(*12*, *27*). It may be that an HP1β molecule in heterochromatin, once released from its binding site, has a higher probability, within a given time period, of encountering another binding site, thereby slowing down its mobility compared with euchromatin. Homodimerization of HP1β molecules may also contribute to the reduced mobility in heterochromatin and possibly to the increased relatively immobile fraction found there.

The high mobility of HP1β suggests that heterochromatin maintenance is dynamic. Rather than statically prohibiting access to chromatin, changes in HP1β mobility could potentially regulate accessibility to factors that modify or displace nucleosomes and molecules involved in DNA function and metabolism.

To investigate this, we examined the kinetics of GFP-HP1β mobility during the nuclear reorganization that accompanies widespread induction of gene expression and entry into the cell cycle during T cell activation. We activated T cells by culturing them for 36 hours with antibodies against the T cell receptor and the costimulatory cell-surface molecule CD28. At this stage, when most cells had not divided but many had entered S phase (measured by bromodeoxyuridine incorporation), FRAP revealed a striking increase in GFP-HP1β mobility, in both heterochromatic clusters and euchromatic regions in activated T cells compared with resting cells (Fig. 2, B, D, and E). Thus, recovery to plateau level took 50 to 80 s after photobleaching heterochromatin in activated T cells compared with 150 to 200 s in unstimulated T cells. The shape of the fluorescence recovery curve obtained after photobleaching heterochromatin in activated T cells now more closely resembles a diffusion curve. Finally, activation resulted in a reduction in the immobile fraction within heterochromatin from ~30% in nonactivated T cells to ~10% in activated T cells. These results suggest that T cell activation has profound effects on the kinetics of GFP-HP1β mobility. Such an increase in HP1β mobility may play a role in the restructuring of heterochromatic regions. Thus, the increased mobility of HP1β that accompanies activation and entry into the cell cycle may reflect or cause a loosening of centromeric clusters, allowing centromeres to detach from each other before mitosis. In contrast, the 10% of HP1β remaining immobile in heterochromatin may be required for sister-chromatid cohesion during mitosis (*28*–*30*). Furthermore, increased HP1β mobility may provide increased opportunity for the transcriptional machinery to gain access to genes previously silenced by heterochromatin and thereby up-regulate their expression. Such mechanisms may modulate the access of transacting factors to chromatin, creating the potential for epigenetic modifications to take place at loci even when packaged into heterochromatin.

Our results suggest dynamic binding of HP1β to heterochromatin and euchromatin. This implies that, even in resting cells, most HP1β does not completely block access to the histone H3 tail but is continually recycling over a period of minutes, allowing intermittent access to other factors that potentially could modify the amino acid residues on the histone tails or displace nucleosomes. In addition, such intermittent associations of HP1β with H3 may potentially regulate H3 exchange, which recently has been invoked as a replication-independent mechanism for replacing epigenetic marks (*31*).

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32. Thanks to H.-J. Delecluse for mathematical advice and to T. Norton, M. Tolaini, and S. Uribe Lewis for technical assistance. This work was supported by the Medical Research Council UK.

Supporting Online Material

www.sciencemag.org/cgi/content/full/299/5607/719/DC1
Materials and Methods
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23 September 2002; accepted 13 December 2002

Maintenance of Stable Heterochromatin Domains by Dynamic HP1 Binding

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One function of heterochromatin is the epigenetic silencing by sequestration of genes into transcriptionally repressed nuclear neighborhoods. Heterochromatin protein 1 (HP1) is a major component of heterochromatin and thus is a candidate for establishing and maintaining the transcriptionally repressive heterochromatin structure. Here we demonstrate that maintenance of stable heterochromatin domains in living cells involves the transient binding and dynamic exchange of HP1 from chromatin. HP1 exchange kinetics correlate with the condensation level of chromatin and are dependent on the histone methyltransferase Suv39h. The chromodomain and the chromoshadow domain of HP1 are both required for binding to native chromatin in vivo, but they contribute differentially to binding in euchromatin and heterochromatin. These data argue against HP1 repression of transcription by formation of static, higher order oligomeric networks but support a dynamic competition model, and they demonstrate that heterochromatin is accessible to regulatory factors.

An important mechanism for epigenetic gene inactivation is packaging of silenced genes into repressive heterochromatin domains (*1*). Methylation of lysine-9 in histone H3 by Suv39h methyltransferases is a characteristic mark for heterochromatin and provides a binding site for HP1, one of the major heterochromatin proteins (*2*–*7*). Heterozygous loss of HP1 results in loss of gene silencing, whereas overexpression of HP1 results in increased gene silencing (*8*). In humans HP1 exists as three isoforms, HP1α, -β, and -γ,

each containing a chromodomain (CD) and a chromoshadow domain (CSD) separated by a hinge region (*8–10*). HP1 is generally believed to act as a structural adaptor by mediating stable macromolecular complexes between nucleosomes, possibly organizing higher order chromatin structures by cross-linking chromatin subunits. To gain insight into the mechanisms by which HP1 mediates formation and maintenance of heterochromatin in living cells, we investigated the kinetic binding of green fluorescent protein–tagged HP1 (GFP-HP1) to native chromatin using in vivo microscopy.

To visualize HP1 in living cells, CHO cell lines stably expressing GFP-HP1α, -β, or -γ under a tetracycline-repressible promoter were established (*11*). The fusion proteins behaved as expected for functional HP1 proteins, as they bound efficiently to a histone H3 peptide methylated on Lys^9, but only weakly to an unmodified or an H3 peptide acetylated on Lys^9 (fig. S1). Like endogenous HP1, the fusion proteins formed homo- and heterodimers with endogenous HP1 isoforms (*12*). An identical fusion protein of HP1α has previously been demonstrated to be fully functional in an in vivo invasion assay (*13*). All fusion proteins localized as expected in intensely labeled nuclear domains set against a diffuse nucleoplasmic background and as previously reported for the endogenous proteins, HP1α and -β formed larger heterochromatin domains than HP1γ, which was found in smaller heterochromatic foci (fig. S1) (*13–15*). Costaining with 4′,6′-diamidino-2-phenylindole (DAPI) and centromeric antibodies confirmed the identity of these domains as heterochromatin (fig. S1).

Exogenous chromatin loci have recently been demonstrated to undergo constrained diffusional movements in the interphase nucleus (*16–18*). Therefore, we investigated the stability of the endogenous heterochromatin domains enriched in GFP-HP1 in the nucleus of living cells. Three-dimensional stacks of optical sections of nuclei in living CHO cells expressing GFP-HP1α, -β, or -γ were acquired every minute for 10 min (Fig. 1). Time-lapse microscopy revealed that the HP1 domains were relatively immobile in position and that the number and the shape of domains were stable over time (Fig. 1A). To quantitatively express the positional changes of heterochromatin domains, we measured the distance between domains in the same focal plane at each time point, and we calculated the mean square changes in distance (Fig. 1B) (*16–18*). For each HP1 isoform, the relative displacement of heterochromatin domains between time points was typically about 0.14 μm per minute (Fig. 1B) (*16–18*). Identical observations were made for time scales of up to 2 hours. These observations demonstrate that heterochromatin domains form long-lasting and positionally stable structures inside the mammalian nucleus.

To study the binding properties of HP1 molecules within these stable heterochromatin domains in living cells, we performed fluorescence recovery after photobleaching (FRAP) on CHO cells stably expressing the GFP-HP1 isoforms. After irreversible bleaching of a spot using a 240-ms pulse with a 488-nm laser, we measured the recovery of fluorescence intensity in the bleached spot (Fig. 2, A and B). The recovery kinetics provide an indication of the binding capacity of the protein to chromatin (*19*, *20*). For these experiments, we defined heterochromatin on the basis of morphological criteria as regions strongly labeled with GFP-HP1, whereas euchromatin was defined as the less brightly labeled regions. For all HP1 isoforms, recovery was rapid and reached 50% of the prebleach intensity after 2.5 s in heterochromatin and after 0.6 s in euchromatin (Fig. 2, C to E). These recovery kinetics are considerably slower than for GFP alone, consistent with the notion that the slowed mobility is a result of the HP1 binding to the chromatin (*19*, *20*). Complete recovery was reached within 5 s in euchromatin, indicating that virtually the entire pool of GFP-HP1 had turned over from binding sites during that period (Fig. 2, C to E; fig. S2B). In heterochromatin, recovery reached 85% within 10 s and reached completion within ~60 s (Fig. 2, F to H). We observed identical recovery kinetics in tetracycline repression experiments, indicating that GFP-HP1 mobility is not affected by expression levels (fig. S2). Furthermore, similar results were observed in transient transfections of CHO, HCT116, and HeLa cells (*14*).

To ensure that the observed transient binding indeed reflected association of GFP-HP1 with its specific target sites in heterochromatin, we transfected GFP-HP1 into mouse embryonic fibroblasts (MEFs) from Suv39h1 and Suv39h2 double-null mice (*5*). The two methyltransferases Suv39h1 and Suv39h2 are responsible for creating HP1 binding sites in heterochromatin by methylation of histone H3-Lys^9 and loss of Suv39h1 and Suv39h2 results in impaired heterochromatin leading to genome instability (*5–7*). In cells from Suv39h double-knockout mice, GFP-HP1 was largely lost from heterochromatin, and FRAP analysis indicated an overall increased mobility of the fusion protein compared with control cells ($P < 0.001$) (Fig. 3, A to C). The observed dependence of GFP-HP1 mobility on Suv39h confirms that the measured mobility of HP1 in heterochromatin is a result of its binding to target sites created by Suv39h, and it confirms in vivo the fundamental role of Suv39h in HP1 binding and distribution.

If HP1 plays a structural role in chromatin organization, one might predict a correlation between chromatin structure and residence time of HP1. To test this prediction, we examined GFP-HP1 binding to altered heterochromatin organization after drug treatments (*21*, *22*). FRAP analyses of CHO cells indicated that GFP-HP1α is more readily exchanged in heterochromatin of cells treated with trichostatin A (TSA) or α-amanitin, which induce chromatin decondensation, compared with control cells ($P < 0.001$) (Fig. 3I; fig. S3). In contrast, GFP-HP1α exchange in heterochromatin is slowed upon chromatin condensation induced by treatment with actinomycin-D or

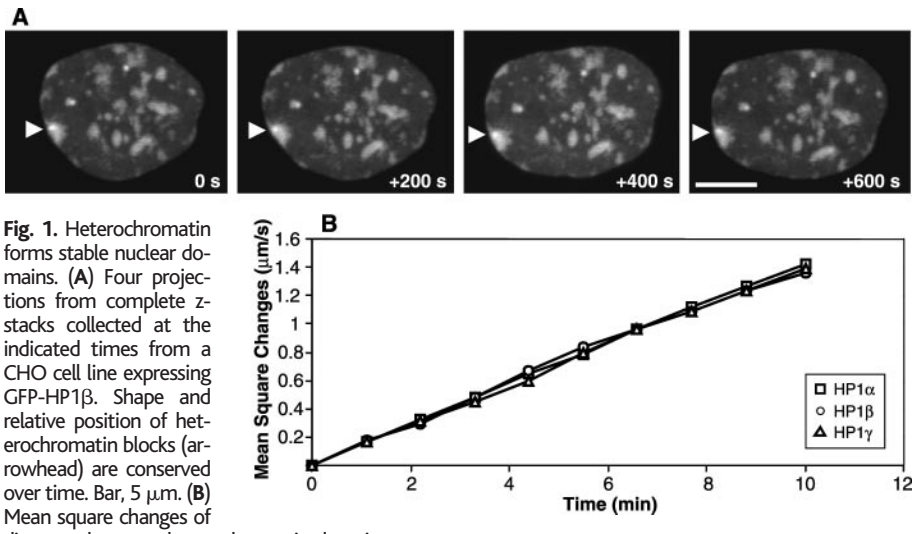

Fig. 1. Heterochromatin forms stable nuclear domains. (**A**) Four projections from complete z-stacks collected at the indicated times from a CHO cell line expressing GFP-HP1β. Shape and relative position of heterochromatin blocks (arrowhead) are conserved over time. Bar, 5 μm. (**B**) Mean square changes of distances between heterochromatin domains.

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in apoptotic cells ($P < 0.001$) (Fig. 3, H and I; fig. S3). Similar results were observed for all HP1 isoforms (*14*). These results indicate that HP1 binding correlates with global chromatin organization, consistent with a structural role of HP1 in heterochromatin formation.

We next analyzed the contribution of the two conserved protein domains, the CD and the CSD, to the dynamics of HP1 binding to native chromatin in living cells. GFP-tagged HP1β mutants were constructed and used to transfect HeLa cells (Fig. 4). GFP-HP1β that lacked the CD (GFP-HP1ΔCD) displayed a localization in small nuclear foci in addition to a diffuse distribution throughout the nucleoplasm (Fig. 4B). These foci do not correspond to heterochromatin as assessed by DAPI staining (*14*). GFP-HP1-V23M, which contains a single point mutation (V23M indicates $Val^{23} \rightarrow Met^{23}$) in the CD and prevents binding of HP1 to histone H3 methylated on Lys^{9} (*12*), displayed an identical localization (Fig. 4C). GFP-HP1 that lacked the CSD (GFP-HP1ΔCSD) localized in heterochromatin and euchromatin as shown by DAPI staining (Fig. 4D). As a control, the hinge region alone (GFP-HP1h) was shown to be a diffuse target to nuclei and showed no accumulation in heterochromatin (*9*) (Fig. 4E). The in vivo binding capacity of these mutants to chromatin was comparable to that of wild-type (wt) GFP-HP1β (Fig. 4, F and G). Whereas deletion of the CD resulted in complete loss of GFP-HP1 from heterochromatin, indicating the absolute requirement for

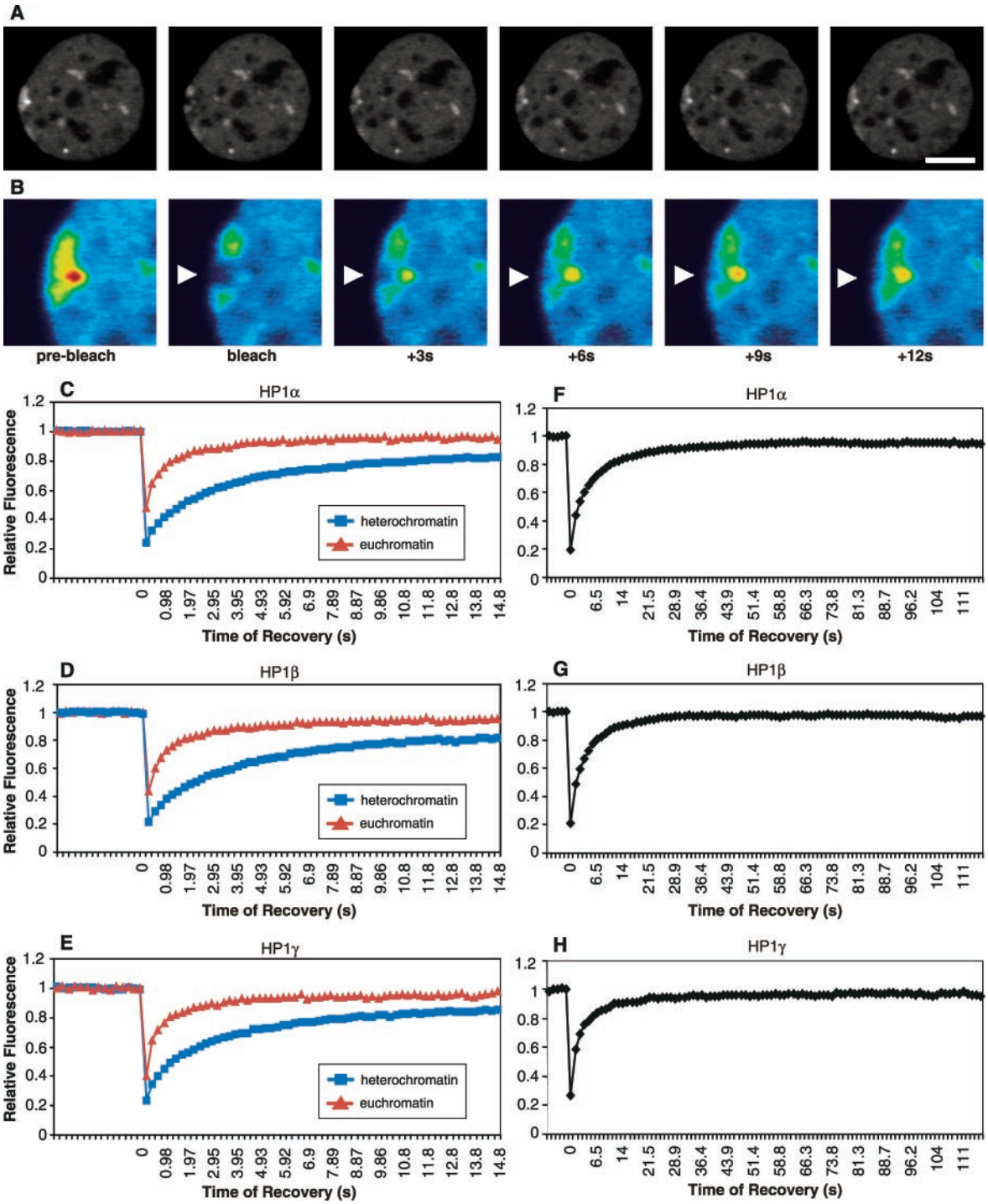


Fig. 2. HP1 dynamically associates with chromatin. (**A** and **B**) FRAP on CHO cells expressing HP1β. (B) Pseudocolored image, showing the fluorescence signal. Bar, 5 μm. Arrowheads point to the bleached area in heterochromatin. (**C** to **H**) Quantitative FRAP analysis of GFP-HP1α [in (C) and (F)], -HP1β [in (D) and (G)], and -HP1γ [in (E) and (H)]. (C) to (E) Short-term recovery; (F) to (H) long-term recovery. All isoforms exhibit similar dynamic properties with a slower recovery in heterochromatin than in euchromatin.

the CD in heterochromatin targeting, deletion of the CSD resulted in significantly faster recovery in heterochromatin ($P < 0.001$), demonstrating that the CSD is also critical for correct binding of HP1 in heterochromatin (Fig. 4F). In euchromatin, all mutants recovered faster than wt HP1, indicating that both domains are required for proper binding (Fig. 4G) ($P < 0.001$). The time to reach 80% of the prebleach intensity (t_{80}) for GFP-HP1ΔCD and GFP-HP1-V23M was 300 ms and 420 ms, respectively, in euchromatin compared with a t_{80} of 780 ms for the wild-type protein ($P < 0.001$). Deletion of the CSD had an even larger effect on the residence time of the HP1 mutant in euchromatin. For GFP-HP1ΔCSD, the t_{80} was 120 ms compared with 300 ms for GFP-HP1ΔCD (Fig. 4G). As expected, expression of the hinge region alone resulted in a highly mobile protein with only weak chromatin binding activity (Fig. 4G). Similar results for all mutants were observed in CHO cells (*14*). We conclude that the contribution of the CD to binding in heterochromatin is stronger than that of the CSD. In contrast, in euchromatin the contribution of the CSD is stronger than that of the CD. These results imply that the mechanism of HP1 binding differs in euchromatin compared to heterochromatin.

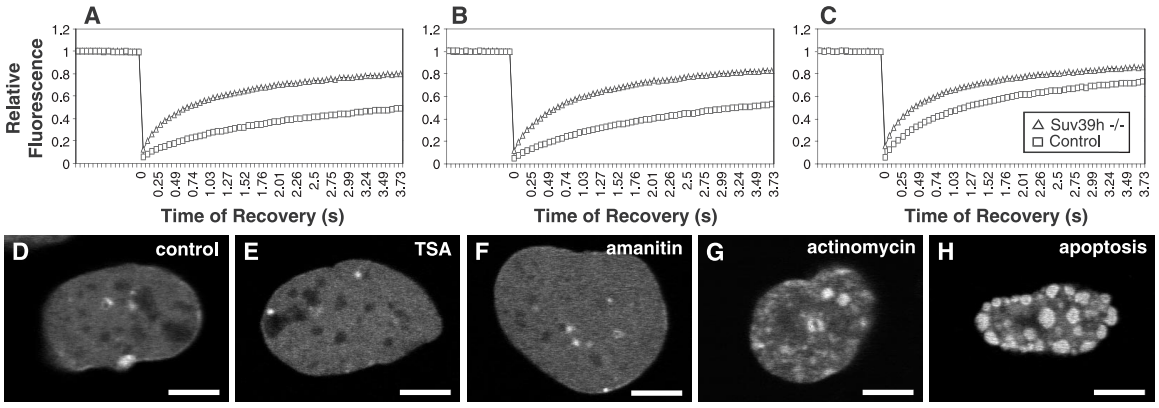
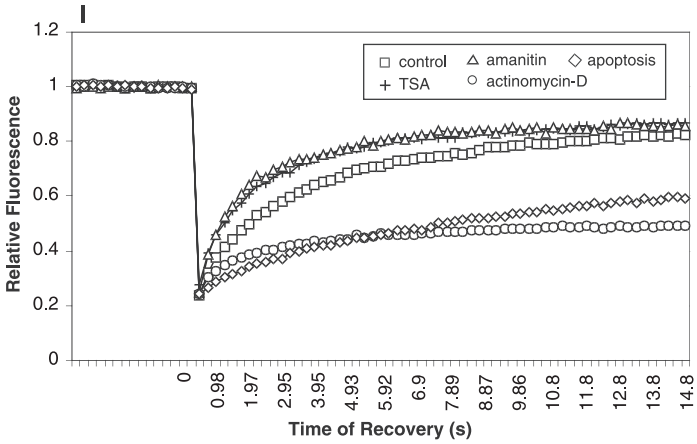


Fig. 3. HP1 mobility depends on Suv39h and chromatin condensation state. (**A** to **C**) FRAP analysis of (A) GFP-HP1α, (B) -HP1β, or (C) -HP1γ in Suv39h double-knockout MEFs and matching control cells. (**D** to **H**) Confocal images of living CHO cells stably expressing GFP-HP1α (D) untreated; treated with (E) TSA, (F) α-amanitin, (G) actinomycin-D; or (H) during apoptosis. Bar, 5 μm. (**I**) Quantitation of FRAP analysis of GFP-HP1α exchange in heterochromatin after drug treatments.



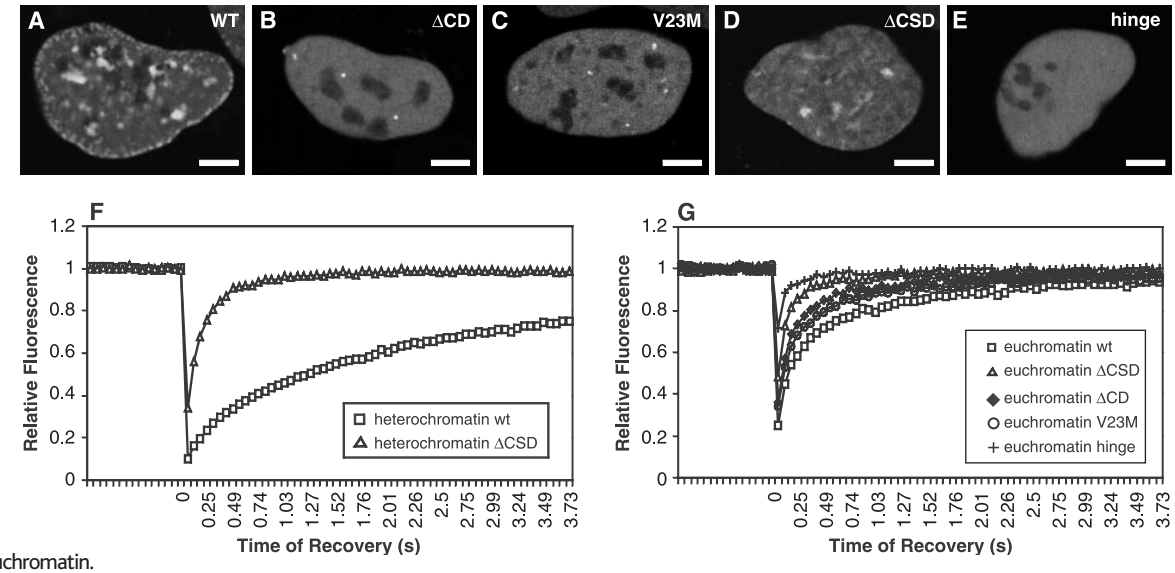


Fig. 4. Contribution of protein domains to HP1 binding to native chromatin in vivo. (**A** to **E**) Confocal images of living HeLa cells expressing (A) GFP-HP1β, (B) GFP-ΔCD, (C) GFP-V23M, (D) GFP-ΔCSD, or (E) GFP-hinge. Only wild-type and GFP-ΔCSD show heterochromatin localization, whereas the other mutants show a diffuse pattern throughout the nucleoplasm. Bar, 5 μm. (**F**) Quantitation of FRAP analysis of GFP-HP1β mutants in heterochromatin. (**G**) Quantitation of FRAP analysis of GFP-HP1β mutants in euchromatin.

We demonstrate here that the major heterochromatin protein HP1 is highly dynamic within stable heterochromatin domains. The transient association of HP1 with heterochromatin argues strongly against a model in which HP1 exerts its function by formation of static oligomeric networks, which prevent access of transcriptional regulators to silenced genes within heterochromatin domains. If HP1 indeed forms extensive higher order structures, they must be inherently dynamic and open to influx of proteins. Our observations are more consistent with a dynamic one-on-one binding model, possibly involving homo- and heterodimers of HP1 (*12*). The fact that HP1 is readily exchanged from heterochromatin also directly demonstrates that heterochromatin domains are not inaccessible to proteins. The dynamic nature of HP1 suggests that continuous exchange of HP1 is instrumental in the maintenance of a stable heterochromatic state. This observation is consistent with the requirement for the continuous presence of silencers in yeast heterochromatin (*23*). The dynamic binding of HP1 to chromatin provides an elegant yet powerful mechanism for the regulation of chromatin states. Each time an HP1 molecule dissociates from heterochromatin, various potential binding factors compete for the available binding site. The fate of the heterochromatin region is, thus, determined by competition among the available binding partners. A probabilistic competition model is supported by the observation that heterochromatin-induced silencing in *Drosophila* can be overcome by simple expression of an activator (*24*). Dynamic rather than static binding of HP1 should facilitate chromatin state transitions, and it may convey the high degree of plasticity to heterochromatin that is required for reorganizations during the cell cycle and differentiation (*25*, *26*). Our observations support a model in which the fate of heterochromatin domains is determined in a stochastic manner by the dynamic competition of activating and repressing factors.

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27. We thank T. Karpova for technical support. Imaging was performed at the NCI Fluorescence Imaging Facility. T.M. is a Keith R. Porter Fellow.

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19 September 2002; accepted 22 November 2002