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Functional Domain Structure of Human Heterochromatin Protein HP1$^{Hs\alpha}$: Involvement of Internal DNA-Binding and C-Terminal Self-Association Domains in the Formation of Discrete Dots in Interphase Nuclei[1]

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Human heterochromatin protein HP1$^{Hs\alpha}$ possesses two evolutionarily conserved regions in the N- and C-terminal halves, so-called chromo and chromo-shadow domains, and DNA-binding domain in the internal non-conserved region. Here, to examine its *in vivo* properties, we expressed HP1$^{Hs\alpha}$ as a fusion product with green fluorescent protein in human cells. HP1$^{Hs\alpha}$ was observed to form discrete dots in interphase nuclei and to localize in the centromeric region of metaphase chromosomes by fluorescence microscopy. Interestingly, this dot-forming activity was also found in the N-terminal half retaining the chromo and DNA-binding domains and in the C-terminal chromo-shadow domain. However, the chromo domain alone stained nuclei homogeneously. To correlate this dot-forming activity with self-associating activity *in vitro*, the chromo and chromo-shadow domain peptides were independently expressed in *Escherichia coli*, affinity purified, and chemically cross-linked with glutaraldehyde. In a SDS-polyacrylamide gel, the former mainly produced a dimer, while the latter produced a ladder of bands up to a tetramer. When passed through a gel filtration column in a native state, these peptides were exclusively separated as a dimer and a tetramer, respectively. These results suggested that the internal DNA-binding and C-terminal chromo-shadow domains are both involved in heterochromatin formation *in vivo*.

Key words: autoantigen, DNA binding, heterochromatin, HP1, self-association.

Heterochromatin is defined as a condensed state of chromatin which persists even in interphase nuclei. It is assumed that certain nonhistone chromosomal proteins constitute a heterochromatic region and regulate the active/inactive state of chromatin. Human heterochromatin protein HP1$^{Hs\alpha}$ was originally identified as one of several closely related antigens recognized by a sub-population of anticentromere autoimmune sera (*1, 2*). The predicted amino acid sequence of this p25 antigen showed a significant homology to a *Drosophila* nonhistone chromosomal protein, HP1, that had been identified as a component of centromeric heterochromatin (*3*). Recently, cDNAs for another p25 (HP1$^{Hs\beta}$) and p23 (HP1$^{Hs\gamma}$) antigens have been isolated by immunoscreening (*4*) or two-hybrid screening in yeast (*5*). These three HP1-related proteins possess two highly conserved regions, chromo and chromo-shadow domains, in the N- and C-terminal halves, respectively (see Fig. 5A, *6*).

We previously reported that HP1$^{Hs\alpha}$ possesses DNA-binding activity *in vitro* that is expected to be directly involved in heterochromatin formation (*7*). Interestingly, its DNA-binding domain was mapped in the internal non-conserved region. In addition, self-associating activity *in vitro* has recently been suggested in the chromo domain and chromo-shadow domain (*8, 9*). Therefore, it is reasonable to assume that these activities have some role in chromatin condensation. To fully understand the molecular mechanism of heterochromatin formation, these biochemical activities should be evaluated further with their corresponding features or functions *in vivo*. However, little is known about the molecular distribution of HP1$^{Hs\alpha}$ in cells, since autoantibodies against it often cross-reacted with other HP1 homologs (*1, 10*) and monoclonal antibodies against HP1$^{Hs\alpha}$ peptide have not yet been produced.

In this study, we expressed human HP1$^{Hs\alpha}$ as a fusion protein with green fluorescent protein (GFP) in human cells and found that it showed discrete dots in nuclei and localized in the centromeric heterochromatin of metaphase chromosomes. Using this phenotype as an *in vivo* marker, we determined the essential regions required for this dot-forming activity. This activity was then correlated with the two biochemical functions, DNA-binding activity and self-associating activity.

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Abbreviations: CENP, centromere protein; GFP, green fluorescent protein; GST, glutathione-S-transferase; HP1, heterochromatin protein 1.

MATERIALS AND METHODS

Plasmid DNAs—Plasmids pGEX-HC, pGEM-HN1, pGEX-HN2 have been described previously (*7*). Plasmid pGEX-HC2 was constructed by introducing the 0.28-kb *Eco*RV-*Not*I fragment of pGEX-HC into the *Sma*I-*Not*I site of pGEX4T-2 (Pharmacia). A series of pEGFP-HP1^{Hsα}-derived plasmids were constructed as follows. The 0.39-kb *Not*I and 0.28-kb *Eco*RV-*Not*I fragments of pGEX-HC were blunt-ended by fill-in reaction with Klenow fragment, then introduced into the *Sma*I and blunt-ended *Hin*dIII site of pEGFP-C1 (Clontech), resulting in pEGFP-HC1 and pEGFP-HC2, respectively. Similarly, pEGFP-HN1 and pEGFP-HN2 were obtained by introducing the 0.56-kb *Eco*RI-*Sal*I fragment of pGEM-HN1 into the same site of pEGFP-C1, and the 0.39-kb *Eco*RI-*Not*I fragment of pGEX-HN2 into the *Eco*RI-*Sma*I site of pEGFP-C1, respectively. The full-length pGEX-HP1 was constructed by simultaneously introducing both the 0.41-kb *Eco*RI-*Mae*III fragment of pGEX-HN1 and the 0.36-kb *Mae*III-*Not*I fragment of pGEX-HC into the *Eco*RI-*Not*I site of pGEX4T-3. The resulting 0.77-kb *Eco*RI-*Not*I fragment encoding the entire open reading frame of human HP1^{Hsα} was partially filled-in, then recloned into the *Eco*RI-*Sma*I site of pEGFP-C1, resulting in pEGFP-HP1.

Cell Culture and Transient Transfection—HeLa S3 and MDA435 cells were grown in MEM containing 10% FCS at 37°C in a 5% CO_2 atmosphere. Cells ($1\text{-}3\times10^5$) were plated on a glass coverslip in a 35-mm plate. Each plasmid DNA (1.5 μg) was dissolved in 150 μl of serum-free MEM, mixed with an equal volume of serum-free MEM containing 15 μl of Lipofectamine (Gibco BRL), and incubated at room temperature for 45 min. The cells on coverslips were incubated at 37°C with 1.5 ml of serum-free MEM containing the above DNA-Lipofectamine mixture for 6 h, then in fresh medium for a further 48 h. The coverslips were fixed with 4% paraformaldehyde dissolved in PBS and observed under a fluorescence microscope (Nikon Optiphoto 2) with a Fluoro-color CCD (Atto, Tokyo), as described elsewhere (*11*).

Stable Expression of GFP-HP1 Fusion Protein—Trypsinized MDA cells (1.2×10^7) were collected and suspended in 500 μl of K-PBS buffer (30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 mM $MgCl_2$), followed by the addition of pEGFP-HP1^{Hsα} DNA (30 μg) dissolved in 100 μl of TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). The cell-DNA mixture was transferred into an electroporation cuvette with a 0.4-cm electrode gap (Bio Rad) and electroporated at 220 V and 960 μF with a Gene Pulser (Bio-Rad). After addition of 500 μl of serum-free MEM, the cell suspension was added to a sufficient volume of MEM containing 10% FCS in several 90-mm plates. After 48 h, G418 (Gibco BRL) was added to each plate to a final concentration of 800 μg/ml. After selection, many transformants were obtained. Mitotic cells were observed by fluorescent microscopy.

Purification of Truncated HP1 Peptides and Gel Filtration—Cell extracts were prepared from IPTG-induced JM109 cells harboring pGEX-HN2 or pGEX-HC2. Each GST-HP1 fusion peptide was purified from 1 ml of extract with glutathione resin, as described (*7*). The HP1 moiety was cleaved by incubating the precipitated resin with 200 μl of 20 mM Hepes-NaOH, pH 7.9, 150 mM NaCl, 2.5 mM $CaCl_2$ containing 5 units of thrombin (Pharmacia) at room temperature for 2 h. HN2 and HC2 peptides in the supernatant (10 μg/50 μl) were each fractionated on a Superdex 75 PC3.2/30 column (Pharmacia) which was equilibrated with 20 mM Hepes, pH 7.9, 150 mM NaCl, 20 mM β-mercaptoethanol. Flow rate (40 μl/min) and fraction size (80 μl) were managed by a SMART system (Pharmacia), as described (*12*).

Chemical Cross-Linking In Vitro—Non-fused HP1 peptides released in the thrombin-cleaved supernatant (9 μl) were cross-linked with 1 μl of each concentration of glutaraldehyde for 30 min at room temperature. The reaction was stopped by adding 1 μl of 1 M Tris-HCl, pH 7.6, and incubating for 15 min. After addition of an equal volume of SDS-sample buffer (104 mM Tris-HCl, pH 6.8, 4.6% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue), the peptides were separated in 12.5% SDS-PAGE and stained with a solution containing 50% (v/v) ethanol, 10% (v/v) glacial acetic acid, and 0.25% (w/v) Coomassie Brilliant Blue R-250.

RESULTS

Expression of HP1^{Hsα} in Human Cells—The localization of heterochromatin protein HP1^{Hsα} in interphase nuclei as well as metaphase chromosomes has not yet been precisely examined. Although this autoantigen was originally identified by anti-chromo-specific autoimmune sera, they simultaneously recognized three HP1-related antigenic peptides (*10*), because of the presence of highly conserved chromo and chromo-shadow domains (see Fig. 5). Thus, we suspected that affinity-purified autoantibodies against HP1^{Hsα} would cross-react with the other HP1s. Also, we failed to obtain specific antibodies against HP1^{Hsα}, when rats were immunized with the recombinant peptides (data not shown). Here, to examine its localization in cells, we rather constructed a fluorescent HP1^{Hsα} by fusing the full-length HP1^{Hsα} to the GFP of *A. victoria* (*13*). As shown in Fig. 1A, GFP itself did not show any specific localization in human cells. When the recombinant plasmid pEGFP-HP1 was transiently expressed in HeLa cells, we found the fusion protein as discrete dots in interphase nuclei (Fig. 1D). The number of dots seemed to vary from cell to cell, possibly dependent on the cell cycle. To examine its localization in mitotic cells, we added G418 to the medium and obtained a mixture of stably transformed cells. As shown in Fig. 1C, the fusion protein was localized in the centromeric region of metaphase chromosomes. This was similar to the staining pattern of another homolog, HP1^{Hsβ} (*4*) and its mouse homolog M31 (*14*). The result suggested that HP1^{Hsα} also constitutes the centromeric heterochromatin, although we did not characterize it further here.

Essential Regions Required for "Dot-Forming" Activity In Vivo—We then determined the essential region(s) required for the above dot-forming activity *in vivo*. HP1^{Hsα} is thought to have a three-domain structure, the N-terminal chromo domain, the internal DNA-binding domain, and the C-terminal chromo-shadow domain (Fig. 2A, 7). As shown in Fig. 2B, HP1^{Hsα} was first dissected into two overlapping peptides, HN1 and HC1. HN1 possesses both the chromo and DNA-binding domains, and HC1 possesses the chromo-shadow domain in conjunction with the C-terminal half of

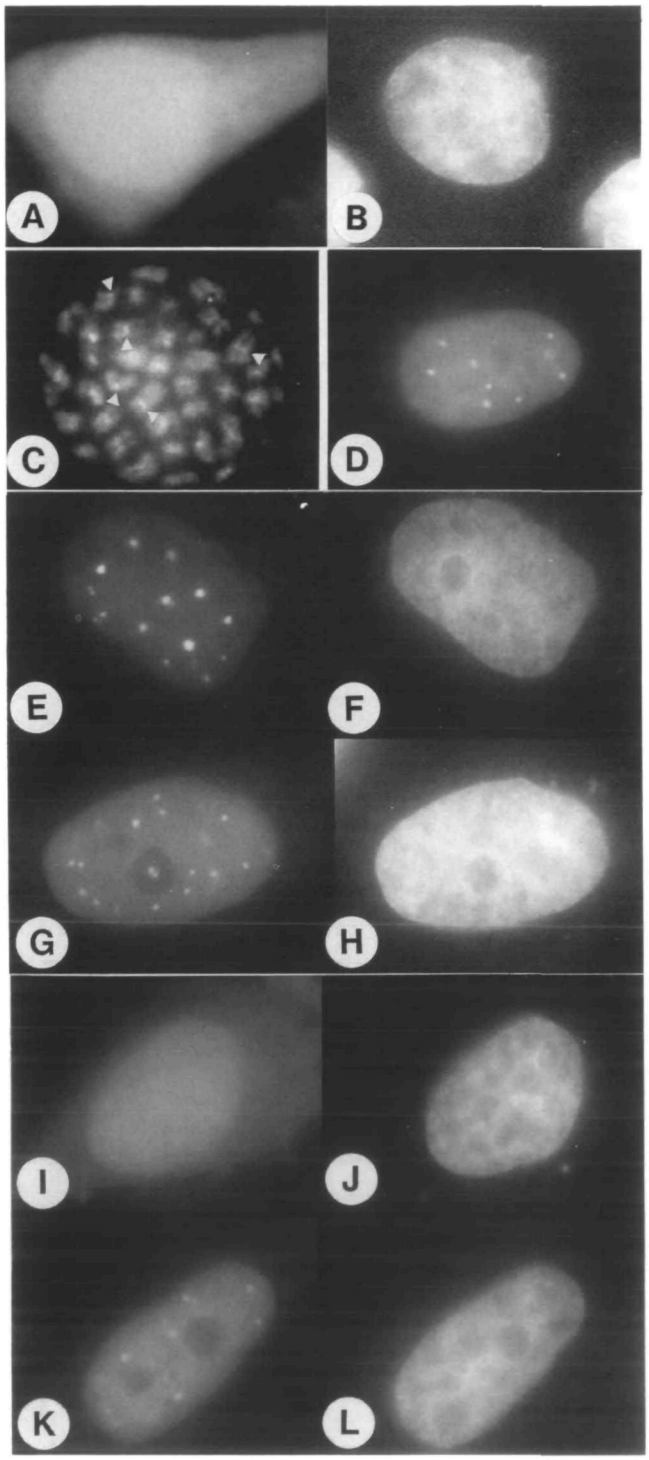

Fig. 1. **Cellular localization of GFP-tagged HP1$^{Hs\alpha}$ and its derivatives.** A, B: HeLa cells transfected with pEGFP-C1. C: Metaphase chromosomes of MDA435 transformants transfected with pEGFP-HP1. D-L: HeLa cells transfected with a series of pEGFP-based plasmids harboring the full or truncated HP1$^{Hs\alpha}$ gene (see Fig. 2B also): pEGFP-HP1 (D), pEGFP-HN1 (E, F), pEGFP-HC1 (G, H), pEGFP-HN2 (I, J), and pEGFP-HC2 (K, L). The cells were fixed with 4% paraformaldehyde. Fluorescence of the intact GFP or GFP-tagged HP1$^{Hs\alpha}$ (A, D, E, G, I, K) and nuclei in the transfected cells which were counterstained with DAPI (B, F, H, J, L) was observed by fluorescent microscopy.

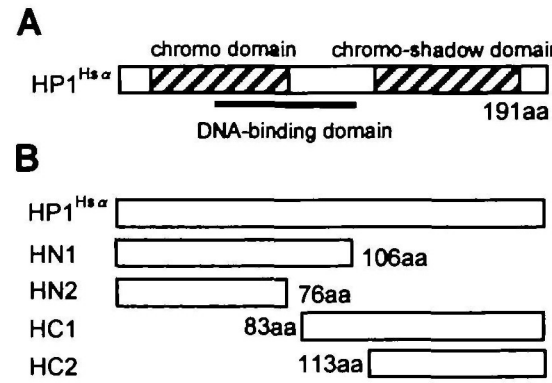

Fig. 2. **Schematic diagram of the truncated HP1$^{Hs\alpha}$ constructs used in this study.** A: The predicted three-domain structure of HP1$^{Hs\alpha}$. Chromo and chromo-shadow domains are presented as hatched boxes. DNA-binding domain is shown as a bar below. B: A series of truncated HP1$^{Hs\alpha}$ constructs. The coding region of each construct is represented as a box with the number of the terminal residue.

TABLE I. **Localization patterns of truncated GFP-HP1$^{Hs\alpha}$ fusion proteins expressed in HeLa cells.**

Constructs	Localization patterns			Total cells
	Nucleus		Cytoplasm	
	Dots	Homogeneity		
HP1$^{Hs\alpha}$	201(98%)	4 (2%)	0(0%)	205
HN1	164(67%)	76(31%)	5(2%)	245
HN2	0 (0%)	110(96%)	4(4%)	114
HC1	214(95%)	11 (5%)	0(0%)	225
HC2	202(92%)	17 (8%)	0(0%)	219

the DNA binding domain. We transiently expressed these two peptides as GFP fusion in HeLa cells. As shown in Fig. 1, E and G, most of the cells transfected with either HN1 or HC1 construct showed a similar dot-staining pattern in nuclei.

As summarized in Table I, the numbers of fluorescent cells were counted under a fluorescent microscope. Sixty-seven percent of the cells expressing HN1 and 95% of the cells expressing HC1 showed similar discrete dots in nuclei. There were two possible explanations for this: that the overlapping region between positions 83 and 106 is responsible for the dot-forming activity; and that two non-overlapping regions, for example, the chromo and chromo-shadow domains, are independently responsible for it (see Fig. 2). To examine this, we prepared two other constructs, HN2 and HC2, which retained the chromo and chromo-shadow domain, respectively, by deleting the internal overlapping region from each parental construct, HN1 and HN2. They were expressed as GFP fusion proteins in HeLa cells.

As shown in Fig. 1I, the HN2 peptide no longer showed the dot-staining pattern. Instead, most cells displayed a homogeneous staining of nucleus. Of 114 transfected cells observed, none displayed the discrete dots (Table I). This indicated that the chromo domain alone was not enough to show the limited localization in nuclei *in vivo*. This was not because of the absence of a nuclear localization signal, since HN2 itself could localize in the nucleus and the addition of a nuclear localization signal did not change the distribution

pattern (data not shown). Here, we interpreted that the internal region between positions 76 and 106 that is missing in HN2 was essential for the dot-forming activity of HN1.

In contrast, as shown in Fig. 1K, the cells transfected with HC2 construct still displayed the dot-staining pattern. Ninety-two percent of cells expressing HC2 showed discrete dots in the nuclei (Table I). The result indicated that, although the internal region was essential for the proper localization of HN1 construct, it was dispensable for the HC1 construct. Therefore, we concluded that HP1^{Hsa} possesses two separate regions responsible for the dot-forming activity *in vivo*. One is the internal DNA-binding domain, since the N-terminal chromo domain alone was not enough to express the limited localization. The other is the C-terminal chromo-shadow domain.

Chemical Cross-Linking of Chromo and Chromo-Shadow Domain Peptides In Vitro—While this work was in progress, self-associating activity *in vitro* was reported to be present in the chromo-shadow domain (*9*) as well as the chromo domain (*8*). This activity of the chromo-shadow domain was well correlated with the dot-forming activity *in vivo*. However, our result indicated that the same activity of the chromo domain was not enough to express the dot-like localization in the cells. Here, we suspected that there might be a difference in the self-association affinity.

To examine this, we did a chemical cross-linking experiment *in vitro*. HN2 and HC2 peptides were then expressed as a fusion protein with glutathione-S-transferase (GST) in

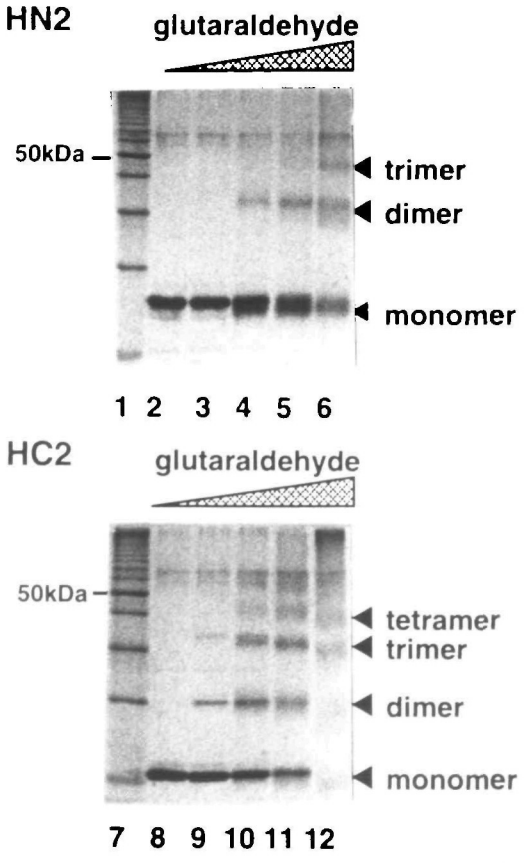

Fig. 3. **Chemical cross-linking of HN2 and HC2 peptides.** The purified HN2 and HC2 peptides were cross-linked with a titrated concentration of glutaraldehyde and separated in a 12.5% SDS-PAGE. The concentration of glutaraldehyde was 0 mM (lanes 2 and 8), 0.1 mM (lanes 3 and 9), 0.5 mM (lanes 4 and 10), 1 mM (lanes 5 and 11), and 5 mM (lanes 6 and 12). A 10-kDa ladder marker (Gibco BRL) was loaded on lanes 1 and 7.

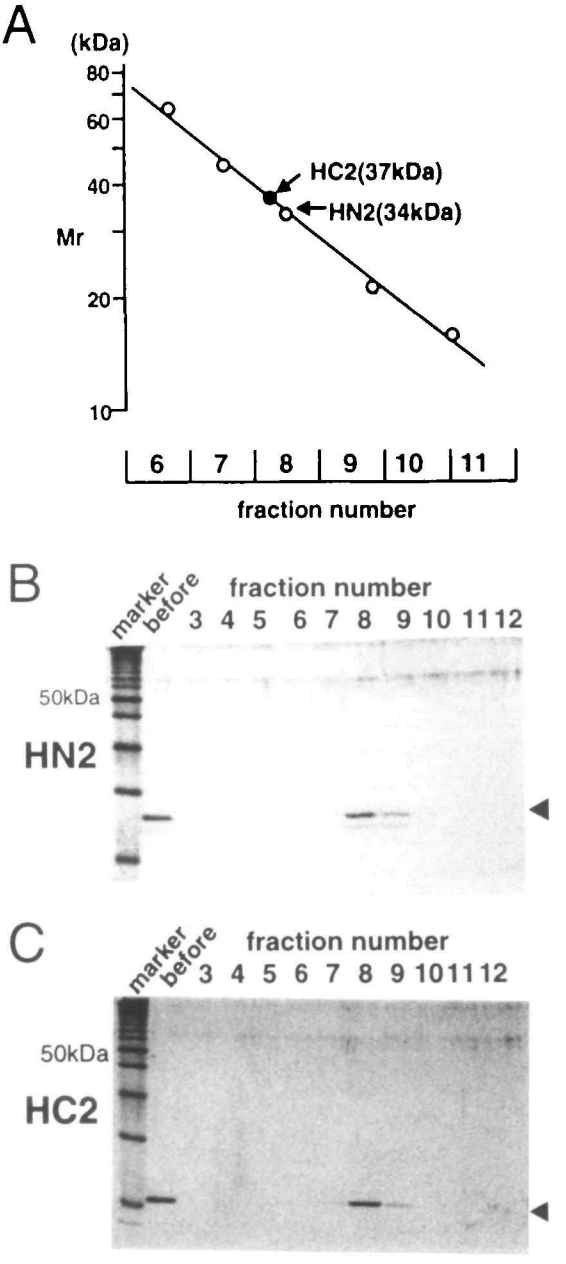

Fig. 4. **Gel filtration of the native HN2 and HC2 peptides.** A: Estimation of apparent molecular mass of the native peptides. HN2 and HC2 peptides were separated by gel filtration on a Superdex 75 precision column (3.2×300 mm) in the native state, as described in "MATERIALS AND METHODS." A Low Molecular Weight Gel Filtration Calibration Kit (Pharmacia) provided molecular mass standards: albumin (64.6 kDa), ovalbumin (46.3 kDa), chymotrypsinogen A (20.7 kDa), and ribonuclease A (15.7 kDa). B: SDS-PAGE of the gel-filtrated fractions of HN2. C: SDS-PAGE of the gel-filtrated fractions of HC2. Fractions 3-12 of each sample were each applied on a 12.5% SDS-polyacrylamide gel. A 10-kDa ladder marker and each sample before fractionation were loaded on the first and the second lanes from the left, respectively. Note that both the peptides were found exclusively in fractions 8.

Escherichia coli and affinity-purified with glutathione Sepharose 4B (*7*). HN2 and HC2 truncated peptides were obtained by cleaving the purified fusion peptides with thrombin, as described previously (*12*). From the predicted amino acid sequences, the molecular weights of HN2 and HC2 were estimated to be 15 and 9.5 kDa, respectively. When separated in a SDS polyacrylamide gel, HN2 peptide migrated at 17 kDa (lane 2, Fig. 3), while HC2 peptide migrated at 11 kDa, slightly larger than the predicted value (lane 8, Fig. 3). These peptides were incubated with titrated amounts of glutaraldehyde and separated in a SDS polyacrylamide gel. HC2 peptide produced a band with a dimer size (20 kDa) at 0.1 mM, and bands of a trimer (32 kDa) and a tetramer (42 kDa) at 0.5 mM (lanes 9 and 10, Fig. 3). In contrast, HN2 peptide displayed a monomer band at 0.1 mM and a dimer band (32 kDa) at 0.5 mM (lanes 3 and 4, Fig. 3). At a higher concentration of glutaraldehyde (5 mM), HC2 showed a smeared ladder or a high molecular weight aggregate, while HN2 showed a clear trimer band (compare lane 6 with lane 12). These results indicated that, although both peptides could be cross-linked with glutaraldehyde, the chromo-shadow domain peptide was more accessible for self-association *in vitro*.

Gel Filtration of N- and C-Terminal Peptides—To further characterize the native form of HN2 and HC2 peptides *in vitro*, they were passed through a gel filtration column in the absence of the chemical cross-linker. As shown in Fig. 4A, the apparent molecular mass of native HN2 form was calculated as 34 kDa. This value was comparable to the dimer size of HN2 peptide. Similarly, the native form of HC2 was calculated as 37 kDa and estimated to be a tetramer, since the predicted molecular weight of HC2 peptide was 9.5 kDa. The protein in each fraction was separated in a SDS-polyacrylamide gel and stained with Coomassie blue (Fig. 4, B and C). We confirmed that HN2 and HC2 peptides had been fractionated in the peak fraction (fraction 8). These results indicated that the native HN2 peptide forms a dimer, while the HC2 peptide forms a tetramer *in vitro*.

DISCUSSION

Heterochromatin protein HP1 was first identified as a component of centromeric heterochromatin of *Drosophila* (*3*). Interestingly, genetic study showed that this nonhistone chromosomal protein was encoded by the *Suvar*2-5 locus, which is responsible for suppression of euchromatic genes that have been located near the heterochromatic region, known as "position-effect variegation" (*15*). Therefore, HP1 homologs in other eukaryotes were predicted to play an important role in the formation as well as the maintenance and regulation of heterochromatin. In human, three homologs, $HP1^{Hs\alpha}$, $HP1^{Hs\beta}$, and $HP1^{Hs\gamma}$, have been identified (*1, 4, 5*). In the previous report, we found that $HP1^{Hs\alpha}$ has DNA-binding activity *in vitro* (*7*). Recently, self-associating activity has also been reported (*8, 9*), although little is known about the molecular behavior in cells. This is the first report that $HP1^{Hs\alpha}$ exists as discrete dots in interphase nuclei and localizes in the centromeric region of metaphase chromosomes in mitotic cells. This dot-forming activity *in vivo* was well correlated with the biochemical activities of the corresponding regions.

Dot-Forming Activity In Vivo vs. Self-Association Activities In Vitro—The putative domain structure of human $HP1^{Hs\alpha}$ so far characterized is summarized in Fig. 5A. The DNA-binding domain has been located in the internal region (*7*) and the self-associating activity has been located in each of the N-terminal chromo and the C-terminal chromo-shadow domains (*8, 9*). In addition, the chromo-shadow domain seems to be involved in the interaction with $HP1^{Hs\gamma}$ and with several nuclear receptors (*5*), and in the epigenetic control of transcription with TIFs (*16*).

We initially suspected that the reported self-associating activity of $HP1^{Hs\alpha}$ *in vitro* was responsible for the dot-forming activity *in vivo*, since the GFP-tagged $HP1^{Hs\alpha}$ would interact with endogenous $HP1^{Hs\alpha}$ molecules in the transfected cells. In fact, the dot-forming activity in the C-terminal half was well correlated with the self-association of the chromo-shadow domain. Nevertheless, the chromo domain peptide did not show the dot-like localization *in vivo*. One explanation is that the truncated fusion peptide is recessive to the intact endogenous molecule. The potential self-associating activity of the chromo domain may be too weak to be maintained in the living cells in the absence of the internal DNA-binding domain. Interestingly, this essential region between positions 76 and 106 of $HP1^{Hs\alpha}$ has a similar sequence to the DNA-binding domain of kinetochore autoantigen CENP-C (*12*).

Hypothetical Model for Molecular Interaction of $HP1^{Hs\alpha}$—How does $HP1^{Hs\alpha}$ display the distinctive localization in nuclei? The DNA-binding and self-associating activities could explain a certain extent of chromatin condensation. We present here one possible hypothetical model in Fig. 5B. $HP1^{Hs\alpha}$ would directly bind to genomic DNA at the internal DNA-binding region (left). We do not know

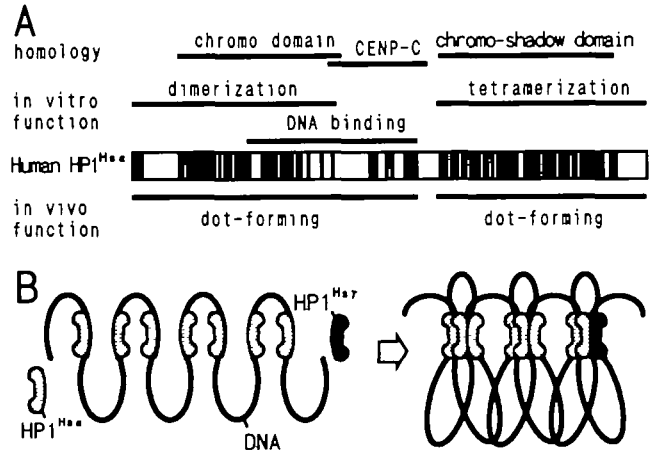

Fig. 5. **Predicted domain structure and molecular interaction of $HP1^{Hs\alpha}$.** A: Domain structure of $HP1^{Hs\alpha}$ characterized *in vitro* and *in vivo*. $HP1^{Hs\alpha}$ molecule is shown in a linear form. The vertical solid bars schematically represent the amino acid residues that are perfectly conserved among $HP1^{Hs\alpha}$ (*1*), $HP1^{Hs\beta}$ (*4*), and $HP1^{Hs\gamma}$ (*5*). The horizontal bars above indicate the two conserved regions, chromo domain (13-78) and chromo shadow domain (114-179) (*6*) as well as the internal region (73-110), which has a weak similarity to the DNA-binding domain of human CENP-C (*7*). Also shown are the biochemical activities characterized *in vitro*: dimerization domain (1-76), DNA-binding domain (43-106), and tetramerization domain (113-191). Shown below are two separate regions (1-106, 113-191) that possess dot-forming activity *in vivo*. B: Hypothetical model for the molecular interaction of $HP1^{Hs\alpha}$. See text for detail.

whether there is a sequence-specific recognition. As illustrated, the mutual association of HP1^{Hsα} and/or further oligomerization with the other HP1 molecules occurs at the C-terminal chromo-shadow domain (right). This will enable the DNA-HP1 complex to become more compact, although it may not necessarily explain the formation of heterochromatin-like dots found in interphase cells. The N-terminal chromo domain will be required for a further chromatin condensation and the regulation of chromatin state, possibly in association with other unidentified chromosomal protein(s).

As shown in Fig. 1C, HP1^{Hsα} localized in the centromeric region of metaphase chromosomes. However, we still do not know whether the region of discrete dots found in nuclei corresponds to centromeric heterochromatin, since the number of dots seems to be less than the chromosome number of human cells. Then, how are HP1^{Hsα} molecules incorporated into the centromere structure? Our preliminary characterization of the GFP fusion protein in stable transformants showed that it drastically changes its morphological features during the cell cycle (Sugimoto, K., unpublished observations). Therefore, the biochemical activities characterized here would be further regulated in a cell-cycle dependent manner.

Our recent studies on human centromere autoantigens, CENP-B, CENP-C, and HP1^{Hsα}, have suggested that DNA-binding and self-associating activities are involved in centromeric heterochromatin formation (*7, 12, 17*). Similar biochemical activities may be shared by other chromosomal autoantigens such as other HP1(s) and CENP-A. Similar evaluation of their biochemical activities *in vivo* will help us to understand the molecular basis of organization and regulation of centromere heterochromatin in the future.

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