclones from the unstable positive colony 119 (Figure 1B), which was either untreated or treated with azaC, detected no stable subclone. Other studies have reported that the effects of azaC are sometimes enhanced by cotreatment with TSA (CAMERON *et al.* 1999). We therefore treated colony 119 with azaC in conjunction with TSA or with TSA alone, as described in MATERIALS AND METHODS. Again, analysis of 24 subclones from each culture of colony 119 detected no stable positive subclone (data not shown).

We also used biochemical tests to examine the state of specific cytidines in the IgH locus of cells in different states. The regions in which cytidine methylation correlates with transcriptional silencing are usually within the expression-activating elements of genes (EDEN and CEDAR 1994). The known expression-activating elements in the IgH expression lie in the promoter, the J_H - $C\mu$ intron, and the 3' α LCR, downstream from the $C\alpha$ exons (Figure 1). CpG dinucleotides occur in the MARs but not in the promoter region, for which the known components lie within 200 bp of the initiator ATG. We used bisulfite sequencing to analyze the state of the CpG's in the MARs and methyl-sensitive and methyl-resistant restriction enzymes with Southern blots to examine other CpG's in the vicinity of the μ gene. This analysis (not shown) indicated that methylation of the MARs and of $C\mu$ correlated (negatively) with transcription but not with stability.

DISCUSSION

Our previous examination of μ expression in recombinant hybridoma cell lines revealed that expression of the LCR-deficient μ gene was variegated, being either highly expressed (positive) or silent (negative), and that cells can switch between these states at rates that are much higher than ordinary mutation rates. Although our initial analysis suggested that variegating cells could be described in terms of these two states, our present analysis of many subclones has detected another variegating feature: the fraction of cells in a colony that have switched their phenotype, termed stability. This analysis has led to the following observations and inferences.

Stability is a clonally inherited feature that is maintained by an epigenetic mechanism: Both positive and negative states occurred with different stabilities, as measured by the extent of switching to the opposite phenotype. The precision of our assay allowed us to distinguish at least three levels of stability of the positive state (Figure 2), and the actual number might be much higher; *i.e.*, stability might be a nearly continuously varying feature.

Stability was a clonally inherited feature, in that subclones resembled their immediate parent more closely than they resembled more distantly related subclones. Although stability was usually a heritable feature, some subclones had a stability different from that of their sibs and parents. The fact that screening of <100 subclones yielded some with different stabilities implies that the rate of conversion from one stability to another was much higher than typical mutation rates. For this reason, we suppose that stability was inherited by an epigenetic rather than a genetic mechanism.

The observation that independent clones make different amounts of a gene product might be diagnostic of clonal variation in stability. Thus, clonal variation in stability might be the reason that independent recombinants expressed a chimeric mouse V/human C κ gene in the endogenous Ig κ locus at different levels (SUN *et al.* 1994).

The stability of the positive state is determined by a cis-acting mechanism: The level and stability of expression of the enhancer-deficient IgH locus are likely to depend on interactions between the locus and cellular factors. However, our analysis of hybrid cells here and previously showed that μ genes in the positive and negative states of expression can persist in the same cell, indicating that the positive and negative states were distinguished by a feature that acted *in cis*. Similarly, our analysis of hybrids formed from cells in the stable and unstable positive states indicated that an unstable positive allele was extinguished much more rapidly than was a stable allele, even when both were in the same nucleus. Therefore, neither the differences between the positive and negative states nor the differences in stability corresponded to a difference in cellular factors. We suspect that the different stabilities of the negative state were also determined *in cis*. Our analysis thus indicates that at least two features—positive/negative and stability—must be specified to define the state of the enhancer-deficient IgH locus.

Although the difference between stable and unstable cells corresponds to a difference in a *cis*-acting feature, the cellular environment might also affect stability. Thus the hybrids from the B(P^s) \times H(P^u) fusion expressed the *H* allele less than would have been expected on the basis of the residual μ expression in the *H* parent. Also, the relatively stable *H* allele was extinguished in one of the hybrids (4) from the B(P^s) \times H(P^s) fusion. These observations suggest that expression of the μ gene might

have been more unstable in the hybrid cell environment than in the parent cell.

Mechanism of variegation in stability and expression:

The model in Figure 4 illustrates how a single graded molecular feature that is self-templating and *cis*-acting can account for the variegation in both the stability and expression of the μ gene. We undertook to test a specific version of this model, in which cytidine methylation is the self-templating, *cis*-acting feature. In this version of the model, expression would be positive or negative when the extent of cytidine methylation in the μ gene was below or above, respectively, a threshold, and expression would be stable or unstable when methylation was greatly or slightly, respectively, beyond the threshold. Our finding that azaC activated more cells in the more unstable subclone argues that stability depended on methylation and is thus consistent with this model. However, azaC treatment did not convert positive cells from the unstable to stable state, and the pattern of cytidine methylation in the μ gene of stable and unstable subclones did not correlate with their stability. The significance of these negative results is uncertain. For example, these negative results might reflect insensitivity in our assays. Thus, stable and unstable positive cells might differ by methylation at multiple sites, but the frequency at which azaC converted unstable positive cells to the stable state might have been too low for us to detect by examining only \sim 24 subclones. Similarly, stability might depend on the state of cytidine methylation in regions that we could not investigate, *e.g.*, multi-copy regions such as the region 5' of the functional VDJ or the 3' LCR. As noted in the RESULTS, another possibility is based on the observation that cytidine methylation can activate gene expression by inactivating insulators and thus allowing access of the gene to a distal enhancer, as in the case of the Igf2 gene (BELL and FELSENFELD 2000; HARK *et al.* 2000). Similarly, variegation of the LCR-deficient IgH locus might have reflected different states of an insulator, and the positive state of μ expression and greater stability might have corresponded to increasing methylation rather than demethylation. This simple form of the model does not fit with our finding that azaC increased the number of μ -positive cells, but perhaps the azaC-induced expression that we observed for the μ gene occurred by an unrelated mechanism, *e.g.*, by activating expression of an ancillary transcription factor.

Some observations made with other systems argue that cytidine methylation is not the graded feature that determines stability. Thus, in an analysis of an Aprt transgene, stability of expression was not obviously correlated with methylation (YATES *et al.* 2003). Also, LORINCZ *et al.* (2002) found that a partially methylated DNA segment was rapidly converted to the extreme states of fully unmethylated or fully methylated. The rapid conversion contrasts with our finding for the μ gene and the findings of others for the Aprt gene (YATES *et*

al. 2003) that intermediate states of stability could be maintained through many cell divisions. The model also implies that the differences in methylation are maintained by a *cis*-acting mechanism and thus contrasts with the finding that methylation of transgenes in mouse spermatocytes and of the *Aprt* locus in kidney cells was transferred from one allele to another (ROSE *et al.* 2000; RASSOULZADEGAN *et al.* 2002). In addition to cytidine methylation, many other features of chromatin vary with expression, *viz.*, histone acetylation and methylation, subnuclear localization, replication timing, and of course, binding of transcription factors. The “histone code” in particular provides a mechanism for determining a multitude of different epigenetic states, such as were seen for the LCR-deficient μ -gene (TURNER 2002). For example, the stable and unstable negative states might correspond to histones that are fully or partially methylated on lysine 9, respectively. However, it is unclear whether histone modifications or other features of chromatin can be self-templating.

Clonal variations in stability of other loci: Analyses with several systems have indicated that an important function of the LCR is to maintain gene expression in a stable, positive state (FIERING *et al.* 2000). Nevertheless, unstable expression might be a physiologically important feature of some genes. As noted in the Introduction, expression of the *IL4* gene in T cells behaves very much like the LCR-deficient IgH locus: *IL4* is expressed in only a fraction of the cells in a T-cell clone; this fraction differs among T-cell clones and is a clonally inherited feature (HU-LI *et al.* 2001; GUO *et al.* 2002). For some genes, different levels of stability might even persist through meiosis. Thus, the level of expression of the *agouti* locus in mice was found to be heritable and to correlate with the extent of methylation in the *agouti* locus (MORGAN *et al.* 1999).

Our results also emphasize that it is not necessary to postulate genetic changes, such as gene rearrangement, to account for allele-specific differences in gene expression that have been seen for other mammalian genes such as the *Ly49* and *NKG2* receptors of natural killer cells and olfactory receptor genes (CHESS *et al.* 1994; HELD *et al.* 1995; TANAMACHI *et al.* 2001; VANCE *et al.* 2002).

Identification of transcription-activating elements—some cautions: Transgene expression is notoriously variable, and the variability is usually attributed to the effects of transgene copy number and neighboring chromatin. An LCR has traditionally been presumed to overcome variability by overcoming the effects of copy number and chromosomal context. We have shown that the LCR-deficient μ gene can exist in multiple distinct states, which range from undetectable to high levels of expression and from high to low stability, even though the μ gene is present as a single copy in a constant chromosomal context in cells that maintain the same set of transcription factors. Our results thus suggest that the different

levels of expression that have been documented for LCR-deficient transgenes might not be due to effects of insertion site and copy number. Our results also emphasize the caution that is needed in using comparative analyses to detect a DNA element that affects gene expression.

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