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Molecular cloning of a human homologue of *Drosophila* heterochromatin protein HP1 using anti-centromere autoantibodies with *anti-chromo* specificity

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SUMMARY

We have identified a novel autoantibody specificity in scleroderma that we term *anti-chromo*. These antibodies recognize several chromosomal antigens with apparent molecular mass of between 23 and 25 kDa, as determined by immunoblots. *Anti-chromo* autoantibodies occur in 10-15% of sera from patients with anti-centromere antibodies (ACA). We used *anti-chromo* antibodies to screen a human expression library and obtained cDNA clones encoding a 25 kDa chromosomal autoantigen. DNA sequence analysis reveals this protein to be a human homologue of HP1, a heterochromatin protein of *Drosophila melanogaster*. We designate our cloned protein HP1^{hs}. Epitope mapping experiments

using both human and *Drosophila* HP1 reveal that *anti-chromo* antibodies target a region at the amino terminus of the protein. This region contains a conserved motif, the *chromo* domain (or HP1/*Pc* box), first recognized by comparison of *Drosophila* HP1 with the *Polycomb* gene product. Both proteins are thought to play a role in creating chromatin structures in which gene expression is suppressed. *Anti-chromo* thus defines a novel type of autoantibody that recognizes a conserved structural motif found on a number of chromosomal proteins.

Key words: heterochromatin, HP1, autoantibodies, chromatin

INTRODUCTION

Although much of the interest in chromosomal centromeres has focused on the role played by these structures in regulating chromosomal movements in mitosis, the structure and function of centromeres during interphase also pose interesting questions. In higher eukaryotes, the centromere consists of constitutive heterochromatin, a specialized type of chromatin that is characterized by a relatively low transcriptional activity and by replication late in S phase (although there may be exceptions to this pattern of late replication - see O'Keefe et al. (1992)). While the DNA of constitutive heterochromatin is known to be composed largely of highly reiterated sequence elements, the proteins that package this DNA and regulate its biological function remain largely unknown.

Genetic experiments in *Drosophila melanogaster* have revealed that if an actively transcribed locus is moved into close proximity to constitutive heterochromatin by a rearrangement or transposition event, transcription from the locus is repressed (Muller, 1930). This so-called position effect demonstrates that the transcriptional inactivity of heterochromatin is not due solely to a lack of genes, but also reflects an active suppression of transcription by some unknown mechanism. The position effect has the further unusual property that the translocated gene may be either transcriptionally active or inactive. This decision is clonally inherited, leading to tissues with clusters of cells expressing the gene adjacent to clusters of cells that do not express it. This response is referred to as position effect variegation. Virtually all *Drosophila* genes are susceptible to position effect variegation, the extent of transcriptional inacti-

vation reflecting proximity to the heterochromatin breakpoint in a given rearrangement. This "spreading effect" of heterochromatin can extend over 80 bands, or 1500 kb (Schultz, 1950; Spofford, 1976).

Relatively little is known about the biochemistry of heterochromatin packaging. However, it has been suggested that the genetically defined suppressors and enhancers of position effect variegation might encode either chromosomal proteins or proteins involved in the modification or assembly of chromosomal proteins (Grigliatti, 1991). Recently we have found that a heterochromatin-associated protein, HP1, fulfills this prediction.

HP1 was first identified as a heterochromatin-associated protein by using monoclonal antibodies to stain the polytene chromosomes of *Drosophila* larvae (James and Elgin, 1986). The gene for HP1 has been cloned (James and Elgin, 1986; Eissenberg et al., 1990). Genetic analysis has shown that mutations in HP1 can be dominant suppressors of position effect variegation: loss of HP1 leads to increased expression of genes subject to position effect. Conversely, production of additional HP1 from a transgene leads to the enhancement of position effect (Eissenberg et al., 1990, 1992). These results suggest that HP1 plays a role in the down-regulation of transcription in heterochromatin, presumably through a packaging mechanism.

HP1 shows similarity over a region of 37 amino acids with a second *Drosophila* protein, the *Polycomb* gene product (Paro and Hogness, 1991). *Polycomb* (*Pc*) is a gene involved in down-regulation of homeotic genes during *Drosophila* development (Paro and Hogness, 1991; Lewis, 1978), and genetic results suggest that its product also functions through a stable modulation of chromatin structure (Paro and Hogness, 1991). The 37-amino acid motif shared by HP1 and the *Pc* protein has been termed the "chromo domain" (chromatin modification organizer, Paro and Hogness, 1991). Because this name assumes a function that is as yet not understood, we prefer to refer to this region of similarity as the "HP1/*Pc* box".

In humans, where considerably less is known about heterochromatin function, two well characterized centromeric autoantigens, CENP-A (17 kDa) and CENP-B (80 kDa) have recently been shown to be components of the centromeric heterochromatin. These proteins were first identified using sera from patients with anti-centromere autoantibodies (ACA) (Moroi et al., 1980; Cox et al., 1983; Guldner et al., 1984; Earnshaw and Rothfield, 1985; Nyman et al., 1986; Kingwell and Rattner, 1987). These sera predominantly recognize four polypeptide antigens termed centromere proteins (CENPs) A, B and C (Earnshaw and Rothfield, 1985) and D (Kingwell and Rattner, 1987). CENP-A has recently been purified from bovine sperm and shown by analysis of tryptic peptides to be a highly divergent isotype of histone H3 (Palmer et al., 1991). CENP-B (80 kDa) binds to a 17-base pair region of centromeric satellite DNA - the CENP-B box - (Masumoto et al., 1989), and immunoelectron microscopy shows it to be distributed through the heterochromatin beneath the kinetochore (Cooke et al., 1990). Thus both CENPs A and B are likely to play a role in the packaging of centromeric DNA. The role of CENP-C in centromere function is unknown at present, although immunoelectron microscopy indicates

that this protein is apparently located in the inner plate of the kinetochore (Saitoh et al., 1992). CENP-D, surprisingly, corresponds to the cell cycle regulatory protein RCC1 (Bischoff et al., 1990). Why this protein should be concentrated at centromeres is completely unknown.

About 10-15% of the patient sera with ACA also contain high titer antibodies to a group of four antigens with apparent molecular mass of between 23 and 25 kDa (Guldner et al., 1984; Earnshaw and Rothfield, 1985; Earnshaw et al., 1986). We will term these antigens p23-25 and the autoantibodies (which always recognize the entire group of 23-25 kDa antigens) anti-chromo (based on our cloning and epitope mapping experiments to be described here). Despite the fact that anti-chromo were detected in sera of patients with ACA, these antigens were originally classified as non-centromeric. Anti-chromo antibodies affinity-purified from nitrocellulose blots of chromosomal proteins recognized the 23-25 kDa proteins in immunoblots, but failed to recognize centromeres (or any other cellular structures) in indirect immunofluorescence experiments (Guldner et al., 1984; Earnshaw and Rothfield, 1985).

In the experiments described below, we have obtained data suggesting that the p23-25 kDa autoantigens constitute a family of human chromosomal proteins possessing the HP1/*Pc* box (chromo domain) motif, and that one of these antigens, p25, is a human homologue of *Drosophila* HP1, which we designate HP1^{HS}. Furthermore, we demonstrate that the region of the p23-25 polypeptides containing the HP1/*Pc* box appears to be the specific target of the autoimmune response in these patients. Thus anti-chromo define a novel class of autoantibodies that recognize a conserved structural motif shared by a number of chromosomal proteins.

MATERIALS AND METHODS

Cells and antibodies

HeLa cells were grown in RPMI-1640 (Gibco Laboratories; Rochester, NY) supplemented with 5% Hyclone calf serum (Sterile Systems; Logan, Utah).

Autoantisera used in these experiments have been characterized in detail elsewhere (Earnshaw et al., 1986). Anti-chromo were found in sera of two individuals with CREST scleroderma one individual with Systemic Lupus Erythematosus and (non-CREST) scleroderma, and one individual with Sjögren's syndrome plus Raynaud's phenomenon. Anti-chromo antibodies were not found by immunoblotting in 70 normal human sera (S. Hildebrand, B. J. Bordwell, N. F. R. and W. C. E., unpublished observations).

Production of antibodies to bacterial fusion proteins in guinea pigs and rabbits was performed as described previously (Saunders et al., 1991; Saunders, 1990). Two sera from rabbits injected with a fusion protein encompassing the entire open reading frame for p25 were used for these studies. The first of these, designated Ra1-anti-HP1^{HS}, recognizes the complete set of cellular p23-25 antigens (Fig. 1, lanes 3, 5) (Saunders et al., 1991; Saunders, 1990). The second serum, Ra2-anti-HP1^{HS}, recognizes only the chromosomal p25 antigen (data not shown) (Saunders et al., 1991; Saunders, 1990). Serum from one guinea pig injected with this antigen also reacted solely with the 25 kDa chromosomal antigen (data not shown).

Both monoclonal and polyclonal antibodies have been prepared that are specific for *Drosophila* HP1. The rabbit antiserum used

here was prepared using a synthetic polypeptide (amino acids 25-47) linked to keyhole limpet hemocyanin (Green et al., 1982). The complexed peptide was injected initially in Freund's complete adjuvant; subsequent booster injections used Freund's incomplete adjuvant. Specificity of the antiserum was established using immunoblots to analyze *Drosophila* nuclear proteins.

Biochemical procedures

HeLa chromosomes were purified through Percoll and sucrose gradients (Earnshaw et al., 1984). Typically, 500 ml of culture yielded sufficient chromosomes for an SDS-polyacrylamide gel with a single 11 cm well. The protocols used for SDS-PAGE and immunoblotting have been described previously (Earnshaw and Rothfield, 1985). Antibody incubations were routinely carried out at a dilution of 1:1,000. Bound antibody was detected with ¹²⁵I-protein A. Affinity-purification of antibodies from proteins immobilized on nitrocellulose was performed as described previously (Earnshaw and Rothfield, 1985).

Induction of a β -galactosidase: 3a fusion protein from a β -gt11 lysogen was carried out by standard methods (Snyder et al., 1988). The fusion protein, which was sequestered in insoluble inclusion bodies, was partially purified by differential centrifugation (Rothfield et al., 1987). The granules remained intact throughout the purification.

These fusion protein granules were used directly as an antibody absorption substrate. Approximately 1 mg of partially purified granules was preincubated for 30 minutes in phosphate buffer with 4% BSA (Earnshaw and Rothfield, 1985). Next, antibody at a dilution of 1:50 was added to the granules and the mixture gently shaken for 8-12 hours at room temperature. The granules were centrifuged for 5 minutes in the microfuge, and the supernatant (containing unbound antibody) removed.

Molecular cloning of the p23-25 kDa antigens

Two cDNA libraries in the β -gt11 expression vector (Young and Davis, 1983a,b) were screened with antibody by standard methods (Snyder et al., 1988). One cDNA library (provided courtesy of M. Chow and R. Axel, Columbia University) was made from poly(A)⁺ RNA from human umbilical vein fibroblasts. The other cDNA library (Clontech) was made from human placental RNA. A total of 2.7×10^6 clones were screened, yielding 6 plaques expressing epitopes present on the p23-25 kDa antigens.

Lysogens were made from plaque pure phage stocks by mixing phage and *E. coli* strain Y1089 at a multiplicity of infection of 5 for 20 minutes at 30°C. Subsequent growth and testing for lysogeny was as described (Snyder et al., 1988).

DNA sequencing

DNA to be sequenced was either subcloned into the M13 vectors mp18 and/or mp19 (Messing and Vieira, 1982), or sequenced directly in pUC9. Subsequent sequencing followed standard methods (Sanger et al., 1977; Tabor and Richardson, 1987), with priming using the -40 M13 sequencing primer (New England Bio-Labs, Beverly, MA). Either polymerase I Klenow fragment (BRL Life Technologies; Gaithersburg, MD) or Sequenase II (United States Biochemical Corporation; Cleveland, Ohio) was used. Sequencing of clone 9A in pUC9 was performed using two synthetic primers. The first oligonucleotide was a 19-mer in the sense orientation starting at nucleotide 317, and consisting of the following sequence: CAATACTTGGGAACCTGAG. The second oligonucleotide was a 17-mer in the antisense orientation starting at nucleotide 611, and consisting of the following sequence: CAGGTCAGCTTCATCTG. These primers were designed to cover the 350-600 bp central region of p25 in both the sense and the anti-sense orientations.

Southern (DNA) and northern (RNA) hybridization

Human lymphocyte genomic DNA was a gift of K. Smith and M. K. McCormick (Johns Hopkins Medical School). HeLa cytoplasmic RNA was isolated from the post-nuclear supernatant using proteinase K digestion and phenol/chloroform extraction (Sambrook et al., 1989). Poly(A)⁺ RNA was isolated from cytoplasmic RNA by two passages over a 1 ml oligo-dT column (Collaborative Research; Lexington, MA).

DNA was electrophoresed in horizontal 0.8% agarose gels in Tris acetate buffer (Sambrook et al., 1989). RNA was electrophoresed in vertical 1.0% agarose gels containing 20 mM MOPS, pH 7.0, 1 mM EDTA, 5 mM sodium acetate and 6.6% formaldehyde. The sample buffer was 19.2 mM MOPS, pH 7.0, 1 mM EDTA, 4.8 mM sodium acetate, 48% formamide (v/v), 6.3% formaldehyde (v/v), 0.1% Ficoll, and a small amount of bromophenol blue. Markers for the RNA gel were 0.24-9.5 kb RNA size standards (BRL Life Technologies; Gaithersburg, MD). Nucleic acids were transferred to nitrocellulose filters as described (Sambrook et al., 1989).

cDNA probes were labeled by nick translation or random primer extension with ³²P (Rigby et al., 1977; Feinberg and Vogelstein, 1983, 1984). Hybridization using 10⁶ cts per min/ml of probe was performed at 42°C for 12-36 hours in 50% deionized formamide (BRL Life Technologies; Gaithersburg, MD), 20 mM HEPES, pH 7.4, 5 × SSC, 0.06% polyvinyl pyrrolidone, 0.06% Ficoll plus 60 µg/ml single stranded herring sperm DNA. All blots were prehybridized for 2-12 hours under the same conditions without probe.

Construction of Lac Z-HP1 fusion genes

An HP1 cDNA was trimmed at the 5' end to the second codon using *Bal31* digestion, a *SalI* linker was ligated and the 5' truncated cDNA was cloned into the *SalI* site at the 3' end of the Lac Z coding sequence in the *Hsp70*-Lac Z promoter fusion vector p194.702T (Simon et al., 1985). To create specific truncated HP1 peptides (see Fig. 5), the HP1 sequence was cleaved at the indicated positions with the following restriction endonucleases: 41, *ScaI*; 95, *StyI*; 151, *BglII*. The ability of these constructs to direct synthesis of β -gal-HP1 fusion protein in embryos was independently verified using anti- β -gal serum in immunoblots (Powers and Eissenberg, 1993). Detailed maps of these constructs are available upon request from J. C. E.

RESULTS

Cloning of the p25 antigen

We have identified a number of human autoimmune sera having both anti-centromere antibodies and autoantibodies to several chromosomal autoantigens of molecular mass 23-25 kDa. An immunoblot using one of these autoantisera is shown in Fig. 1, lane 1. This serum recognizes CENPs A-C in addition to the p23-25 antigens.

When this serum was used to screen a human cDNA library in β -gt11, we obtained a group of six related cDNA clones. Affinity-purification of the autoantibodies from an immunoblot of a bacterially expressed fusion protein demonstrated that these clones encode at least one auto-epitope shared by all of the p23-25 antigens (Fig. 1, lane 2). Immunoabsorption experiments using rabbit antisera raised to a bacterial fusion protein demonstrated that these clones encode the 25 kDa antigen.

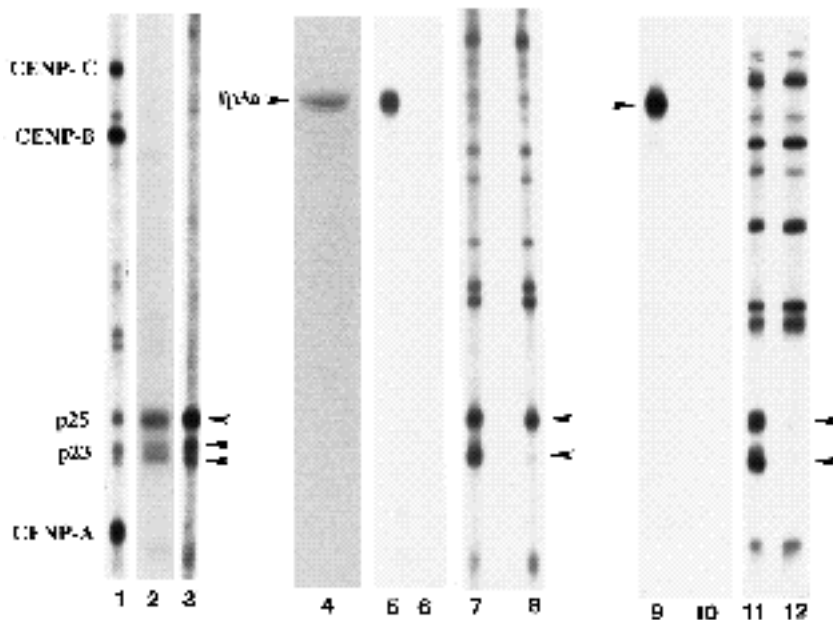


Fig. 1. (Lanes 1-3) Autoantiserum and experimental rabbit serum identify the p23-25 antigens. Purified human chromosomes were subjected to SDS-PAGE and immunoblotting (Earnshaw and Rothfield, 1985) with the following sera: lane 1, autoantiserum; lane 2, anti-p23-25 antibodies affinity-purified from the autoantiserum (Earnshaw and Rothfield, 1985); lane 3, rabbit serum Ra1-anti-HP1^{Hsa}. Arrowheads indicate the p23-25 kDa antigens. (Lanes 4-8) Cloned and chromosomal p25 share at least two independent epitopes recognized by the rabbit serum. A short cDNA (3a - see Fig. 3) encoding 56 amino acids from the amino terminus of p25 was expressed in *E. coli* and the resultant β -galactosidase fusion protein was purified by differential centrifugation. Lane 4 shows a Coomassie-blue-stained gel of the purified 3a fusion protein. (Lanes 5,6) Immunoblot of the 3a fusion protein with the rabbit antibodies used in lane 3. Lane 5 shows a blot with whole serum. Lane 6 shows a blot with this serum following absorption with the 3a fusion

protein granules. The absorption removes all antibodies reactive with the 3a fusion protein. (Lanes 7, 8) Immunoblot of human chromosomal proteins with the rabbit sera, as in lanes 5 and 6, respectively. The absorption removes all antibodies reactive with the p23 antigens (lower arrowhead), but antibodies to the p25 antigen remain (upper arrowhead). (Lanes 9-12) Autoantiserum adsorbed with 3a fusion protein no longer binds to the p23-25 antigens. Lanes 9 and 10 demonstrate that the absorption of autoantiserum with 3a fusion protein was complete. Lane 9, immunoblot of 3a protein with autoantiserum prior to absorption. Lane 10, identical blot probed with adsorbed serum. Lanes 11 and 12 show the use of these sera to probe human chromosomal proteins. Lane 11, immunoblot probed with autoantiserum prior to absorption. Lane 12, identical blot probed with adsorbed serum.

Two rabbit antisera were obtained following immunization with bacterially expressed fusion protein. One serum, Ra1-anti-HP1^{Hs}, recognized the complete set of cellular p23-25 antigens in immunoblots of chromosomes (Fig. 1, lanes 3, 5) (Saunders, 1990; Saunders et al., 1991). The second, Ra2-anti-HP1^{Hs}, recognized only p25 in immunoblots of human chromosomal proteins (data not shown).

Ra1-anti-HP1^{Hs} recognized at least two epitopes shared by chromosomal p25 and the bacterial fusion protein. When this serum was absorbed with isolated β -galactosidase fusion protein derived from clone 3a (see Fig. 3), all antibodies recognizing the fusion protein were eliminated (Fig. 1, lanes 5, 6). In blots of chromosomal proteins, the absorbed serum continued to recognize p25, but no longer bound to the p23 polypeptides (Fig. 1, lanes 7, 8).

This experiment defines at least two epitopes shared by the bacterial fusion protein and chromosomal p25. The first of these, 1, is present on the p25 antigen(s), but not in the region encoded by clone 3a. Thus incubation of this serum with the 3a fusion protein does not abolish binding to epitope 1 on chromosomal p25. The second epitope, 2, is present within the 56-amino acid region encoded by clone 3a. 2 must be the sole epitope present on the 23 kDa antigen(s) that is recognized by this serum. Thus, removal of antibodies to 2 by absorption with the fusion protein abolishes the ability of this serum to recognize the chromosomal p23 antigen(s).

These observations provide strong suggestive evidence that the cloned cDNA encodes the cellular p25 protein.

p25 is a human homologue of *Drosophila melanogaster* heterochromatin protein 1

The two longest cDNA clones obtained by antibody screening (9a and 1d) were both 876 bp in length. Four additional clones, all shorter, were contained within clone 9a, and were identical in sequence to it. Interestingly, the 5'-most 85 base pairs of clone 1d were completely divergent in sequence from the other clones. The reason for the divergence of the sequence at the 5' end of clone 1d is unknown: it could reflect expression from distinct genes or alternative splicing of a single transcript, or it could result from a cloning artifact. We cannot at present distinguish between these three possibilities. If our interpretation of the cDNA sequence is correct, this variation would have no effect on the structure of the encoded protein.

Northern (RNA) blotting reveals that the cDNAs hybridize predominantly to a low-abundance poly(A)⁺ mRNA of 700-800 bases in length (Fig. 2). Thus it is likely that the longer clones encompass essentially the full length of the mRNAs. (In some experiments, weaker hybridization was also noted to a 9.8 kb RNA species.) The results of Southern hybridization are consistent with the presence of at least two genes complementary to these cloned cDNAs, since two distinct fragments were recognized in each of three restriction digests probed with clone 3A (Fig. 2).

The nucleotide sequence of the cDNAs was determined by standard procedures (Sanger et al., 1977). The clones contain 607 bp of open reading frame, followed by a 3 untranslated region 169 base pairs in length (Fig. 3). The first ATG occurs at position 135. The sequence flanking

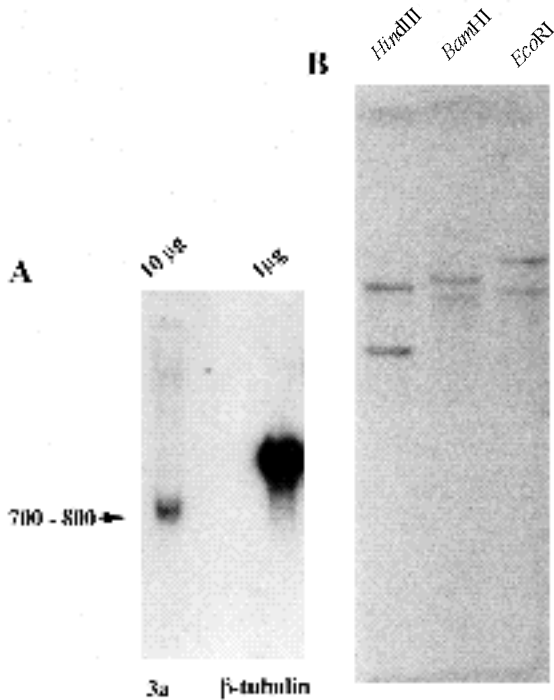


Fig. 2. Northern (RNA) and Southern (DNA) hybridization with a cDNA clone for HP1^{HS} (clone 3a). (A) HP1^{HS} is encoded by a relatively non-abundant polyadenylated mRNA. The figure compares the hybridization of a labeled 3a probe to 10 µg of poly(A)⁺ RNA from HeLa cells with that of a mouse β-tubulin probe to 1 µg of the same RNA (4-day exposure). (B) Clone 3a hybridizes to two different DNA bands in restriction digests of human DNA with three different enzymes. The bands shown range between 4.3 and 17 kb.

this ATG shows a 4/7 match to the Kozak consensus, and includes the most highly conserved features (purine at position -3 and G at position +4 (Kozak, 1987)). The striking similarity between the cloned protein and the HP1 proteins from *Drosophila* and mouse (see below) also suggests strongly that the ATG at position 135 is the initiation codon, and this assignment has been used in the preparation of Fig. 3.

The deduced sequence of p25 shares significant similarity with that of the HP1 proteins of *Drosophila melanogaster* (70%, James and Elgin, 1986; Eissenberg et al., 1990) and *Drosophila virilis* (74%, Clark and Elgin, 1993). The sequence contains both the conserved amino-terminal and carboxyl-terminal domains found in HP1s from *Drosophila* (Clark and Elgin, 1992), and we therefore designate the protein HP1^{HS}. We suggest the use of the term HP1^{HS} to designate clone HSM1 previously isolated in a screen for mouse and human proteins containing the HP1/*Pc* box (Singh et al., 1991). Interestingly, the HP1/*Pc* box homology is entirely contained within the 56-amino acid region of p25 encoded by clone 3a.

HP1^{HS} is predicted to be an acidic polypeptide of MW 22,222 and pI 5.6. This corresponds closely to the isoelectric point of the human p23-25 kDa proteins observed in two-dimensional gel electrophoresis (Cox et al., 1983; Saunders, 1990).

Mapping of the autoepitope to the region of HP1^{HS} containing the chromo domain (HP1/*Pc* box)

The target of the autoimmune response against the p23-25 antigens is entirely located within the 56-amino acid region of HP1^{HS} encoded by clone 3a. Thus, when the patient's

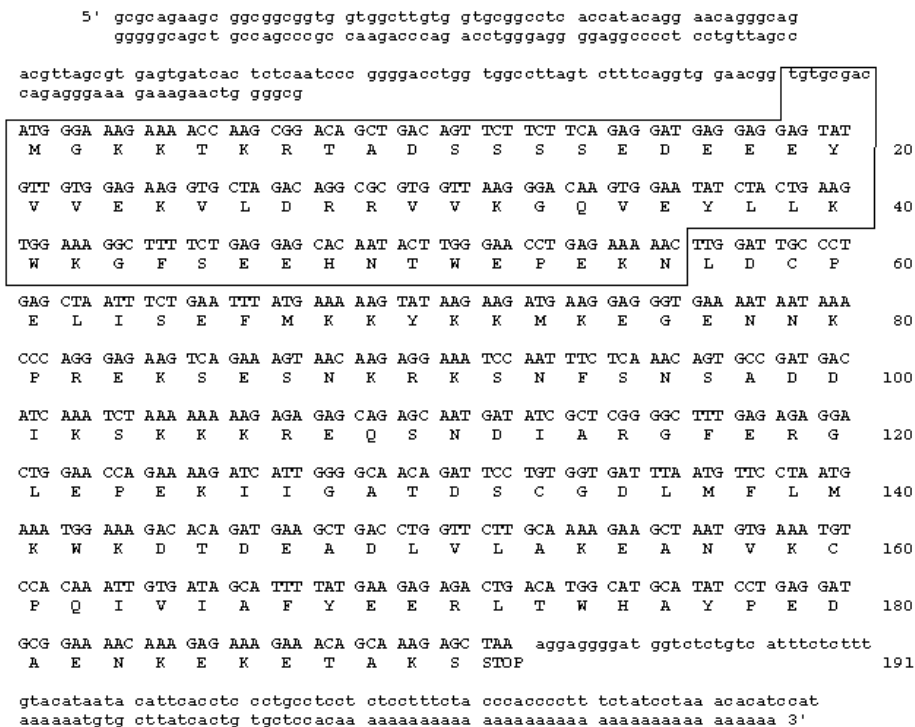


Fig. 3. Nucleotide and deduced amino acid sequence of HP1^{HS}. The position of the ATG translation initiation codon is inferred by comparison with homologous HP1 sequences. Coding sequence is in capitals. The position of the 3a cDNA is boxed. Both sequences obtained for the 5' region are shown. The upper sequence corresponds to that shared by 9a and the four shorter clones.

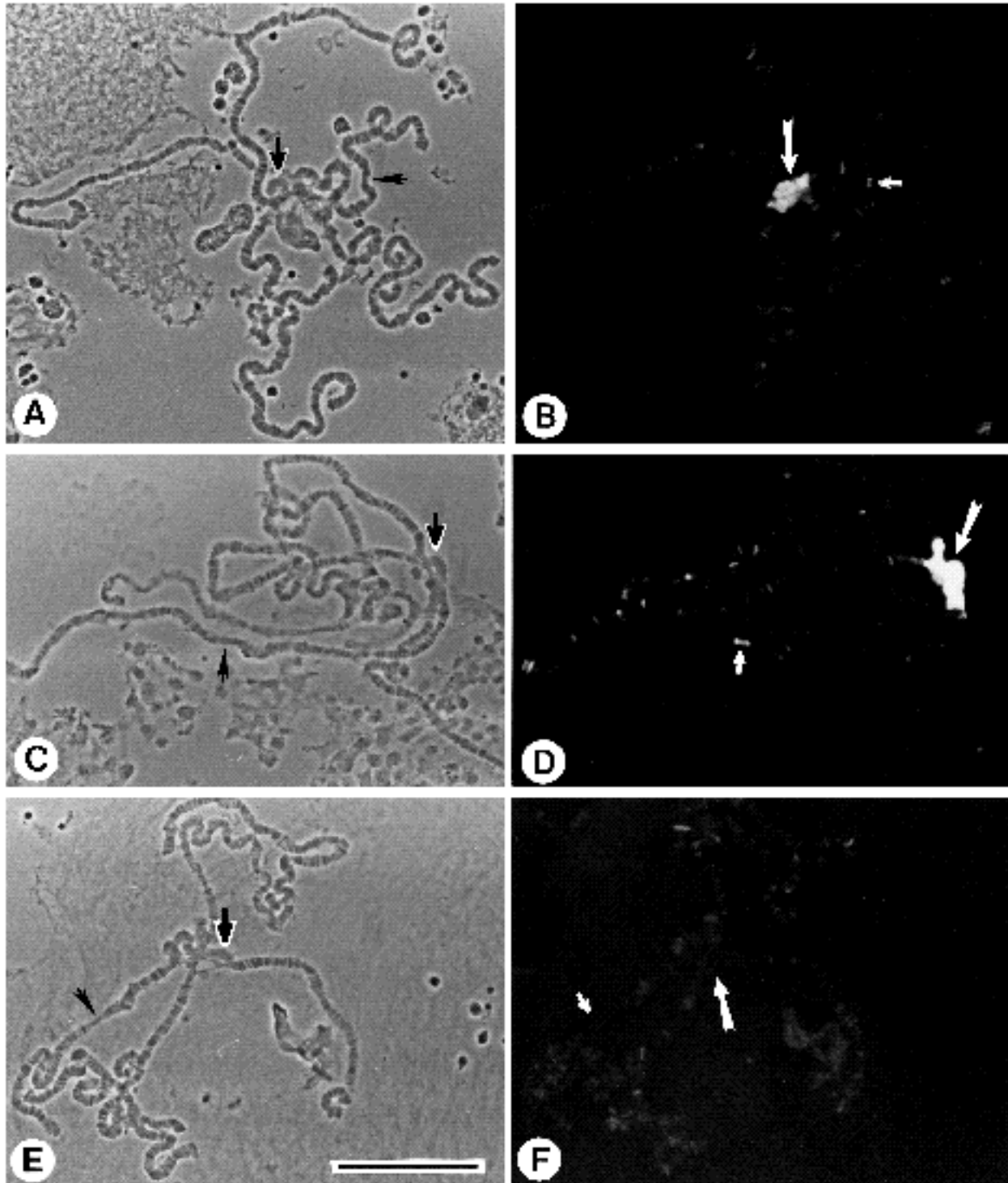


Fig. 4. Human autoimmune serum containing anti-chromo autoantibodies reacts with heterochromatic regions of *Drosophila* polytene chromosomes. Immunofluorescence staining is shown using the following sera: (B) autoimmune serum containing both anti-centromere and anti-chromo autoantibodies (as in Fig. 1); (D) polyclonal serum raised against cloned *Drosophila* HP1 (James et al., 1989); (F) anti-centromere serum containing antibodies to the CENPs but lacking anti-chromo autoantibodies. (A, C and E) are phase contrast images. (B, D and F) are fluorescence images. Anti-chromo and anti-HP1 antibodies label region 31 on chromosome arm 2L (indicated by small arrows) as well as the heterochromatin at the chromocenter (indicated by large arrows). Anti-centromere serum lacking anti-chromo autoantibodies weakly labels a number of loci on polytene chromosomes, but shows no labeling of heterochromatic regions. Immunofluorescence was performed as described (James et al., 1989). Bar, 10 μ m.

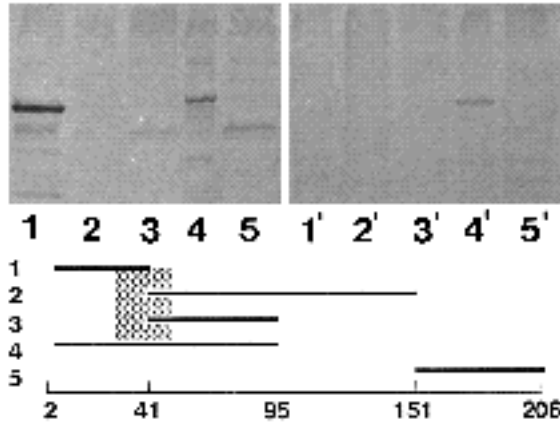


Fig. 5. Human anti-chromo autoantibodies target the HP1/Pc box (chromo domain) motif of *Drosophila* HP1. *Drosophila* strains expressing -galactosidase fusion proteins containing various portions of the HP1 molecule were subjected to immunoblotting with (lanes 1-5) rabbit antibodies raised against a peptide consisting of residues 25-47 of *Drosophila* HP1 or (lanes 1'-5') anti-chromo autoantibodies. The portions of HP1 present in each fusion protein are shown in the diagram at the bottom of the figure. The shaded area between amino acids 25 and 61 represents the HP1/Pc box motif. Anti-chromo autoantibodies recognize the fusion protein containing residues 2-95 of *Drosophila* HP1, but none of the other fusion proteins. The rabbit anti-peptide antibody recognizes, as expected, fusion proteins containing residues 2-95 and 1-41 of HP1. The identity of the anomalous bands recognized in control lanes 1 and 5 is not known.

serum was adsorbed with 3a fusion protein granules as described above, all antibodies recognizing the p23-25 antigens on immunoblots were removed (Fig. 1, lanes 11, 12). No change in binding to any other chromosomal antigens was observed, indicating that the absorption of anti-p23-25 was specific. This result was not peculiar to the patient serum shown in Fig. 1: identical results were obtained with sera from three unrelated patients with anti-p23-25 (data not shown).

These results raised the possibility that the human anti-p23-25 autoantibodies might be directed against the HP1/Pc box itself. Two independent lines of evidence have been obtained in support of this hypothesis.

First, when two human autoantisera containing anti-p23-25 were used to stain polytene chromosomes, immunore-

activity was detected at the chromocenter, the fourth chromosome and region 31 on chromosome arm 2L (Fig. 4A,B). A similar staining pattern was obtained when chromosomes were stained with anti-HP1 antibodies, including those directed against part of the HP1/Pc box motif (amino acids 25-47 of *Drosophila* HP1 - Fig. 4C,D) (James et al., 1989). In addition, a rabbit serum Ra2-anti-HP1^{Hs} that reacts only with the p23-25 antigens by immunoblotting (see Fig. 1) also stained the chromocenter (data not shown). In control experiments, sera from three patients with ACA but without anti-p23-25 antibodies all failed to stain the chromocenter (Fig. 4E,F and data not shown).

Secondly, human anti-p23-25 autoantibodies recognize the HP1/Pc box region of *Drosophila* Hp1 in immunoblots (Fig. 5). For this experiment, fly stocks were obtained that express various regions of the *Drosophila* HP1 molecule fused to -galactosidase. Embryo lysates were prepared from these strains and subjected to immunoblotting. In all cases, the autoantibodies recognized a species comigrating with the endogenous HP1 (not shown). In addition, the autoantibodies also recognized a fusion protein containing residues 2-95 (Fig. 5 lane 4) of *Drosophila* Hp1 fused to -galactosidase, but neither of two fusion proteins containing residues 2-41 or 41-95 of HP1 (Fig. 5, lanes 1 and 3). This suggests either that the autoantisera may recognize a determinant at or near residue 41 (in the HP1/Pc box), or that the epitope is unable to renature in these short fusion proteins. In addition, the autoantibody failed to recognize either of two fusion proteins containing residues 41-151 or 152-206 of *Drosophila* HP1 (Fig. 5, lanes 2 and 5), confirming that no auto-epitopes are found outside the HP1/Pc box region of the protein.

In recognition of the reactivity of these autoantibodies with the HP1/Pc box motif, we propose to call this novel autoantibody specificity anti-chromo.

DISCUSSION

The present results reveal that some patients with anti-centromere autoantibodies have antibodies against a human homologue of the *Drosophila* heterochromatin protein HP1. We have used these autoantibodies to clone several cDNAs encoding this protein, which we designate HP1^{Hs}, and here report the complete sequence of these cDNAs. Analysis of

MGKKTAKRTADSSSS-----EDEEE	YVVEKVLDRRVVVGKQVEYLLKWKGFSEEHNTWEPEN	LDCEPELISEF HP1 ^{Hs} a
MGKKIDNPFSSAKV--SDAEEEEEE	YAVKRIIDRRVRKGGKVEYLLKWRGYPETENTWEPENN	LDCCDLIQQY HP1
MGKQGN-K-KKVEEVL--EEEEEE	YVVEKVLDRRVVVGKQVEYLLKWKGFSDDEDNTWEPENN	LDCPDLIAEF M31
MGKQNGK SKKVEE-----AEPEE	FVVEKVLDRRVVVGKQVEYFLKWKGFDTADNTWEPENN	LDCEPELIDF M32
VLEEEEE	YVVEKVLDRRVVVGKQVEYLLKWKGFSDDEDNTWEPENN	LDCPDLIAEF HSM1
MTGRGKGSKGLGRDNATDDPVDLV	YAAEKIIQKRVKQVVEYRVLKWKGNQRYNTWEPENN	ILDRLRLIDY Pc
MGKK.....E.EE	YVVEKVLDRRVVVGKQVEY.LLWKWGF....NTWEPEN	LDCEPELISEF OVERALL CONSENSUS

Fig. 6. Conservation of the HP1/Pc box between the *Homo sapiens* HP1^{Hs}, *Drosophila melanogaster* HP1 (Eissenberg et al., 1990), *Mus musculus* M31 and M32 (Singh et al., 1991), *Homo sapiens* HSM1 (Singh et al., 1991) and *Drosophila melanogaster* Pc (Paro and Hogness, 1991) deduced protein sequences. Amino acid identities between the various proteins and p25 are indicated by colons. Dashed lines

represents gaps introduced in the sequence to maximize the homology. The HP1/Pc box (chromo domain) is indicated by the boxed amino acids. The consensus sequence given at the bottom indicates residues conserved in at least four of the proteins. The underlined residues are conserved in all six sequences.

the autoimmune response against HP1^{Hs} reveals that this is an unusual response, with the autoantibodies recognizing a localized region shared by several chromosomal proteins. Previous studies with cloned chromosomal autoantigens have revealed a tendency to recognize multiple sites distributed across individual proteins (Earnshaw et al., 1987; D'Arpa et al., 1990). We designate this novel autoantibody specificity in scleroderma **anti-chromo**.

The region of HP1^{Hs} recognized by the patient sera used here is of considerable interest in its own right, since this region, termed the HP1/*Pc* box (chromo domain) motif was first identified in the *Drosophila* HP1 and *Pc* proteins (Pao and Hogness, 1991). This motif is thought to be a hallmark of proteins that produce stable alterations in chromatin structure. The precise function of the HP1/*Pc* box motif is not known. It does not appear to be directly involved in DNA binding, but may be tightly complexed with another factor (or factors) *in vivo*. This is suggested by the observation that affinity-purified anti-chromo autoantibodies (shown here to specifically target the HP1/*Pc* box motif) are completely inactive in immunofluorescence experiments on human cells, even though they react well in immunoblots and bind to native fusion protein in solution (Guldner et al., 1984; Earnshaw and Rothfield, 1985). We suggest that the presence of other tightly bound factors may mask the HP1/*Pc* box motif from recognition by the autoantibodies.

The HP1/*Pc* box motif appears to be a novel type of autoantigen in human rheumatic disease. The great majority of autoantibodies are highly specific for particular proteins: hence their great importance as reagents in cell and molecular biology. In contrast, the anti-chromo autoantibodies apparently recognize a family of chromosomal antigens containing the HP1/*Pc* box motif. This is consistent with the observation that anti-chromo autoantibodies all recognize several chromosomal antigens of 23-25 kDa molecular mass. That these are distinct proteins, and not simply different modification states of a single polypeptide, is supported by our observations that two rabbit polyclonal antibodies (Ra1-anti-HP1^{Hs} and Ra2-anti-HP1^{Hs} following absorption with the 3A fusion protein) recognize only the 25 kDa species.

Comparison of the sequence of HP1^{Hs} with that of *Drosophila* HP1 reveals that the two proteins are closely related (70% similarity). Within the amino-terminal domain (residues 19-67, indexed on the *Drosophila* sequence) p25 and HP1 share 71% amino acid identity. Within the carboxyl-terminal domain (residues 141-200) they share 55% identity. This extensive similarity is the basis for our suggestion that p25 is a human homologue of HP1. This suggestion is supported by the observation that rabbit antibodies to *Drosophila* HP1 recognize several proteins of 23-25 kDa in immunoblots of nuclear lysates (R. L. C. unpublished data).

Two murine cDNAs encoding HP1 homologues were recently obtained by screening a mouse embryo cDNA library with a probe corresponding to the HP1/*Pc* box region of *Drosophila* HP1 (Singh et al., 1991). These cDNAs, designated M31 and M32, exhibit 82% and 85% similarity to HP1^{Hs}, respectively (Singh et al., 1991). The same authors then screened a human cDNA library with the M31 clone, obtaining a clone that they termed HSM1. The

sequence of HSM1, though not shown in its entirety, was stated to be identical to that of M31 (Singh et al., 1991). Because HP1^{Hs} and the protein encoded by clone M31 are only 81% identical to one another, we infer that HP1^{Hs} and the HSM1 protein are two distinct human homologues of HP1. We suggest that p25 be referred to as HP1^{Hs} and the HSM1 protein be referred to as HP1^{Hs}. The sequence of the HP1/*Pc* box motif in HP1^{Hs} is compared to those from the *Drosophila*, mouse and human (HP1^{Hs}) HP1 homologues as well as to that from the *Pc* protein in Fig. 6.

The chromosomal location of HP1^{Hs} is unknown. As stated above, affinity-purified, anti-chromo autoantibodies do not recognize cellular structures when used in indirect immunofluorescence (Guldner et al., 1984; Earnshaw and Rothfield, 1985) (W.S.S., unpublished observations). Nonetheless, two factors lead us to suggest that HP1^{Hs} is likely to be a component of the centromeric heterochromatin. Firstly, HP1 is known by both cytological and genetic criteria to be associated with centromeric heterochromatin in *Drosophila* (James and Elgin, 1986; Eisenberg et al., 1990, 1992; James et al., 1989). Secondly, autoantibodies to HP1^{Hs} have thus far only been seen in sera of patients with anti-centromere antibodies (Guldner et al., 1984; Earnshaw and Rothfield, 1985). Autoantibodies in rheumatic diseases often recognize components of macromolecular complexes, including snRNPs, nucleosomes, nucleoli and centromeres (Hardin, 1986; Tan, 1989). The linkage between anti-chromo and anti-centromere antibodies suggests that HP1^{Hs} might be a component of the centromeric complex, albeit one that is only recognized by ~10-15% of patients who mount an anti-centromere autoimmune response.

While HP1^{Hs} may be associated with heterochromatin, others of the p23-25 proteins may have a different localization within the nucleus. Rabbit antiserum Ra1-anti-HP1^{Hs} stains a novel nuclear domain termed the PIKA (polymorphic interphase karyosomal association) in interphase human cells (Saunders et al., 1991). The PIKA staining was seen when the antibodies were affinity-purified from either a clone 3a-*galactosidase* fusion protein or the chromosomal p23-25 antigens. The functional significance of the PIKA is not known. Immunofluorescence experiments suggested that this region contains significantly reduced levels of hnRNP and snRNP antigens relative to the surrounding nucleoplasm, suggesting that there may be a low level of transcriptional activity within the PIKA regions (Saunders et al., 1991). Therefore, proteins containing the HP1/*Pc* box motif may be associated with inhibition of transcriptional activity outside of the constitutive heterochromatic regions of the cell, as is true for the *Polycomb* gene product in *Drosophila*.

As noted above, our data suggest that the p23-25 antigens represent a family of chromosomal proteins having in common the HP1/*Pc* box. Because anti-chromo specifically target this structural motif, these autoantibodies may provide a simple means of identifying other human proteins containing the HP1/*Pc* box motif. Our results, together with those of Singh and coworkers (Singh et al., 1991) reveal that at least two different homologues of HP1 are present in human cells. However, other proteins that are not homol-

ogous overall to HP1 may also contain the HP1/*Pc* box motif. Such proteins could, for example, include human homologues of the *Pc* protein. It will be interesting to determine in future experiments whether the remaining p23-25 kDa antigens represent other HP1 homologues or distinct polypeptide species with the HP1/*Pc* box motif.

The function of the HP1/*Pc* box (chromo domain) is not known at present, but the characteristics of the proteins in which it has been found are consistent with the suggestion that this domain may be a binding site for one or more proteins to form a complex that is involved in the down-regulation of transcription. We anticipate that the results and reagents reported here may contribute to the future determination of the role of HP1^{Hs} and the HP1/*Pc* box in heterochromatin structure.

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