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## DNA triplet repeats mediate heterochromatin-protein-1-sensitive variegated gene silencing

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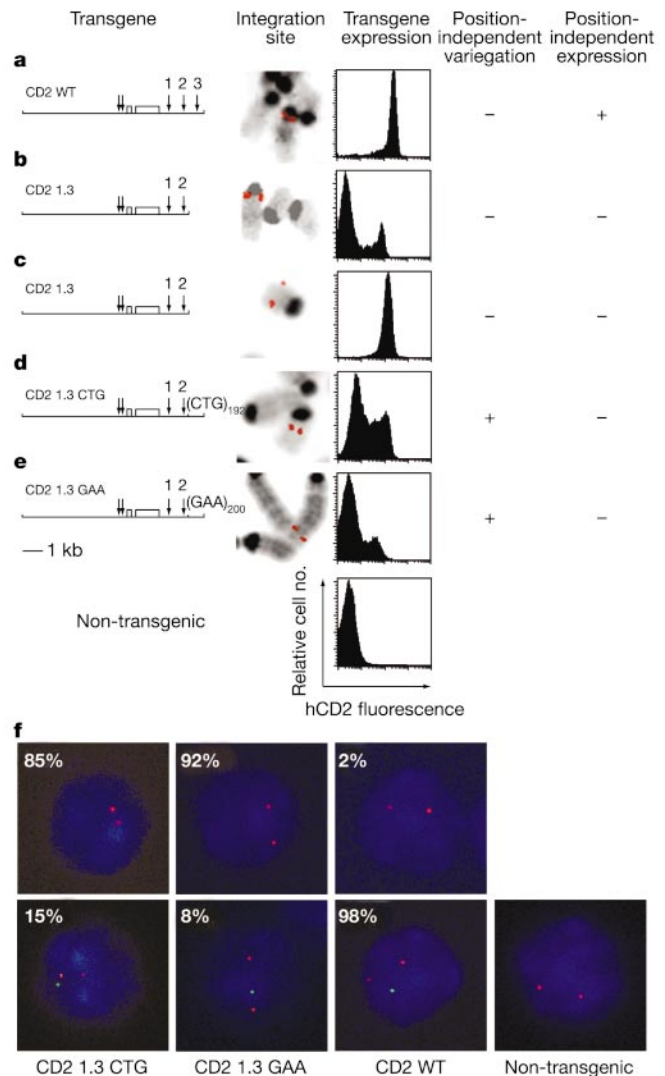
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Gene repression is crucial to the maintenance of differentiated cell types in multicellular organisms, whereas aberrant silencing can lead to disease. The organization of DNA into chromatin and heterochromatin<sup>1</sup> is implicated in gene silencing. In chromatin, DNA wraps around histones, creating nucleosomes. Further condensation of chromatin, associated with large blocks of repetitive DNA sequences, is known as heterochromatin. Position effect variegation (PEV) occurs when a gene is located abnormally close to heterochromatin, silencing the affected gene in a proportion of cells<sup>1</sup>. Here we show that the relatively short triplet-repeat expansions found in myotonic dystrophy and Friedreich's ataxia confer variegation of expression on a linked transgene in mice. Silencing was correlated with a decrease in promoter accessibility and was enhanced by the classical PEV modifier heterochromatin protein 1 (HP1). Notably, triplet-repeat-associated variegation was not restricted to classical heterochromatic regions but occurred irrespective of chromosomal location. Because the phenomenon described here shares important features with PEV, the mechanisms underlying heterochromatin-mediated silencing might have a role in gene regulation at many sites throughout the mammalian genome and

modulate the extent of gene silencing and hence severity in several triplet-repeat diseases.

Although recent studies on modifiers of PEV have identified molecules (such as SUV39 and HP1) that participate to propagate heterochromatic structures<sup>2,3</sup>, very little is known about the DNA *cis*-acting requirements for heterochromatin formation. Certain transgenes are prone to variegation when repeated in tandem arrays<sup>4,5</sup>; however, some transgenes do not variegate despite being repeated in tandem<sup>1</sup>. Gene regulatory elements known as locus control regions (LCRs)<sup>6</sup> can overcome pericentromeric PEV and disabling them renders the expression of linked genes PEV-sensitive<sup>7,8</sup>. Aberrant gene silencing can occur in diseases—for example, deletion of an LCR results in thalassaemia<sup>9</sup>—whereas the expansion of GAA or CTG triplet repeats leads to gene silencing in Friedreich's ataxia<sup>10</sup> and myotonic dystrophy<sup>11,12</sup>, respectively. Consistent with the regular spacing of nucleosomes found in heterochromatin



**Figure 1** CTG or GAA triplet-repeat expansions confer position-independent variegation on a heterochromatin-sensitive reporter. **a**, CD2 wild type, the hCD2 minigene with LCR<sup>7</sup>, expressed hCD2 on all T cells despite a pericentromeric location. **b, c**, Transgenes lacking LCR HSS3 (CD2 1.3) showed variegation when pericentromeric (**b**) and unimodal expression when in the chromosomal long arm (**c**). **d, e**, However, when linked to 192 CTG (**d**) or 200 GAA (**e**) repeats, expression of the CD2 1.3 transgene was variegated irrespective of location. kb, kilobase. **f**, Primary-transcript RNA FISH. Hybridization of thymocytes from CD2 1.3 CTG and CD2 1.3 GAA lines with mouse CD2 (red) and hCD2 (green) probes revealed hCD2 variegation at the transcriptional level.

Table 1 **CTG and GAA repeats confer variegation on a linked hCD2 transgene irrespective of its chromosomal location**

	CD2 with full LCR†	CD2 1.3‡	CD2 1.3 CTG	CD2 1.3 GAA
Pericentromeric integration	U (n = 3)	V (n = 4)	V (n = 5)	V (n = 1)
Peritelomeric integration	– (n = 0)	U (n = 1) V (n = 1)	V (n = 2)	V (n = 2)
Integration in long arm of chromosome	U (n = 5)	U (n = 4) V (n = 2)	V* (n = 3)	V* (n = 5)
Variegation	None	Position-dependent variegation	Position-independent variegation	Position-independent variegation

V, variegates; U, unimodal expression; n, number of transgenic lines.

\* $P < 0.05$ , exact one-sided midpoint  $P$  values. Contingency tables were constructed to compare numbers of CD2 1.3 variegating lines (with chromosomal long-arm transgene integration sites) that showed variegation with and without triplet repeats. The STATXACT program (Cytel Corporation, Massachusetts, USA) was used to analyse the results and obtain exact probabilities.

† The figures shown for these transgenic lines were obtained in a previous study<sup>7</sup>.

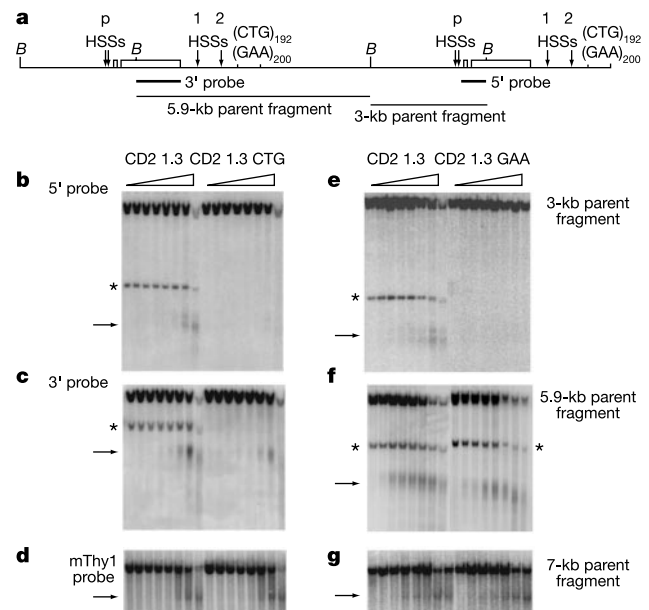
‡ These figures combine the results obtained from six lines analysed previously<sup>7</sup> with the results obtained after an identical analysis of six new lines.

*in vivo*<sup>13</sup> is the observation that CTG repeats ‘position’ nucleosomes *in vitro*<sup>14</sup>. Here we investigate the hypothesis that such triplet repeat expansions might act to stimulate heterochromatin formation. In myotonic dystrophy, a CTG expansion in the untranslated part of the dystrophin myotonia protein kinase (*DMPK*) gene, which is located immediately upstream of the *Six 5* gene, results in *Six 5* downregulation and decreased DNase I accessibility at its promoter<sup>15</sup>. Mice deficient in *Six 5* develop cataracts—a feature of myotonic dystrophy<sup>16</sup>. Although *DMPK* might also be downregulated in patients with myotonic dystrophy<sup>17,18</sup>, the CUG-containing RNA forms nuclear aggregates<sup>19</sup> and has a toxic gain-of-function effect resulting in the disease phenotype<sup>20</sup>. In Friedreich’s ataxia, GAA expansion in the intron of the *X25* gene causes its downregulation. Although studies *in vitro* suggest that expanded GAA repeats can form a triplex DNA structure<sup>21</sup>, thereby impeding transcriptional elongation<sup>22</sup>, their effect on chromatin packaging is unknown.

To determine whether CTG or GAA expansions could silence genes at the level of chromatin packaging, we used a heterochromatin-sensitive human CD2 (hCD2) transgene as a reporter. There are three DNase I-hypersensitive sites (HSSs) within the hCD2 LCR (Fig. 1a), which directs hCD2 expression to all T cells in transgenic mice irrespective of the chromosomal location of the transgene<sup>7,23</sup>. The omission of HSS3 (Fig. 1b and c) renders the transgene (CD2 1.3) susceptible to silencing by heterochromatin (for example by pericentromeric integrations) resulting in variegation of hCD2 expression (Fig. 1b). In contrast, CD2 1.3 transgenes, which integrate in euchromatic locations (such as the chromosomal long arm; Fig. 1c), express hCD2 on all T cells<sup>7</sup> over a range (4–25 copies) of transgene copy numbers<sup>7</sup>. By generating and analysing (as described previously<sup>7</sup>) six additional CD2 1.3 transgenic lines, the data presented here extend the initial observation that the CD2 1.3 transgene is sensitive to chromosomal location (see Table 1) and that large CD2 1.3 tandem arrays (up to 25 copies) are not sufficient to induce variegation. The variegation observed in some non-centromeric locations suggests that this reporter can be used as a ‘gene trap’ to identify heterochromatic sites in the genome that cannot be identified cytogenetically<sup>24</sup>. These properties of the CD2 1.3 transgene allowed us to assay the heterochromatin-forming potential of DNA sequences *in vivo*.

We reasoned that if expanded triplet repeats were capable of mediating a heterochromatin-like effect on a juxtaposed CD2 1.3 transgene, the outcome would be variegation of hCD2 expression irrespective of the chromosomal location of the transgenes. We linked 192 CTG or 200 GAA repeats to the CD2 1.3 reporter gene (Fig. 1d and e) to generate ten CD2 1.3 CTG and nine CD2 1.3 GAA transgenic mouse lines. Southern blot analysis revealed each line to contain ‘head-to-tail’ (5′ → 3′) tandem arrays with a range of transgene copy numbers from 3 to 25 for the CD2 1.3 CTG lines and from 3 to 27 for the CD2 1.3 GAA lines. Each line contained about 190 CTG repeats or 200 GAA repeats as well as some expanded and contracted repeats that remained in the pathological range (namely 70–350 CTG repeats, or 40–500 GAA repeats); somatic repeat instability was undetectable at 4–8 weeks of age.

Transgene location was determined by fluorescence *in situ* hybridization (FISH) and hCD2 expression by flow cytometry using fluorescently labelled anti-hCD2 antibody<sup>7</sup>. The mean fluorescence detected on the surface of the T cells was previously shown to be directly correlated with the steady-state hCD2 messenger RNA levels (see Supplementary Fig. S1a). Strikingly, expression of hCD2 on thymocytes and mature T cells was variegated in all lines and occurred at pericentromeric, peritelomeric and long-arm locations, and was therefore not dependent on chromosomal position (see Table 1). Three CD2 1.3 CTG lines and five CD2 1.3 GAA lines that had their transgenes located at different sites in the chromosomal long arm all showed hCD2 variegation (this was statistically significant at  $P < 0.05$  when compared with CD2 1.3 lines lacking the repeats with long-arm integration sites; see Table 1). Examples of this are shown in Fig. 1d and e. The histograms demonstrate bimodal hCD2 expression on T cells typical of variegation. That the variegation in these lines was occurring at the level of transcription was shown by using primary-transcript RNA FISH on cells from two independent CD2 1.3 CTG and CD2 1.3 GAA lines (examples are shown in Fig. 1f); northern blot analysis from these lines confirmed marked transcriptional repression (Supplementary

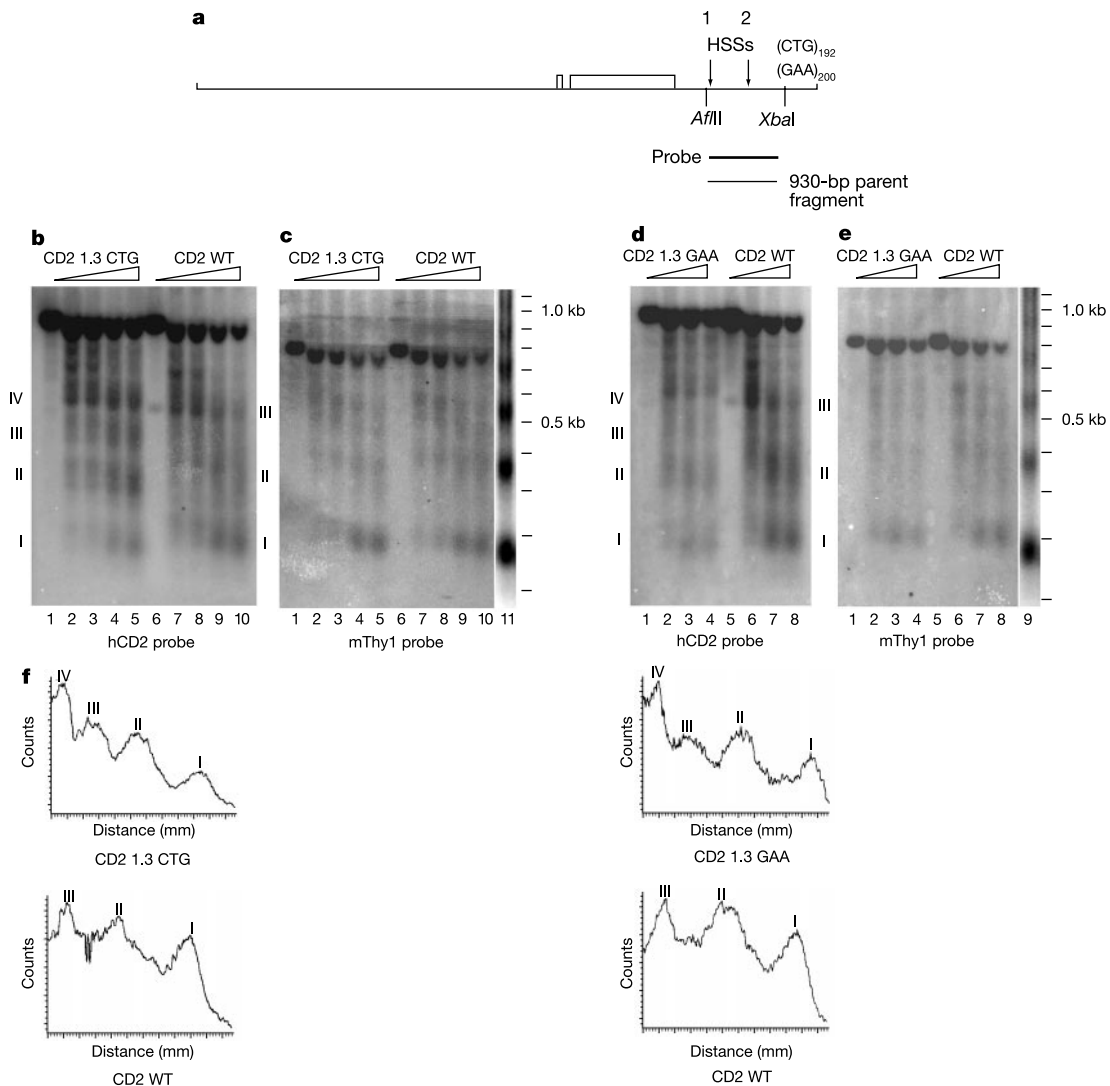


**Figure 2** Triplet-repeat-associated gene silencing correlated with inaccessible chromatin at the promoter but not the enhancer. **a**, Transgene map. *B*, *Bgl*III. **b–g**, DNase I hypersensitivity analysis<sup>7</sup> on thymocyte nuclei from CD2 1.3 CTG transgenics (**b**, **c**, **d**) or CD2 1.3 GAA transgenics (**e**, **f**, **g**) compared with CD2 1.3 nuclei lacking the triplet repeats. Transgenes were all in the chromosomal long arm. **b**, **e**, Promoter (p) HSS analysis; **c**, **f**, enhancer HSS analysis; **d**, **g**, *Thy1* HSS analysis as an internal control<sup>7</sup>. Arrows indicate bands resulting from treatment with DNase I. Asterisk indicates transgene end-fragments, the length of which was determined by mouse genome *Bgl*III sites.

Fig. S1b). Southern blot analysis on DNA obtained from T cells sorted according to hCD2 expression excluded the possibility that this bimodal hCD2 expression pattern resulted either from deletion of the transgene in the cells not expressing hCD2 or from truncation of triplet repeats in the hCD2-expressing cells (data not shown). Thus, the inclusion of CTG or GAA repeats within the CD2 1.3 transgene leads to chromosomal position independent variegation.

To investigate whether CTG or GAA repeat expansions could be mediating their effect by modifying chromatin structure, DNase I (Fig. 2) and micrococcal nuclease (MNase) (Fig. 3) accessibility studies were undertaken on transgenics with chromosomal long arm transgene locations. The former, to examine the DNase I hypersensitive sites, and the latter to assess the arrangement of nucleosomes proximal to the repeats. Aliquots of nuclei from transgenic thymocytes (of which less than 10% expressed the hCD2 transgene) were exposed to increasing concentrations of nuclease and the DNA obtained analysed by Southern blot as previously described<sup>7</sup>. Thymocyte nuclei from hCD2 transgenic mice (CD2 1.3), which express hCD2 on all T cells, were used as

controls (first 8 lanes in each Southern blot – Fig. 2b–g). The top blots (Fig. 2b and e) were hybridized with the 5' probe (Fig. 2a), thereby identifying the promoter HSS in CD2 1.3 transgenic thymocytes. Strikingly, this HSS was undetectable in nuclei obtained from either the CD2 1.3 CTG or the CD2 1.3 GAA transgenic mice (Fig. 2b and e). However, hybridization with the 3' probe revealed that the HSS located within the truncated LCR was formed irrespective of whether the CTG or GAA repeats were present (Fig. 2c and f). (The analysis represented in Fig. 2 was reproduced for an additional CD2 1.3 CTG and CD2 1.3 GAA line.) Thus, these repeats appear to specifically inhibit the formation of the promoter HSS, leaving the LCR HSS1 (previously shown to be an enhancer<sup>25</sup>) relatively unaffected. In contrast, both the promoter and enhancer HSS were undetectable when a CD2 1.3 transgene was silenced by proximity to pericentromeric heterochromatin<sup>7,24</sup>. This result may reflect differences in the 'chromatin packaging' ability of different repetitive DNA sequences (triplet repeats vs pericentromeric repeats) and is consistent with a probability/competition model for chromatin packaging<sup>9</sup> in which the enhancer is more



**Figure 3** Increased nucleosomal density in triplet-repeat proximal regions. **a**, DNA from MNase-treated thymocyte nuclei was digested to release a 930-bp fragment adjacent to the triplet-repeat insertion, and nucleosomal density was estimated by Southern blotting. WT, wild type. **b**, A more distinct and compact nucleosomal ladder is found adjacent to the CTG repeat (lanes 1–5) than in its absence (lanes 6–10). **c**, The same DNA probed for

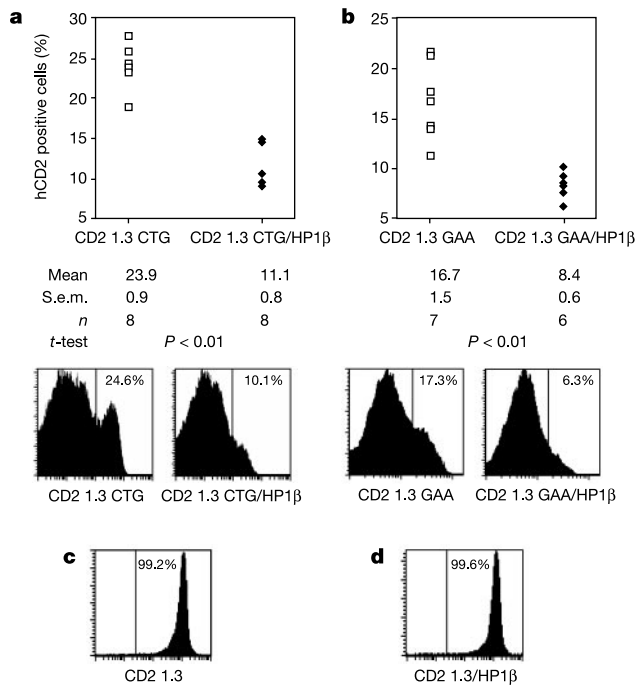
endogenous *Thy1*. **d**, DNA adjacent to the GAA repeat had a similar pattern to that in **b**, **e**. The same DNA analysed with the *Thy1* probe. The average nucleosomal density is shown by ethidium bromide staining at the far right. **f**, Phosphorimager quantification of lines (origin 0.6 kb) drawn downwards through lanes 4 and 9 in **b** and 3 and 7 in **d**.

effective than the promoter at competing with silencing factors to establish and maintain an open chromatin configuration. In addition, hypersensitivity at the promoter may be dependent on its interaction with the LCR hypersensitive site. The abnormal chromatin packaging induced by triplet repeats may inhibit this interaction. We therefore examined the chromatin structure of the region immediately adjacent to the repeats which contains the LCR HSS. Consistent with the finding that CTG repeats are very efficiently packaged into nucleosomal arrays *in vitro*<sup>14</sup>, our MNase studies indicated that, in the presence of the triplet repeats, the adjacent DNA had a more dense and ordered nucleosomal array than the wild type hCD2 transgene (Fig. 3b and f). More distinct bands were obtained with less background signal in the region proximal to the CTG repeats (Fig. 3b) compared to the same region when adjacent to the GAA repeats (Fig. 3d). This suggests that CTG repeats induce more precise nucleosome positioning than GAA repeats. The endogenous *Thy1* gene (which is transcribed in T cells and was used as a control for the extent of MNase digestion) showed a lower density of nucleosomal packaging (Fig. 3c and e) similar to the hCD2 wild type transgene which contained no triplet repeats. Notably, a recent study in *Drosophila* used the same approach to demonstrate that a transgene located in constitutive heterochromatin had a more compacted nucleosomal structure compared with an identical transgene located in euchromatin<sup>13</sup>. Thus, the inclusion of CTG or GAA repeats within the CD2 1.3 transgene not only leads to position-independent variegation but also correlates with a 'closed' chromatin structure at the promoter of the transgene. It may be that the promoter hypersensitive site cannot form because the triplet-repeat-associated heterochromatin inhibits the interaction of the enhancer hypersensitive site with the promoter. Such direct 'looping' may be essential for activation of the promoter and

has recently been shown to occur between the LCR and the  $\beta$ -globin locus<sup>26</sup>.

Recently, investigation of PEV modifiers has revealed mechanisms involved in heterochromatin formation. A powerful modifier of PEV is the methyltransferase Suv3-9 that establishes the binding site for HP1 by methylating Lys 9 on the tail of histone H3. Suv3-9 and HP1 are thought to associate with each other, thereby propagating heterochromatin along the chromosome<sup>2,3</sup>. Most PEV studies use genes located near cytogenetically defined heterochromatin such as the centromere. A key question is therefore whether similar heterochromatin-mediated silencing can occur elsewhere in the mammalian genome. We had previously shown that overexpression of the classical PEV modifier HP1 $\beta$  enhanced the silencing of pericentromeric CD2 1.3 transgenes in transgenic mice<sup>24</sup>. Because transgenes containing CTG or GAA repeats *in cis* exhibit variegation of expression even when located in the chromosomal long arm, we investigated whether HP1 $\beta$  dosage could influence the extent of this silencing. CD2 1.3 CTG and CD2 1.3 GAA transgenic mouse lines that carried their transgenes in the chromosomal long arm were crossed with transgenic mice overexpressing HP1 $\beta$  (fivefold increase over endogenous concentrations at the protein level; data not shown). Analysis of the offspring showed that HP1 $\beta$  overexpression, in both CD2 1.3 CTG and CD2 1.3 GAA transgenic mouse lines, significantly decreased the percentage of hCD2-expressing T cells (Fig. 4a and b), whereas in the absence of triplet repeats and variegation, the overexpression of HP1 $\beta$  had no effect on the proportion of expressing cells<sup>24</sup> (Fig. 4c and d). This result further implicates heterochromatin formation as the mechanism underlying this GAA or CTG repeat-mediated gene silencing.

Thus, CTG and GAA trinucleotide repeats, which expand pathologically in human disease, might exert their gene-silencing effect by a mechanism resembling heterochromatin-mediated PEV. Although it remains to be seen whether other examples of the many repeats found in vertebrate genomes are capable of similar effects, it is tempting to speculate that the opposing effects of LCRs and repetitive DNA on chromatin structure and gene expression are a reflection of their evolutionary interdependence. That HP1 has a role in gene repression is suggested by two recent studies: one correlated the binding of HP1 to retinoblastoma protein with the silencing of the cyclin E gene<sup>27</sup>, and the other correlated methylation of H3 Lys 9 with gene silencing<sup>28</sup>. A recent study in *Drosophila* suggested that HP1 participated in gene repression at two euchromatic locations<sup>29</sup>. To our knowledge the findings presented here provide the first evidence that PEV-like silencing can occur at multiple sites in the mammalian chromosomal long arm where most genes are located and therefore indicate that the modifiers of pericentromeric PEV might participate in physiological gene silencing in mammals. Our findings show that heterochromatin-like effects can be mediated by short CTG and GAA triplet repeats. Furthermore, because the repeats studied here cause gene silencing in human disease it is possible that the modifiers of PEV might modify clinical severity. □



**Figure 4** Triplet-repeat-associated variegation is enhanced by overexpression of HP1 $\beta$ . **a, b**, Scatter plots showing proportions of hCD2-expressing T cells. Representative histogram plots are shown below. **a**, CD2 1.3 CTG mice were compared with siblings overexpressing HP1 $\beta$ . **b**, A similar analysis of CD2 1.3 GAA mice. The result was reproduced in two independent CD2 1.3 CTG and CD2 1.3 GAA lines. **c, d**, Overexpressing HP1 $\beta$  (**d**) in a CD2 1.3 line (i.e. lacking triplet repeats) that does not variegate had no effect on the proportion of hCD2-expressing T cells in comparison with the control (**c**).

Methods

Mice

All recombinant DNA manipulations were performed with standard techniques. The plasmids containing the expanded CTG and GAA sequences were a gift from Mauro Santibanez-Koref. The CTG or GAA repeat was subcloned at the 3' end of the hCD2 1.3 construct<sup>12</sup> by using the *KpnI-XbaI* or *KpnI-BamHI* sites, respectively. Sequencing confirmed that about 190 CTG repeats and 200 GAA repeats were contained within the plasmids and the joints were intact. The resulting hCD2 1.3 CTG and hCD2 1.3 GAA constructs were lifted out with the *SalI* and *NotI* enzymes, purified with Elutip-D columns (Schleicher & Schuell) and injected into mouse C57Black6/CBA Ca fertilized eggs. Founder transgenic mice were crossed with CBA Ca mice to establish transgenic lines. The project was given ethical approval by Imperial College and the UK Home Office.

Flow cytometric and FISH analysis

Flow cytometric and FISH analyses of second-generation transgenic mice were performed as described previously<sup>7</sup>. Primary-transcript RNA FISH was done essentially as described

previously<sup>30</sup>. In brief, thymocyte suspensions were prepared and left to settle for 90 s on poly(L-lysine)-coated slides (Sigma), which were then fixed in 4% formaldehyde, 5% acetic acid in normal saline for 20 min at 23 °C. This was followed by three sequential 5-min washes in PBS. Slides were then equilibrated with 70% ethanol for 5 min followed by 5 min in 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl. This was followed by digestion in 0.01% pepsin in 0.01 M HCl for 5 min at 37 °C, a brief rinse in water and fixation in 3.7% formaldehyde in PBS at 23 °C. They were then washed and dehydrated sequentially in 70%, 90% and 100% ethanol (3 min in each) and air-dried. Single-stranded biotinylated mouse CD2 intron probe (originally cloned after PCR of a mouse CD2 cosmid—a gift from D. Kioussis—using the primer pair 5'-GTTACTCCACCTCTTCAA-3' and 5'-GGCACTGTTTCCTGTTACTC-3') and a digoxigenin-labelled human CD2 cDNA probe were prepared by *in vitro* transcription followed by reverse transcription. The probes were denatured by heating at 80 °C for 5 min. Hybridization overnight in a humidified chamber was followed by sequential washes in 2 × SSC, then by blocking and detection with several layers of antibodies. For digoxigenin the antibodies were sheep anti-digoxigenin (Roche), rabbit anti-(sheep fluorescein isothiocyanate (FITC)) (Calbiochem) and goat anti-(rabbit FITC); for the biotinylated probe the antibodies were avidin D-Texas Red (Vector), goat anti-(avidin D-biotin) (Vector) and a final step with avidin D-Texas Red. Both intron and exon probes have been shown to identify primary transcripts generated at the site of transcription<sup>30</sup>. This procedure strips most of the cytoplasm from the cell, and leaves intact nuclei that were detected by staining with 4,6-diamidino-2-phenylindole. For microscopy, images were acquired with a Deltavision system (Applied Precision) and a cooled charge-coupled device camera (Photometrics CH350L) on an Olympus IX70 inverted microscope, with a 100 × 1.35 numerical aperture UPlanApo objective lens.

**Nuclease sensitivity**

Aliquots of nuclei obtained from 10<sup>8</sup> cells<sup>24</sup> were digested either for 4 min at 37 °C with increasing amounts of DNase I (0–6 µg ml<sup>-1</sup>) or for 5 min at 23 °C with MNase (Roche 107921; 0–500 U ml<sup>-1</sup>). After inactivation of nuclease, nuclei were treated with proteinase K and DNA was purified, digested and analysed by Southern blotting. The *Thy1* probe used for MNase analysis was a 749-base-pair *Pst*I–*Bam*HI fragment from the fourth exon of the endogenous *Thy1* gene.

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