Locus Control Region Function and Heterochromatin-Induced Position Effect Variegation

Richard Festenstein,* Mauro Tolaini, Paola Corbella, Clio Mamalaki, Jenny Parrington, Margaret Fox, Antigoni Miliou, Margaret Jones, Dimitris Kioussis*

Human CD2 locus control region (LCR) sequences are shown here to be essential for establishing an open chromatin configuration. Transgenic mice carrying an hCD2 minigene attached only to the 3′ CD2 transcriptional enhancer exhibited variegated expression when the transgene integrated in the centromere. In contrast, mice carrying a transgene with additional 3′ sequences showed no variegation even when the latter integrated in centromeric positions. This result suggests that LCRs operate by ensuring an open chromatin configuration and that a short region, with no enhancer activity, functions in the establishment, maintenance, or both of an open chromatin domain.

Locus control regions (LCRs) direct high-level and tissue-specific expression of linked genes in transgenic mice. This expression is independent of the site of integration in the host genome (*1*–*8*). Such a region was identified within the 3′ flanking region of the human CD2 (hCD2) gene with the use of transgenic mice (*2*). Because chromosomal position usually affects transgene expression negatively, it is thought that LCRs act by establishing open chromatin domains (*9*). To understand the mechanism of action of the hCD2 LCR, we established both the chromosomal position and composition of the integration site as well as the specific DNA sequences within the hCD2 LCR necessary to overcome negative position effects.

Deletional analysis has shown that 2 kb of 3′ flanking region is sufficient for hCD2 LCR function (*10*). We used high-resolution deoxyribonuclease I (DNase I) hypersensitivity assays to obtain our data (Fig 1C) (*11*). Three clusters of sites were identified within the 2-kb 3′ flanking region (HSS 1–3). The upstream HSS cluster (HSS 1) of strong hypersensitive sites coincides with the region that functions as a classical enhancer, whereas the weak HSS 2 and HSS 3 have no enhancer activity in transient transfection assays (*12*). We investigated the role of HSS 3 in vivo by generating transgenic mouse lines with constructs in which these sites were deleted (Fig. 1D).

To assess expression patterns in these mice, we measured with flow cytometry the amount of CD2 protein on the surface of individual cells (*13*). This allowed us to distinguish between two types of position effects: (i) decreased expression in all cells and (ii) variegated expression, in which a gene is silenced only in a proportion of cells of the same lineage (*14*). Variegated gene expression in mice has previously been associated with integration at the X (*15*) and Y (*16*) chromosomes. Transgenic lines carrying the full LCR showed position-independent expression at the protein level (*11*) and showed a characteristic unimodal distribution of CD2 on all thymocytes and T cells (Fig. 1A). In contrast, thymocytes from a proportion of transgenic mouse lines carrying the hCD2 minigene linked to either 1.5 kb or 1.3 kb of immediate 3′ flanking DNA (in which HSS 3 was partially deleted or omitted) showed a mosaic expression pattern. Thus, five out of seven CD2–1.5 kb transgenic lines showed the expected unimodal distribution of expression. The remaining two produced a bimodal distribution indicated by thymocyte populations either positive or negative for hCD2 (Fig. 1A). Three out of six CD2–1.3 kb transgenic lines showed a similar bimodal distribution of hCD2 expression (Fig. 1A).

In contrast to a study showing that concatamerization of a P element would induce silencing (*17*), the effect described here is apparently independent of transgene copy number [that is, all lines are variegating irrespective of copy number (ranging from 4 to 40 copies)]. This effect is also independent of orientation because two out of four additional transgenic lines carrying the 1.3-kb deletion in the reverse orientation also showed bimodal distributions of hCD2 ex-

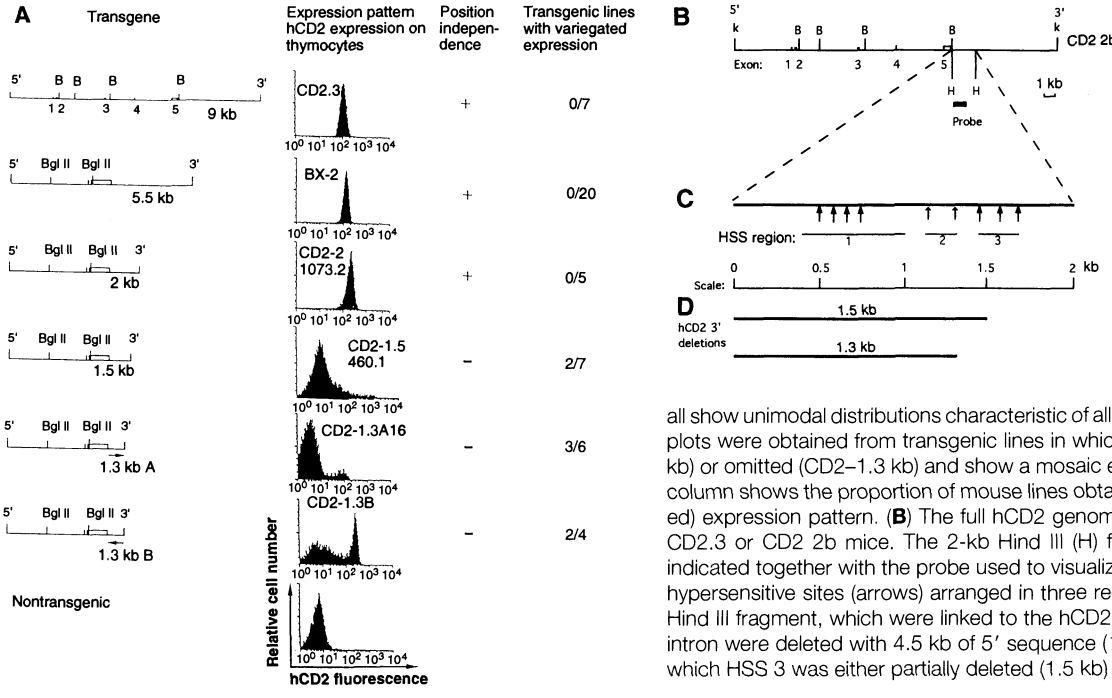

Fig. 1. Mosaic expression is a consequence of deletion of hCD2 LCR downstream DNase I hypersensitive sites. (**A**) Fluorimetric analysis of hCD2 expression on T cells from hCD2 transgenic mice. The constructs carried by each of the transgenic lines are depicted on the left. Levels of hCD2 expression are shown as histogram plots of fluorescence intensity; B, Bam HI. The upper three plots (obtained from transgenic lines with the complete LCR) all show unimodal distributions characteristic of all the lines analyzed. The lower three plots were obtained from transgenic lines in which HSS 3 was truncated (CD2–1.5 kb) or omitted (CD2–1.3 kb) and show a mosaic expression pattern. The right-hand column shows the proportion of mouse lines obtained with such a mosaic (variegated) expression pattern. (**B**) The full hCD2 genomic transgene construct carried by CD2.3 or CD2 2b mice. The 2-kb Hind III (H) fragment with full LCR function is indicated together with the probe used to visualize it. (**C**) Map showing the DNase I hypersensitive sites (arrows) arranged in three regions. (**D**) 3′ deletions of the 2-kb Hind III fragment, which were linked to the hCD2 minigene [in which all but the first intron were deleted with 4.5 kb of 5′ sequence (*10*)] to generate transgenic lines in which HSS 3 was either partially deleted (1.5 kb) or omitted (1.3 kb).

pression on thymocytes and peripheral T cells (CD2–1.3B; Fig. 1A). This mosaic expression pattern was apparent in all thymocyte and peripheral T cell subsets identified by concomitant staining with CD4 and CD8 antibodies (*11*). This suggests that the decision to express is made early in differentiation.

Thus, the loss of position independence of the hCD2 LCR deletion transgene is manifested as a mosaic expression pattern rather than as a lower expression level in all cells. This phenotype is similar to position effect variegation (PEV) as described in *Drosophila* (*14*) and yeast (*18*), in which cell to cell variation of gene expression within a cell lineage is correlated with the translocation of a normally euchromatic gene to centromeric heterochromatin. To further investigate the analogy with PEV, we immunostained thymus sections (*19*) from the CD2–1.3 kb transgenic lines, which show mosaic expression for hCD2. Such staining showed thymocytes clustered in groups that were either positive or negative for hCD2 (Fig. 2A), which confirmed a variegated phenotype. It also implies that the observed phenotype may be clonally stable.

Fluorescence in situ hybridization (FISH) (*20*) showed that the transgene in each of the variegating strains is located within pericentromeric heterochromatic regions (Fig. 3). Thus, these mice exhibit the two characteristic features of classical PEV: (i) variegated expression within the tissue and (ii) variegation associated with transgene location at the centromere. In contrast, integration at the centromere of transgenes with a complete LCR did not show variegation of expression (Fig. 3C and Table 1).

One explanation for gene inactivation in PEV is variability in chromatin condensation (*21*). We tested this hypothesis by separating thymocytes from variegated thymuses according to hCD2 expression status (*22*). Nuclei from hCD2-positive ($hCD2^+$) or $hCD2^-$ thymocytes from variegating mice were subjected to DNase I hypersensitive site analysis (*23*). Southern (DNA) blot analysis showed that with increasing DNase I concentration, specific bands appeared in the DNA from the $hCD2^+$ cells, indicating cleavage at HSS 1. In contrast, DNA from the $hCD2^-$ cells (97% pure) revealed no such DNase I sensitivity (Fig. 4). Thus, gene inactivation associated with classical PEV in mammals is also associated with a closed chromatin configuration. This result is in agreement with recent experiments on PEV in *Drosophila* (*24*).

Positive cells with the highest expression obtained from the variegating lines express hCD2 in a manner dependent on transgene copy (*11*). This is consistent with the findings of Robertson *et al.* (*25*) and indicates that the hCD2 enhancer (HSS 1)

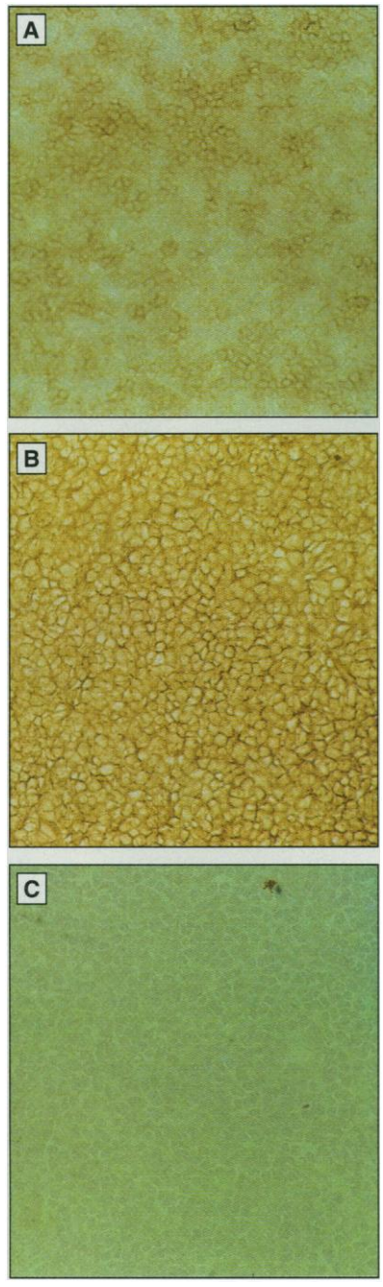

Fig. 2. Omission of DNase I HSS 3 results in variegated expression of hCD2 within the transgenic mouse thymus. (**A**) Cryostat section from a CD2–1.3 kb transgenic thymus stained with hCD2 monoclonal antibody reveals clusters of cells either positive or negative for hCD2 expression. This result was repeated for two other variegating strains (CD2–1.3A9 and CD2–1.3A16). (**B**) Cryostat section from a hCD2 transgenic thymus from a mouse carrying a hCD2 transgene with full LCR (CD2 2b). The uniform pattern of staining indicates that all thymocytes are expressing the transgene. (**C**) Cryostat section from a nontransgenic thymus stained with hCD2 monoclonal antibody.

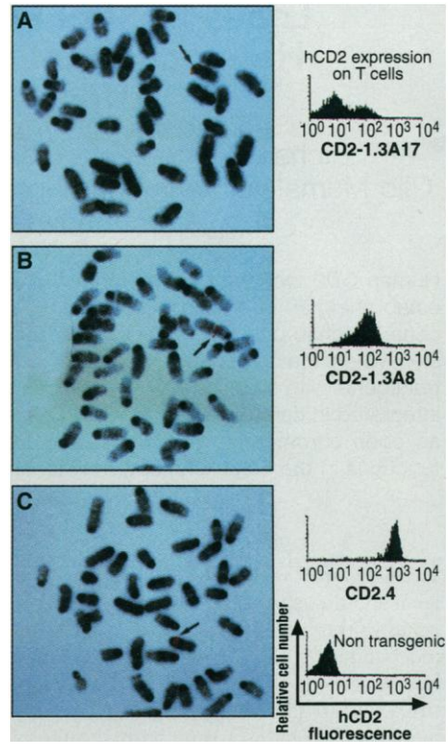

Fig. 3. Variegation of expression is associated with location of LCR deletion transgenes at the centromere. Left: images of mouse metaphase chromosomes, shown reversed on a white background after FISH (*20*). Right: the hCD2 expression pattern on peripheral T cells from these mice (*13*). Transgene signals are shown in red (arrow). (**A**) Transgene signal from CD2–1.3A17, a transgenic mouse showing variegated hCD2 expression (right) with the transgene signal located on centromeric heterochromatin in a telocentric position; this result was reproduced with other variegating transgenic mice: CD2–1.3A16, CD2–1.3B, and CD2–1.3A14 (Table 1) (*33*). (**B**) Transgene signal from CD2–1.3A8, a mouse carrying an LCR deletion construct with a unimodal pattern of expression (right). The transgene signal was found midway down the long arm of chromosome 1. Another transgenic line with unimodal expression carrying the LCR deletion construct also had its transgene located in euchromatin (CD2–1.3A3) (*33*). (**C**) Transgene signal from CD2.4, a line carrying an hCD2 transgene with full LCR and unimodal expression (right), with the signal found in the centromeric region. This result was reproduced with other transgenic lines carrying the full LCR (Table 1) (*33*). Five other lines carrying the hCD2 transgene with full LCR were analyzed, and in each case the transgene was found within euchromatic regions (*33*). Images were made from five metaphases per transgenic mouse. The FACS analysis was done as described (*13*).

R. Festenstein, M. Tolaini, P. Corbella, C. Mamalaki, D. Kioussis, Division of Molecular Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.
J. Parrington, M. Fox, A. Miliou, Medical Research Council Human Biochemical Genetics Unit, Galton Laboratory, University College London, Wolfson House, 4 Stephenson Way, London NW1 2HE, UK.
M. Jones, Nuffield Department of Pathologgy, John Radcliffe Hospital, Oxford OX3 9DU, UK.
*To whom correspondence should be addressed.

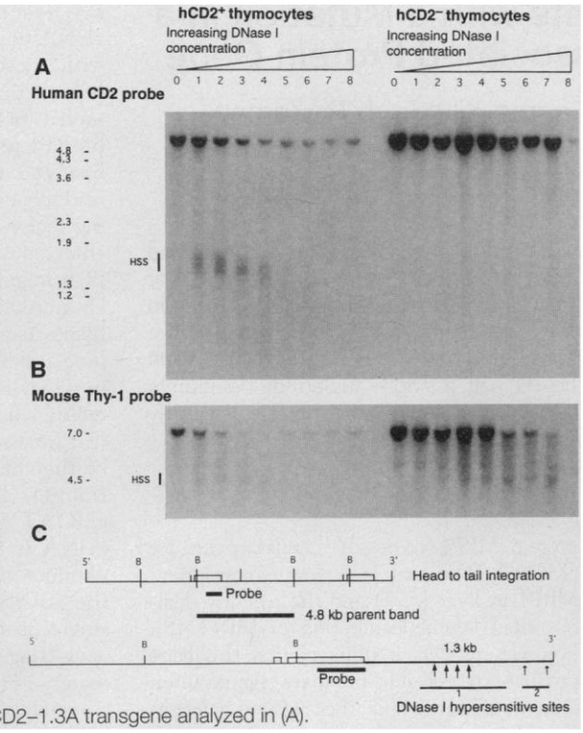

Fig. 4. The LCR in hCD2$^+$ thymocytes is more sensitive to DNase I than that in hCD2$^-$ thymocytes. (**A**) Southern blot analysis of DNase I–treated thymocytes from variegating mice (CD2–1.3A16). Nuclei from hCD2$^+$ or hCD2$^-$ cells were treated with DNase I. Treated DNA was digested with Bgl II and subjected to Southern blot analysis. Hypersensitive sites (HSSs) were detected only in the hCD2$^+$ nuclei. This result was reproduced with mice from another transgenic line, showing variegation of hCD2 expression (CD2–1.3B). Size markers are shown on the left in kilobases. (**B**) Southern blot analysis of DNA in (A) hybridized to a Thy-1–specific probe. DNase I hypersensitivity associated with the endogenous Thy-1 gene was detected in both the hCD2$^+$ and hCD2$^-$ cells. We loaded 30% more DNA from the hCD2$^-$ thymocytes, thereby increasing the sensitivity of the assay. (**C**) Top: The predicted 4.8-kb DNA Bgl II fragment and the probe that was used to visualize this fragment; B, Bgl II restriction site. Bottom: The positions of HSS shown within the CD2–1.3A transgene analyzed in (A).

Table 1. Position effect variegation in mice transgenic for the hCD2 gene attached to a truncated LCR depends on the chromosomal location of the site of integration; *n*, number of independent transgenic lines analyzed; "−," no variegated expression; "+," variegated expression.

Site of integration	Variegation in mice	
	With full LCR	CD2–1.3 kb
Euchromatin	− (*n* = 5)	− (*n* = 2)
Centromere	− (*n* = 3)	+ (*n* = 4)

can function provided the transgene in these thymocytes is in an active chromatin configuration. These results also indicate that HSS 3 is required for each cell to establish an active transcriptional unit.

Although DNA elements (insulators) that can prevent chromosomal position effects have been described, all those tested are incapable of overcoming centromere-associated PEV (*26*). In addition, the hCD2 LCR probably does not act simply as an insulator (*27*) because similar expression patterns have been observed in mice carrying a transgene with the immunoglobulin (Ig) enhancer placed 3′ of the full hCD2 LCR (*28*). In that case, variegation seemed to be the result of a cis effect made possible by the Ig enhancer and could not be prevented by the presence of HSS 3. Finally, recent experiments suggest that insulators or border elements do not propagate changes in chromatin structure (*29*). Here, these effects are clearly associated with chromatin configuration changes. Thus, we propose that early in T cell development tissue-specific "chromatin opening" proteins or transcription factors would compete with heterochromatin-associated proteins in the establishment of an open chromatin domain (*30*). Without HSS 3, the outcome of this competition is uncertain. These results are consistent with the hypothesis that enhancers act by increasing the probability of forming a stable transcription complex at the promoter (*31*). Our system suggests that additional LCR sequences turn this probability into a certainty, so that an active transcriptional complex is established in every cell even if the gene is located within a heterochromatic region. Because the variegation described here is an early event in thymocyte development, we propose that this mode of gene regulation may be involved in the acquisition and maintenance of a particular phenotype (lineage commitment), particularly at those branch points in differentiation where randomness appears to underlie decisions taken by the cell.

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19. Cryostat sections were stained with biotinylated monoclonal antibody [anti-hCD2, MAR206; A. Moretta *et al.*, *Eur. J. Immunol.* **16**, 1427 (1986)] and secondary antibody [StreptABComplex, horse radish peroxidase–conjugated (DAKO K377)] as described (*2*).
20. Metaphase spreads were obtained from transgenic mouse spleens cultured for 2 days after lipopolysaccharide stimulation (Sigma; final concentration, 20 μg/ml). The hCD2 DNA probe was labeled and hybridized with the metaphase spreads following procedures previously described [D. Pinkel, T. Staume, J. W. Grey, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2934 (1986); B. Gharib *et al.*, *Ann. Hum. Genet.* **57**, 9 (1993)] and analyzed with a Zeiss Axiophot fluorescence microscope. The images were collected with a cooled charge-coupled device camera (Photometrics) with capture software (Digital Scientific).
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23. Nuclei from both hCD2$^+$ or hCD2$^-$ thymocytes were subjected to DNase I digestion (Sigma) (*2*). Extracted DNA was subjected to Southern analysis (*32*) after digestion with Bgl II. The probe used was a ^{32}P-labeled hCD2 3′ flanking probe extending 500 bp downstream from the polyadenylation signal. The blots were stripped and rehybridized with a 700-bp 3′ endogenous Thy-1 probe, which is an Apa I fragment from the fourth exon [E. Spanopoulou, thesis, University of London (1990)].
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