J. Biochem. 120, 153-159 (1996)

Human Homolog of *Drosophila* Heterochromatin-Associated Protein 1 (HP1) Is a DNA-Binding Protein Which Possesses a DNA-Binding Motif with Weak Similarity to That of Human Centromere Protein C (CENP-C)[1]

Kenji Sugimoto,* Taku Yamada,* Yoshinao Muro,† and Michio Himeno*

*Laboratory of Applied Molecular Biology, Department of Applied Biochemistry, University of Osaka Prefecture, 1-1 Gakuen-cho, Sakai, Osaka 593; † Department of Dermatology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466; and all concurrently Division of Marginal Science, Research Institute for Advanced Science and Technology, University of Osaka Prefecture

Received for publication, February 28, 1996

Heterochromatin-associated protein 1 (HP1) is a nonhistone chromosomal component tightly associated with the pericentromeric heterochromatic region of fruit fly, mouse, and human throughout the cell cycle. *Drosophila* HP1 has been shown to be involved in position effect variegation and to be required for the correct chromosome segregation *in vivo*, while the biological activity of human homolog ($HP1^{Hsα}$) has not yet been characterized. We previously reported that human CENP-B and CENP-C, two major centromere heterochromatin autoantigens often recognized by autosera in scleroderma patients, possess DNA-binding activity *in vitro*. Here, we show that human HP1, which is also an autoantigen targeted by some types of anticentromere autosera, is a DNA-binding protein. Human HP1 was expressed as a GST-fusion in *Escherichia coli* and purified with glutathione-Sepharose. The DNA-binding activity of the recombinant HP1 was demonstrated by gel mobility shift assay and South-Western-type blotting. The minimum DNA-binding region was further limited to the internal 64-amino acid stretch that is less-conserved between human and fruit fly but retains a helix-enriched motif with weak similarity to CENP-C. This suggests that HP1 is involved in the pericentromeric heterochromatin formation by directly associating with genomic DNA.

Key words: autoantibody, centromere, DNA binding, heterochromatin, HP1.

Certain nonhistone chromosomal proteins and satellite DNAs consisting of tandem repeat units are intensively located in the heterochromatic regions of *Drosophila* and human chromosomes. It is likely that specific chromosomal proteins are involved in packing satellite DNA into the heterochromatin structure of the centromeric region of the mitotic chromosome. However, relatively little is known about the molecular interaction between these proteins and DNAs.

Heterochromatin-associated protein 1 (HP1) was originally identified as a nonhistone chromosomal protein that is primarily located in the heterochromatic regions of the *Drosophila* interphase nuclei or the "chromocenter" of polytene chromosomes (*1, 2*). Recently, a mammalian homolog of *Drosophila* HP1 has also been shown to be a component of the constitutive heterochromatin that is localized in the pericentromeric region of metaphase chromosomes (*3*). Genetic study further indicated that *Drosophila* HP1, encoded by the *Suvar2-5* locus, is one of the chromatin proteins responsible for the modification of chromatin structure, or "position-effect variegation," a suppression of euchromatic genes that have come to be located near the heterochromatic region (*4*). These studies suggest that HP1 plays a direct role in the formation and/or maintenance of the constitutive heterochromatin. Almost all of these studies have treated *Drosophila* HP1, while few have dealt with characterization of human or other homologs, partly because of difficulty in the genetic analysis. An alternative approach to understand the molecular mechanisms is to establish an assay system to detect a biochemical activity *in vitro*. This type of analysis will cooperatively accelerate the characterization of the whole functional domain structure of *Drosophila* HP1 as well.

HP1 is tentatively estimated to have a three-domain structure from several recent studies (see Fig. 5 for a reference). Two highly conserved regions have been identified at the both ends by comparing the *Drosophila* HP1 (*5*) with the homologs of mealybug (*6*), mouse (*7*), and human (*8*). Near the N-terminus is located a "chromo domain," which is similar to the *Drosophila* Polycoumb (*9*) and *Shizosaccharomyces swi6* proteins (*10*), both of which are also involved in mediating gene repression. Another evolutionarily conserved region, called the "chromo shadow domain," exists at the C-terminus (*11*). Cytological analy-

[1] This work was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan.

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

sis of HP1-β-gal fusions of *Drosophila* HP1 showed that the latter corresponds to the nuclear localization domain (*12*). In addition, a central less-conserved region is important for the "heterochromatin binding" of the β-gal fusion in the cell nucleus in conjunction with the C-terminus (*12*). Nonetheless, the DNA-binding activity of HP1 has not been tested yet, probably because the sequence analysis revealed no apparent homology to any known DNA binding motif.

We previously reported that human centromere heterochromatin components centromere protein B (CENP-B) and centromere protein C (CENP-C), when expressed as recombinant proteins in *Escherichia coli*, possess DNA-binding activities *in vitro* (*13, 14*). These centromere proteins, together with CENP-A, are often recognized by autosera produced by autoimmune diseases patients suffering from scleroderma (*15*). Interestingly, human HP1 ($HP1^{Hs\alpha}$) was also an autoantigen targeted by certain types of these anticentromere autosera (*8*). Simultaneous microscopic observation showed that these three centromere autoantigens were localized on the centromere heterochromatin throughout the cell cycle (*16*). Direct molecular interaction was subsequently demonstrated *in vitro* between CENP-B and the centromeric satellite DNA called "alphoid" (*17*). Similarly, recent independent microscopic studies by Fernandez *et al.* (*18*) and Nicol and Jeppesen (*19*) suggested that human HP1 is located in the vicinity of another satellite DNA, called "classical satellite," since both components were precisely localized on the chemically-stretched pericentromeric heterochromatic region of certain chromosomes. Therefore, we assumed that the HP1 autoantigen also possess DNA-binding activity.

In this study, we first expressed human HP1 antigen as a fusion protein with a glutathione-S-transferase in *E. coli* and affinity-purified it using glutathione-conjugated resin. The DNA-binding activity of the purified HP1 was then assayed by two different sensitive methods: gel mobility shift assay, and South-Western blotting. Finally, we determined the minimum region required for this activity. We also speculate on the whole domain structure of human HP1 by epitope mapping and comparison with the *Drosophila* homolog.

MATERIALS AND METHODS

Cloning and Amplification of Human HP1 cDNAs—The human $HP1^{Hs\alpha}$ clone (λ50-1), harboring the 5′-noncoding region and encoding the N-terminal 106 amino-acid residues, was isolated from human λgt11 cDNA library by immunoscreening with an anticentromere autoserum with anti-chromo specificity (Iwai *et al.*, submitted), as described (*13, 14*). A forward λgt11 primer (5′-ATCGACGGTTTCCATATGGGGATTG-3′) and the reverse λgt11 primer (5′-TTGACACCAGACCAACTGGTAATGG-3′) were used to amplify the 0.54-kb cDNA insert of the λ50-1 clone. To amplify the HN5 and HN6 coding sequences (see Fig. 2A for reference) using the λ50-1 DNA as a template, the forward λgt11 primer was replaced by an HPchromo primer (5′-GATGAGGAGGAGTATGTTGTGGAG-3′) or an HPfd18 primer (5′-GGCTTTTCTGAGGAGCAC-3′), respectively, both of which were derived from the DNA sequence of our clone. To amplify the HC coding sequence, another 5′ primer (HPfd25: 5′-GGAGAAGTCAGAAAGTAACAAGAGG-3′) and a 3′ primer (HPrv18: 5′-GAGCTAAAGGAGGGGATG-3′) were designed based on our sequence data and the published sequence (*8*). Polymerase chain reaction (PCR) was performed according to the manufacturer's instructions with *Tth* DNA polymerase (Toyobo, Tsuruga) or Expand High Fidelity (Boehringer Mannheim). All PCR products were first cloned into the pGEM-T vector (Promega). To express the recombinant protein, the plasmid pGEX-HN1 was constructed by inserting the 0.54-kb *Eco*RI-*Sal*I fragment, excised from the pGEM-T recombinant, into the *Eco*RI-*Xho*I sites of pGEX4T-3. Similarly, pGEX-HN5, pGEX-HN6, and pGEX-HC were constructed by inserting the *Eag*I fragment of each pGEM-T-derived recombinant into the *Not*I site of pGEX5T-2. To construct pGEX-HN2, pGEX-HN3, and pGEX-HN4, the parental pGEX-HN1 was double-digested with *Spe*I and *Pst*I and treated with exonuclease III using a Deletion Kit (Nippon Gene, Toyama). The nucleotide sequence of all the constructs was analyzed using an ALF DNA sequencer (Pharmacia). The DNA sequence of our clones was identical to that of human HP1 clone 9a previously isolated by Saunders *et al.* (*8*), except for one G insertion at the position between 9G and 10C in the noncoding region.

Expression and Purification of GST-Fusion Proteins—*E. coli* JM109 cells harboring a pGEX-derived plasmid were grown at 37°C in 150 ml of LB medium. The expression of the GST-fused HP1 proteins was induced in the presence of 1 mM isopropyl β-D(−)-thiogalactopyranoside (IPTG) for 2 h. Crude extract (4 ml) was prepared from the induced cell pellets, as described (*20*), except that the final concentration of EDTA in the buffer was adjusted to 50 mM and leupeptin and pepstatin were added to the final concentration of 5 and 0.5 μg/ml, respectively. To purify each GST-fusion protein, 200 μl of extract was diluted with three volumes of dilution buffer (10 mM Hepes-NaOH, pH 7.9, 80 mM KCl, 10 mM β-mercaptoethanol, 10% glycerol) and mixed with 40 μl of pre-equilibrated glutathione-Sepharose. After incubation for 2 h, the resin was recovered by centrifugation (10,000 rpm, 1 min, 0°C) and washed three times with buffer (20 mM Hepes-NaOH, pH 7.9, 60 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 0.05% Triton X-100). For South-Western blotting, the affinity-purified protein on the resin was eluted by heating in 40 μl of SDS-sample buffer for 1 min at 100°C. Alternatively, the resin was incubated with 40 μl of elution buffer [10 mM reduced glutathione (Kohjin) in 50 mM Tris-HCl, pH 8.0] for 30 min at room temperature. After centrifugation, the supernatant was used for gel mobility shift assay.

Gel Mobility Shift Assay, Immunoblotting, and South-Western Blotting—*Hin*fI-digested human alphoid clone, cos2 (*21*) or *Hin*fI- or *Sau*3AI-digested satellite III clone, pUC1.77 (*22*), were labeled with [α-^{32}P]dATP (111 Tbq/mmol, 370 Mbq/ml, ICN Biochemicals) by use of Klenow fragment (Nippon Gene) and used as a probe for gel mobility shift assay, as described (*23*). Immunoblotting and South-Western blotting procedures were performed as described previously (*14*), except that Renaissance chemiluminescence reagents (Dupont NEN) were used instead of ECL reagents. Rabbit anti-GST antibodies were obtained from Sigma Chemicals.

RESULTS

DNA-Binding Activity of Human HP1—The original characterization of *Drosophila* HP1 by James and Elgin (*1*) suggested its tight binding to DNA. Nevertheless, no direct evidence had been presented that HP1 is a DNA-binding protein. To examine the DNA-binding activity of the human homolog in this study, an $HP1^{Hs\alpha}$ cDNA clone (λ50-1) was isolated by immunoscreening with an anti-chromo-positive anticentromere autoserum, essentially as described (*8*). Although our clone encoded the only 106-amino acid residues of the N-terminal half of human HP1, we tested the DNA-binding activity before cloning the remaining C-terminal portion. To obtain HP1 as a fusion with glutathione-S-transferase, the cDNA insert was introduced into a pGEX bacterial expression vector (*24*) to yield pGEX-HN1. The recombinant human HP1 was expressed in *E. coli*, purified with glutathione resin and then applied to gel mobility shift assay. As a positive control, we used another construct in which the GST tag was fused to the DNA-binding domain of CENP-B (*13*), a well characterized human heterochromatin protein which possesses a sequence-specific DNA-binding activity (*17*). The two GST-fusions were separately incubated with each probe and loaded on a native polyacrylamide gel. As probe DNAs, we used a human alphoid DNA clone, cos2, which contained alphoid repeats enriched with authentic CENP-B binding motifs (*21*), for the CENP-B fusion, and a human classical satellite DNA clone, pUC1.77, which contains a 1.77-kb repeat of classical satellite III (*22*), a candidate for the target sequence which is predominantly localized at the secondary constriction of chromosome 1, for the HP1 fusion. As shown in Fig. 1A, all the restriction fragments of the probes were retarded severely in the presence of the CENP-B product or the HP1 product. This preliminary result encouraged us to continue the characterization further.

Although the result suggested that the recombinant HN1 protein possesses DNA-binding activity *in vitro*, this activity might be provided by the amino acid stretch compulsorily translated from the long 5′-noncoding region of this clone. To exclude this possibility and to examine the activity of the C-terminal half further, another two constructs, pGEX-HN5 and pGEX-HC, were prepared (see Fig. 2A). The HN5 protein retains the chromo domain as well as the internal region, and the HC protein has the C-terminal half of HP1, sharing the 24 amino acid residues between positions 83 and 106 with the HN5 protein. The two GST-fusions affinity-purified were again applied to the gel mobility shift assay. As shown in Fig. 1B, the restriction fragments of the probe were again retarded in proportion to the amount of the HN5 protein in the presence either of poly(dI-dC) or herring sperm DNA as a competitor. No retardation was observed in the reaction with the C-terminal half or the GST tag alone (data not shown), indicating no effect of the GST portion on this assay and no detectable activity of the C-terminal half. We concluded that the N-terminal half of human HP1 possessed DNA-binding activity, although we could not clarify whether human HP1 possessed sequence-specific DNA-binding activity in this experiment.

Mapping of the DNA-Binding Domain of Human HP1—The sequence analysis of HP1 revealed no apparent homology to any known DNA-binding motif. While this work was in progress, Platero *et al.* (*25*) reported that the chromo domain of *Drosophila* HP1 was important for its localization on heterochromatin. Therefore, we suspected that this domain might be involved in the DNA-binding activity. To

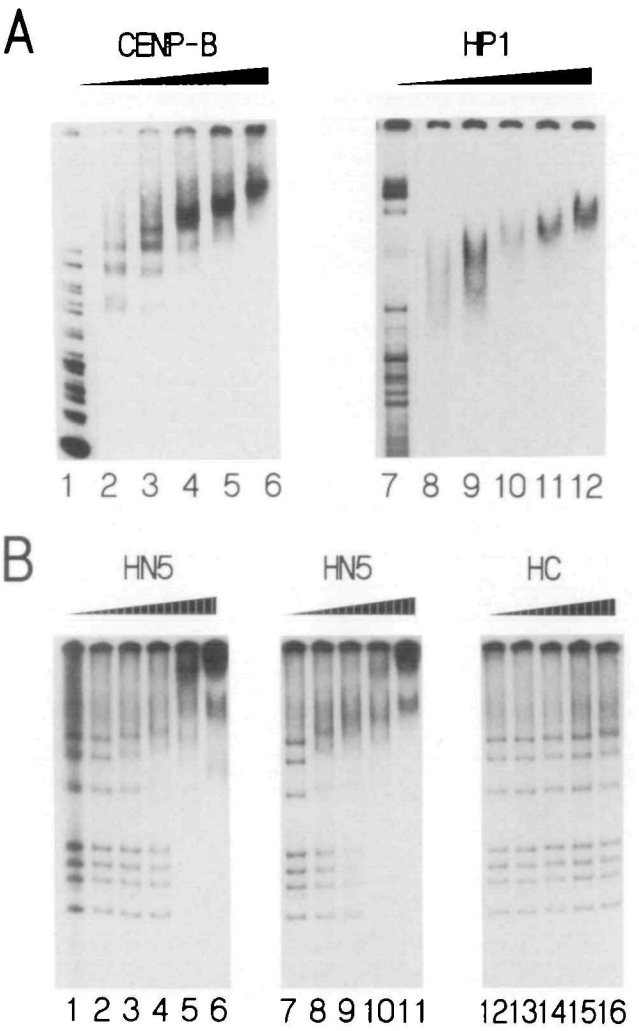

Fig. 1. **Identification of the DNA-binding activity of human HP1.** A: Gel mobility shift assay of the recombinant human CENP-B and HP1 proteins. The insert DNAs of λ1-11, human CENP-B clone (*13*), and λ50-1, human $HP1^{Hs\alpha}$ clone, immunoscreened with anti-centromere sera, were each recloned into a pGEX4T-3 vector. Each recombinant was expressed as a GST-fusion in *Escherichia coli* and affinity-purified (see "MATERIALS AND METHODS"). *Hin*fI-digested alphoid clone, cos2 (*21*), and satellite III clone, pUC1.77 (*22*), were used as probes for CENP-B and HP1, respectively. The titrated amount of the affinity-purified CENP-B or HP1 was included in the binding reaction: 0 μl (lanes 1 and 7), 0.25 μl (lanes 2 and 8), 0.5 μl (lanes 3 and 9), 1 μl (lanes 4 and 10), 2 μl (lanes 5 and 11), and 4 μl (lanes 6 and 12). B: DNA-binding activity of the N-terminal or C-terminal half of human HP1. The N-terminal half (HN5) and C-terminal half (HC) of HP1 were separately expressed in *E. coli*. The titrated amount of the affinity-purified HN5 (0.2 μg/μl) or HC (0.4 μg/μl) was incubated with ^{32}P-labeled *Sau*3AI-digested pUC-1.77: 0 μl (lanes 1, 7, 12), 0.25 μl (lanes 2, 8, 13), 0.5 μl (lanes 3, 9, 14), 1 μl (lanes 4, 10, 15), and 2 μl (lanes 5, 11, 16). As a competitor DNA, 0.25 μg of poly(dI-dC) for lanes 1 to 6 and 12 to 16 or herring sperm DNA (Promega) for lanes 7 to 11 was included in the reaction.

characterize the relationship between the chromo domain and the DNA-binding activity further, we next tried to determine the essential region for this activity. We used a South-Western-type blotting procedure which we had previously used to limit the DNA-binding domains of human CENP-B (*13*) and CENP-C (*14*).

We prepared three new constructs, pGEX-HN2, pGEX-HN3, and pGEX-HN4, which were deleted stepwise from the C-terminal side of pGEX-HN1, as shown in Fig. 2A. Both the HN2 and HN3 products retained the whole chromo domain, while the HN4 protein had lost its C-terminal half. The recombinant proteins were purified and identified by immunoblotting with anti-GST antibodies (Fig. 2B). The amount of the intact full-length products in the affinity-purified proteins was roughly adjusted to be equal, judging from the intensity of the uppermost bands of each lane. The proteins transferred to the membrane were denatured in the presence of guanidine-hydrochloride solution, gradually renatured, then incubated with ^{32}P-labeled genomic DNA of HeLa cells. As a positive control, we used another construct (CB2G) in which the GST tag was fused to the DNA-binding domain of human CENP-C (*14*). As shown in Fig. 2C, in addition to CB2G, the 48-kDa band of the HN1 protein (lane 2) and the 39-kDa band of HN5 protein (lane 6) were labeled, consistent with the above result, while no band of the deleted constructs (lanes 3-5) nor the GST moiety alone (lane 9) was detected. Therefore, any deletion of the internal region from position 106 completely abolished the DNA-binding activity, indicating the importance of this region. To further examine the DNA-binding domain, we expressed the HN6 protein, in which the N-terminal half of the chromo domain had been deleted. As shown in lane 7, the DNA-binding activity was retained by the HN6 protein. These results clearly showed that the evolutionarily less-conserved internal region between positions 43 and 106 was essential for the DNA-binding activity. No binding activity of other portions such as chromo domain alone (HN3) or the C-terminal half of HP1 (HC) was detected, even in the absence of the competitor DNA (data not shown).

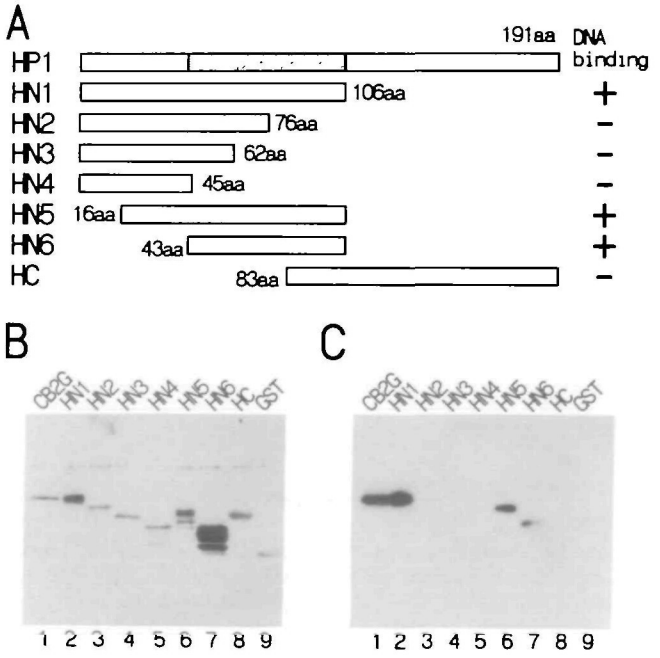

Fig. 2. **Mapping of the DNA-binding domain of human HP1.** A: Schematic diagram of the truncated HP1 constructs used in this experiment. The coding regions are presented as open boxes with the number of the terminal residues. The DNA-binding domain determined here is presented as a shaded area. B: Immunoblotting of the truncated HP1 products. The affinity-purified GST-fusions were separated in 10% SDS-PAGE, transferred to the membrane and detected by immunoblotting with anti-GST antibodies. C: South-Western blotting of the truncated HP1 products. The DNA-binding activity of the recombinant HP1 transferred on the membrane was examined by South-Western-type blotting with a human genomic DNA as a probe, as described (*14*). CB2G, the GST-fusion harboring the DNA-binding region (396-552 residues) of human CENP-C (lane 1), HN1 (lane 2), HN2 (lane 3), HN3 (lane 4), HN4 (lane 5), HN5 (lane 6), HN6 (lane 7), and HC (lane 8). Note that only the uppermost band of HN6 in lane 7 possesses the DNA-binding activity.

Putative Secondary Structure of the DNA-Binding Domain—Figure 3 shows the predicted secondary structure of the limited DNA-binding domain of human HP1. Homology search of this sequence against the Swiss Prot database revealed no significant similarities to any known sequences. However, this region appeared to be very hydrophilic and high in helical content, as was the DNA-binding domain of human CENP-C which we had previously determined (*14*). When the two sequences were compared, a weak similarity was found between the DNA-binding domains of human HP1 and human CENP-C. This presumptive helix-enriched short stretch with about 40% similarity to CENP-C might be important for the interaction with DNA.

Localization of Autoepitopes—We previously clarified the functional domain structure of human centromere autoantigen, CENP-B, and found that the autoepitopes were precisely located at the functionally active sites (see Fig. 1 of Ref. *23*). Since human HP1 is also an autoantigen recognized by certain types of anticentromere autosera, this prompted us to assign the autoepitope(s) on the HP1 molecule. Saunders *et al.* showed that one autoepitope is located within the N-terminal 56-amino acid region (*8*). To search for another autoepitope, the same set of truncated

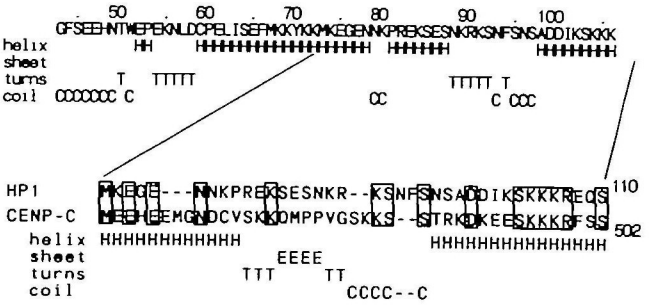

Fig. 3. **Secondary structure prediction of human HP1 DNA-binding domain.** Above is the amino acid sequence of the DNA-binding domain of HP1^{His6} determined in this study. Garnier prediction was performed with a Fujitsu Bioresearch analyzing system. The predicted alpha helix (H), beta sheet (E), turns (T), and coil (C) are shown under the deduced amino acid sequence. A helix-enriched stretch of human HP1 homologous to that also found in the DNA-binding domain of human CENP-C (*14*) is depicted below with the secondary structure prediction. The common residues conserved between human HP1 and CENP-C are boxed.

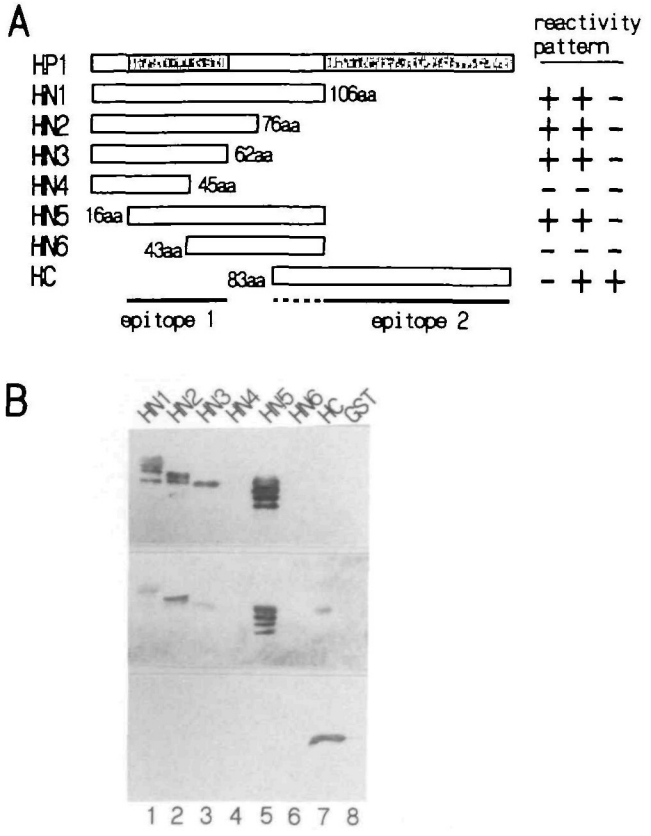

Fig. 4. **Epitope mapping of human HP1 with anticentromere autosera with anti-chromo specificity.** A: Schematic diagram of the truncated HP1 constructs. The same set of the human HP1 constructs as shown in Fig. 2 was used here. The hatched area indicates the epitope location. B: Three patterns of reactivity of patient sera to the truncated HP1 products. Antigenicity of the series of truncated HP1 constructs was tested by immunoblotting with six antichromo-positive anticentromere sera obtained from autoimmune disease patients (Iwai *et al.*, submitted). Four sera showed the pattern in the top panel, one showed that in the middle panel, and the rest showed that in the bottom panel.

HP1 products as used in Fig. 2 were transferred to the membrane and probed with six anti-chromo-positive anticentromere sera. As shown in Fig. 4B, three different patterns of reactivity were observed. The top panel shows one pattern in which four patient sera exclusively recognized the all the N-terminal constructs except for HN4 and HN6, in which the chromo domain was half deleted. From this result, the epitope was precisely mapped on the chromo domain between positions 16 and 62, as indicated previously (*8*). In addition, as shown in the bottom panel, one serum recognized the C-terminal half alone, as recently predicted by Iwai *et al.* (submitted). The middle panel showed the result with another serum indicating the mixture of the two types of autoantibodies. However, none recognized the HN6 protein, the minimum DNA-binding portion, although the number of samples tested here was limited. This region might correspond to "a minor epitope" of human HP1, which is only recognized by a minority of patient sera.

DISCUSSION

Heterochromatin is thought to be formed as the result of cooperative DNA-protein and protein-protein interactions. This assumption is supported by the genetic study of *Drosophila* "suppressor-of-variegation" [*Su(var)*] mutants as well as the recent biochemical characterization of human nuclear autoantigens: the molecular analysis of *Su(var)* genes indicates that some encode structural chromatin components including DNA-binding proteins, and others encode enzymes that could modify them (*26*). On the other hand, biochemical analysis of human centromere heterochromatin autoantigens showed that at least two possess DNA-binding activity (*13, 14, 17*).

DNA-Binding Activity of Human HP1^{Hsa}—This is the first report that heterochromatin-associated protein 1 (HP1) is a DNA-binding protein. So far, several kinds of repetitive sequences have been identified on the centromeric region of human metaphase chromosomes. Classical satellite DNAs are well presented in the constitutive heterochromatin of human chromosomes 1, 9, 16, and Y or alpha satellite is situated in the centromeric region of all human chromosomes. It is known that the preculture of human cells with 5-azacytidine or other chemicals induces undercondensation of pericentromeric heterochromatin of certain chromosomes. Recently, fluorescence *in situ* hybridization by Fernandez *et al.* showed that a classical satellite DNA (D9Z1) was specifically located on the 5-azacytidine undercondensed region of chromosome 9 (*18*). At the same time, Nicol and Jeppesen reported that human HP1 is predominantly localized on the decondensed heterochromatin (*19*). This tight association of HP1 with heterochromatin suggests that HP1 possesses DNA-binding activity.

We limited the minimum DNA-binding domain to the internal 64-amino acid stretch of human HP1 which is less homologous to other HP1 homologs. Although the overall amino acid sequence showed no significant homology to other DNA-binding motifs, we found that it partly possessed weak similarity to that of the DNA-binding domain of human CENP-C (Fig. 3). This common motif might constitute the core part of the DNA-binding domain structure of human HP1 and CENP-C, and this type of "microstructural" analysis of the functional domain should help the interpretation of the DNA-binding of other proteins in the future.

To detect the DNA-binding activity of human HP1, we used the *Hin*fI- or *Sau*3AI-digested satellite III (*22*) as a probe. The gel mobility shift assay in Fig. 1, however, showed no obvious bands retarded specifically in the presence of the recombinant HP1. So far, we do not know whether human HP1 recognizes a specific DNA sequence. The sequence specificity might be achieved by associating with other proteins or, alternatively, HP1 might bind to a type of classical satellite DNA other than that in this study. Since we have previously reported an affinity-precipitation procedure to enrich the recognition sequences of DNA-binding proteins (*20*), this type of approach might be useful to characterize the target sequence, if any, of human HP1.

Hypothetical Domain Structure of Human HP1—In contrast to *Drosophila* HP1, the whole domain structure of human HP1 has not been clarified, except for the DNA-binding domain characterized in this study. When the amino acid sequence of human HP1 was compared with that of the well-characterized *Drosophila* HP1, two separate regions were found where the sequence was extensively

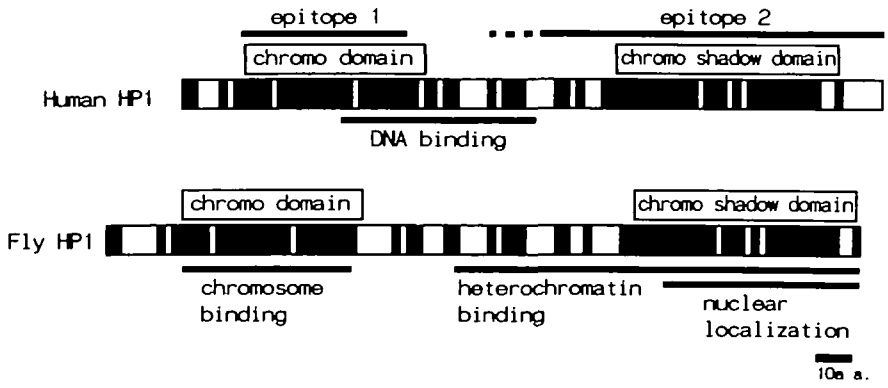

Fig. 5. **Predicted domain structure of human HP1.** Human HP1^{Hsα} and *Drosophila* HP1 are presented in linear form. The vertical solid bar schematically represents the region where the amino acid sequence was relatively conserved between human and fruit fly HP1. The chromo domain and the chromo shadow domain have been located by Aasland and Stewart (*11*). The chromosome-binding domain, the heterochromatin-binding domain, and the nuclear localization domain of *Drosophila* HP1 have been determined by Eissenberg's group (*12, 25*).

conserved: the chromo domain (*7*) and the chromo shadow domain (*11*), as schematically illustrated in Fig. 5. These two regions have been predicted to be the active sites, since the functionally important region should be evolutionarily conserved. In fact, the cytological analysis of *Drosophila* HP1 demonstrated that the chromosome-binding activity (*25*) and the nuclear localization activity (*12*) were located in the N-terminal and the C-terminal homology domain, respectively.

In this study, to estimate the whole domain structure of human HP1, we did epitope mapping using anticentromere autosera with anti-chromo specificity. The recent epitope analyses of human CENP-B (*23*) and CENP-C autoantigens (Sugimoto *et al.*, manuscript submitted) indicated that the autoepitopes were located at the functionally important sites, although the functional regions were not always targeted by autoantibodies (see Fig. 1 of Ref. *23*). In the previous preliminary study, Saunders *et al.* suggested that the chromo domains of human HP1 as well as *Drosophila* HP1 were recognized by human anti-chromo autoantibodies (*8*). As shown in Fig. 4, the antigenic region was further limited to the 47-amino acid stretch between positions 16 and 62, just covering the conserved chromo domain. In addition, the C-terminal half of HP1 also seemed to be targeted by anticentromere sera, although further analysis is required to confirm this. Therefore, these two antigenic regions of human HP1 recognized by autoimmune sera were also estimated to be functionally important sites. Although the DNA-binding domain between positions 43 and 106 was not recognized by the above anticentromere sera, the DNA-binding activity was clearly demonstrated in the internal region by the functional analysis (Figs. 1 and 2). It is possible that, if we increase the number of the patient sera, it might be found that this region is targeted by a minority of autosera, as previously shown for epitope 2 of human CENP-B (*28*). Alternatively, this region might not be recognized by the autoantibodies at all, as in the case of the internal self-association domain of CENP-B *in vitro* (*23*).

The putative three-domain structure of human HP1 is illustrated in Fig. 5. The possibility cannot be excluded, however, that the chromo domain might also be involved in the DNA-binding activity, since the DNA-binding domain could not be separated from the chromo domain and, in fact, the intensity of the band of HN6 seemed to be slightly lower than that of HN5 (lanes 6 and 7, Fig. 2C). On the other hand, when the domain structure of *Drosophila* HP1 was compared to the human homolog, it appeared that the internal less-conserved region of *Drosophila* HP1 responsible for heterochromatin binding might correspond to the DNA-binding domain. It will be interesting to know whether *Drosophila* HP1 also possesses a DNA-binding activity.

Recently, Aasland and Stewart pointed out that the N- and C-terminal halves of HP1 have a structural homology (*11*). Therefore, the mechanism of chromosomal localization of HP1 may be through similar macromolecular contacts. However, since the C-terminus of *Drosophila* HP1 alone is not enough to localize to heterochromatin, it is conceivable that the real activity of the internal heterochromatin localization domain might be the "DNA-binding activity," as predicted above. If so, how is the "heterochromatinization" state formed and maintained? We previously proposed a model by which centromeric repetitive DNA could be condensed into centromeric heterochromatin through the multimeric self-association of CENP-B (see Fig. 7 of Ref. *23* in detail). A similar model presented by Platero *et al.* (*25*) can also be applied to HP1. In either case, HP1 is predicted to have two interactive sites with other HP1 molecules or other chromosomal proteins. Moreover, phosphorylation was recently suggested to be involved in the assembly and maintenance of heterochromatin in *Drosophila in vivo* (*27*). The DNA-binding activity might be affected by the phosphorylation, since serine and threonine residues are rich in the central portion. We must wait for the further biochemical study on HP1.

We have described here the detection of DNA-binding activity of human HP1. The same approach will be applied to other candidates for heterochromatin components encoded by other *Su(var)* loci in the future. In addition, this type of analysis seems to be necessary to complement the genetic and cytological study of HP1 and its related proteins to clarify the molecular basis of heterochromatin condensation.

We would like to thank Ms. Mayumi Matsushita, Mr. Masato Kitajima, and Mr. Tatsuya Shiobara (Computer Chemistry System Dept., Science Systems Div., Fujitsu Ltd.) for the secondary structure prediction of human HP1 and Prof. Cooke, H.J. for permission to use pUC1.77 clone.

REFERENCES

1. James, T.C. and Elgin, S.C.R. (1986) Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. *Mol. Cell Biol.* **6**, 3862-3872

2. James, T.C., Eissenberg, J.C., Craig, C., Dietrich, V., Hobson, A., and Elgin, S.C.R. (1989) Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of *Drosophila*. *Eur. J. Cell Biol.* **50**, 170-180
3. Wreggett, K.A., Hill, F., James, P.S., Hutchings, A., Butcher, G.W., and Singh, P.B. (1994) A mammalian homologue of *Drosophila* heterochromatin protein 1 (HP1) is a component of constitutive heterochromatin. *Cytogenet. Cell Genet.* **66**, 99-103
4. Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V., and Elgin, S.C.R. (1990) Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **87**, 9923-9927
5. Clark, R.F. and Elgin, S.C.R. (1992) Heterochromatin protein 1, a known suppressor of position-effect variegation, is highly conserved in *Drosophila*. *Nucleic Acids Res.* **20**, 6067-6074
6. Epstein, H., James, T.C., and Singh, P.B. (1992) Cloning and expression of *Drosophila* HP1 homologs from a mealybug, *Planococcus citri*. *J. Cell Sci.* **101**, 463-474
7. Singh, P.B., Miller, J.R., Pearce, J., Kothary, R., Burton, R.D., Paro, R., James, T.C., and Gaunt, S.J. (1991) A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res.* **19**, 789-794
8. Saunders, W.S., Chue, C., Goebl, M., Craig, C., Clark, R.F., Powers, J.A., Eissenberg, J.C., Elgin, S.C.R., Rothfield, N.F., and Earnshaw, W.C. (1993) Molecular cloning of a human homologue of *Drosophila* heterochromatin protein HP1 using anti-centromere autoantibodies with anti-chromo specificity. *J. Cell Sci.* **104**, 573-582
9. Paro, R. and Hogness, D.S. (1991) The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl. Acad. Sci. USA*, **88**, 263-267
10. Lorentz, A., Ostermann, K., Fleck, O., and Schmidt, H. (1994) Switching gene *swi6*, involved in repression of silent mating-type loci in fission yeast, encodes a homologue of chromatin-associated proteins from *Drosophila* and mammals. *Gene* **143**, 139-143
11. Aasland, R. and Stewart, A.F. (1995) The chromo shadow domain, a second chromo domain in heterochromatin-binding protein 1, HP1. *Nucleic Acids Res.* **23**, 3168-3173
12. Powers, J. and Eissenberg, J.C. (1993) Overlapping domains of the heterochromatin-associated protein HP1 mediate nuclear localization and heterochromatin binding. *J. Cell Biol.* **120**, 291-299
13. Sugimoto, K., Muro, Y., and Himeno, M. (1992) Anti-helix-loop-helix domain antibodies: Discovery of autoantibodies that inhibit DNA binding activity of human centromere protein B (CENP-B). *J. Biochem.* **111**, 478-483
14. Sugimoto, K., Yata, H., Muro, Y., and Himeno, M. (1994) Human centromere protein C (CENP-C) is a DNA-binding protein which possesses a novel DNA-binding motif. *J. Biochem.* **116**, 877-881
15. Muro, Y., Sugimoto, K., Okazaki, T., and Ohashi, M. (1990) The heterogeneity of anticentromere antibodies in immunoblotting analysis. *J. Rheumatol.* **17**, 1042-1047
16. Masumoto, H., Sugimoto, K., and Okazaki, T. (1989) Alphoid satellite DNA is tightly associated with centromere antigens in human chromosomes throughout the cell cycle. *Exp. Cell Res.* **181**, 181-196
17. Masumoto, H., Masukata, H., Muro, Y., Nozaki, N., and Okazaki, T. (1989) A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* **109**, 1963-1973
18. Fernandez, J.L., Goyanes, V., Pereira, S., Lopez-Fernandez, C., and Gonsalvez, J. (1994) 5-Azacytidine produces differential underdecondensation of alpha, beta and classical human satellite DNAs. *Chromosome Res.* **2**, 29-35
19. Nicol, L. and Jeppesen, P. (1994) Human autoimmune sera recognize a conserved 26 kD protein associated with mammalian heterochromatin that is homologous to heterochromatin protein 1 of *Drosophila*. *Chromosome Res.* **2**, 245-253
20. Sugimoto, K. (1994) DNA immunoprecipitation: Application to characterization of the target sequences for a human centromere DNA-binding protein (CENP-B) in *Cell Biology: A Laboratory Handbook* (Celis, J.E., ed.) pp. 335-343, Academic Press, San Diego
21. Sugimoto, K., Furukawa, K., and Himeno, M. (1994) Functional cloning of centromere protein B (CENP-B) box-enriched alphoid DNA repeats utilizing the sequence-specific DNA binding activity of human CENP-B *in vitro*. *Chromosome Res.* **2**, 453-459
22. Cooke, H.J. and Hindley, J. (1979) Cloning of human satellite III DNA: Different components are on different chromosomes. *Nucleic Acids Res.* **6**, 3177-3197
23. Sugimoto, K., Hagishita, Y., and Himeno, M. (1994) Functional domain structure of human centromere protein B: Implication of the internal and C-terminal self-association domains in centromeric heterochromatin condensation. *J. Biol. Chem.* **269**, 24271-24276
24. Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptide expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40
25. Platero, J.S., Hartnett, T., and Eissenberg, J.C. (1995) Functional analysis of the chromo domain of HP1. *EMBO J.* **14**, 3977-3986
26. Reuter, G. and Spierer, P. (1992) Position effect variegation and chromatin proteins. *BioEssays* **14**, 605-612
27. Eissenberg, J.C., Ge, Y.-W., and Hartnett, T. (1994) Increased phosphorylation of HP1, a heterochromatin-associated protein of *Drosophila*, is correlated with heterochromatin assembly. *J. Biol. Chem.* **269**, 21315-21321
28. Sugimoto, K., Migita, H., Hagishita, Y., Yata, H., and Himeno, M. (1992) An antigenic determinant on human centromere protein B (CENP-B) available for production of human-specific anticentromere antibodies in mouse. *Cell Struct. Funct.* **17**, 129-138