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CHD1 interacts with SSRP1 and depends on both its chromodomain and its ATPase/helicase-like domain for proper association with chromatin

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Abstract. CHD1, an M_r ~200,000 protein that contains a chromodomain (C), an ATPase/helicase-like domain (H) and a DNA-binding domain (D), was previously shown to be associated with decompacted interphase chromatin in mammalian cells and with transcriptionally active puffs and interbands in *Drosophila* polytene chromosomes. We now show by transient transfection experiments with genes expressing wild-type and mutant forms of CHD1 that both the C and H domains are essential for its proper association with chromatin. We also present evidence for an in vivo interaction between CHD1 and a novel HMG box-containing protein, SSRP1, which involves an amino-terminal segment of CHD1 that does not include the chromodomain. Immunocytochemical analyses indicated that CHD1 and SSRP1 colocalize in both mammalian nuclei and *Drosophila* polytene chromosomes.

Introduction

Cell growth and development depend on the selective expression of genetic information encoded in DNA. The first step in this process involves recognition of a particular subset of genes by RNA polymerase and other components of the transcriptional apparatus. For this to occur, the chromatin structure in which DNA is embedded must be locally remodeled so as to render the relevant genes accessible to the transcriptional machinery. Remodeling entails an unfolding and reorganization of the densely packed arrays of nucleosomes, which comprise the fundamental building blocks of chromatin. It has become evident that cells are endowed with a wide variety of mechanisms for altering chromatin structure, including the modification of histones by acetylation/deacetylation and interactions with specialized nonhistone proteins that either promote chromatin compaction or facilitate its decompaction (Kingston et al. 1996; Tsukiyama and Wu 1997). A myriad of such specialized proteins has been identified by both genetic and biochemical studies; however, a clear picture of how they cause changes in chromatin structure has yet to emerge.

A few years ago, we discovered an M_r ~200,000 protein, termed CHD1, whose structural properties suggested that it might play an important role in chromatin remodeling (Delmas et al. 1993). The hallmark of CHD proteins, which is signified by their name, is their novel combination of structural domains. Approximately 300 amino acids from the amino-terminus of these proteins is a sequence of about 60 amino acids (C) that resembles the so-called chromatin-organization-modifier (chromo) domain of proteins that have been implicated in chromatin compaction and transcriptional silencing (Paro 1993). In the central region, there is a sequence of about 500 amino acids (H) that is closely related to the ATPase/helicase-like domain of proteins that participate in nucleosome disruption and transcriptional activation (Pazin and Kadonaga 1997). Toward the carboxy-terminus, there is a 230 amino acid segment (D) with DNA-binding capability (Stokes and Perry 1995). In previous studies of mouse CHD1, we observed that the protein is associated with noncompacted interphase chromatin and that it is released into the cytoplasm when chromosomes condense during mitosis (Stokes and Perry 1995). More recent studies of *Drosophila* CHD1 have shown that it is preferentially located in the transcriptionally active, decompacted regions of polytene chromosomes (Stokes et al. 1996). Collectively, these observations favor the idea that CHD1 helps to maintain chromatin in a transcriptionally active state.

Proteins that appear to be orthologs of mouse and *Drosophila* CHD1 have been identified in a broad range of organisms from yeast to human, either as a consequence of genome-sequencing projects or from studies designed

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for other purposes (see Stokes et al. 1996; Woodage et al. 1997 for references). In mammals, *Drosophila* and *Caenorhabditis elegans*, there are one to three additional proteins that are clearly related to CHD1 (Woodage et al. 1997). Thus, in metazoan cells, the CHD family of proteins is encoded by multiple genes, some of which have a long evolutionary history. Conceivably, each CHD variant may be associated with a distinct remodeling complex, as is known to be the case with variant forms of other H-domain-containing proteins (Cairns et al. 1996; Wang et al. 1996).

The experiments described below have extended our characterization of CHD1 in two ways. Firstly, we demonstrate by transfection experiments with genes expressing wild-type and mutant forms of CHD1 that the C and H domains are essential for its proper association with chromatin. Secondly, we present evidence that CHD1 is associated with an interesting HMG box-containing protein, presumably as part of a large multiprotein complex. This evolutionarily conserved protein, termed SSRP1 because it specifically recognizes structurally modified DNA (Bruhn et al. 1992), colocalizes with CHD1 in both mammalian nuclei and *Drosophila* polytene chromosomes.

Materials and methods

Constructs and transient transfections. The parental expression vector pWS4 (Sheay et al. 1993) contained a 684 bp segment encompassing the cytomegalovirus (CMV) promoter, a 368 bp segment bearing the adenovirus tripartite leader and a spliceable intron, the polylinker of Bluescript II SK and a 236 bp segment bearing the SV40 poly(A) site. Standard recombinant DNA procedures were used to insert segments encoding the IgG-binding region of protein A (Pro A, 780 bp) or the green-fluorescent protein (gfp) ST65 (729 bp) (Schaar et al. 1997), and then, in frame, segments encoding the wild-type and mutant forms of CHD1 (beginning at amino acid no. 2). The inducible expression plasmids were made by replacing the EcoRI cassette containing the CMV promoter with an EcoRI cassette containing five tandemly linked 35 bp glucocorticoid response elements and a 67 bp segment embracing the adenovirus major-late promoter (Mader and White 1993). The critical regions of all constructs were sequenced to verify their authenticity.

For transfection experiments, monolayer cultures of U2OS or HeLa cells on coverslips or 100 mm petri dishes were grown to 60%–70% confluency in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 10% heat-treated fetal bovine serum, incubated in fresh medium for 2–6 h and then for 16–20 h with a calcium phosphate suspension containing the expression plasmids. The cells were washed, incubated with fresh medium for 24 h, washed again, and then incubated for various periods of time with medium containing dialyzed serum and 250 nM dexamethasone. The cells were then either fixed in 3.5% paraformaldehyde for immunocytochemical analysis or harvested for nuclear extraction.

Immunological procedures. Immunocytochemical analysis of mammalian cells and *Drosophila* polytene chromosomes and immunoblotting of nuclear extracts were carried out as described elsewhere (Stokes and Perry 1995; Stokes et al. 1996). The primary antibodies were as follows: mCHD1, a polyclonal rabbit antiserum described by Stokes and Perry (1995), used at 1/2,000 dilution; hSSRP1, a polyclonal rabbit antiserum raised against the protein produced by the Pt2 cDNA clone of SSRP1 (Bruhn et al. 1992), generously supplied by S. Lippard and D. Zamble and used at 1/200 dilution (coverslips) or 1/1,000 dilution (immunoblots); dCHD1, an affinity-purified antibody described by Stokes et al. (1996), used at 1/100 dilution; hsRPB7, a polyclonal rabbit antiserum to human Pol II subunit 7 (Khazak et al. 1998), a gift from Erica Golemis, used at 1/200 dilution; p54, a mixture of polyclonal rabbit antisera raised against peptides A, B and C (Chaudhary et al. 1991), a gift from Nilabh Chaudhary, used at a 1/300 dilution of each; SC-35, a mouse monoclonal antibody, a gift of Gordon Chan; and TFIIEα(C-17), an affinity-purified antibody, purchased from Santa Cruz Biotechnology. The secondary antibodies, biotinylated anti-rabbit or anti-mouse IgG (Jackson Labs), were reacted with Texas Red- or fluorescein-coupled streptavidin for visualization.

Nuclear extracts and fractionation. The preparation of nuclear extracts from monolayer cultures of mammalian cells and *Drosophila* embryos and the assay of their protein content were as previously described (Stokes and Perry 1995; Stokes et al. 1996). The nuclear extracts used for the phosphocellulose/Superose 6 fractionation study were prepared as described elsewhere (Abmayr and Workman 1993). The nuclear extract was applied to a P11 phosphocellulose column in a solution containing 20 mM HEPES-OH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and the protease inhibitors apoprotinin, leupeptin and pepstatin. Fractions were stepwise eluted with 0.3, 0.5, 0.7, and 1 M KCl in the same solution. The KCl concentration of the 0.7 M eluate was adjusted to 0.35 M and the sample was passed through a Superose 6 column together with a set of size markers (dextran blue, 2,000 kDa; thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa).

Interaction trap/two-hybrid system. Interaction trap assays were carried out as described by Golemis et al. (1996). The principal reagents were pEG202 for the bait vector, pJG4–5 as the activation domain fusion vector, pJK103 for the LacZ reporter plasmid, and pRFHM1 and pSH17-4 for negative and positive controls, respectively. All of these reagents, the appropriate yeast and bacterial strains and a human cDNA library cloned into pJG4–5 were generously provided by Erica Golemis.

Results

Effect of mutations in the C, H and D domains on the intranuclear distribution of CHD1

To examine the importance of the three signature domains of CHD1 for its association with chromatin, we carried out a series of transfection experiments with plasmid constructs designed to produce high levels of suitably tagged wild-type or mutant proteins (Fig. 1). These constructs have the following features: (i) a strong constitutive promoter from CMV or an inducible promoter consisting of five tandem glucocorticoid response elements linked to a minimal adenovirus major-late promoter (Mader and White 1993); (ii) a segment containing a spliceable intron and the adenovirus tripartite leader, which acts as a translational enhancer (Sheay et al. 1993); and (iii) an amino-terminal recognition tag of either the IgG-binding region of Pro A or gfp (Schaar et al. 1997). Six CHD1 mutants were studied: a 165 amino acid deletion that encompasses the chromodomain (C^-), a 35 amino acid deletion that removes the nucleotide-binding pocket of the ATPase/helicase domain (H^-), a 200 amino acid deletion that encompasses most of the DNA-binding domain (D^-), double mutants bearing either

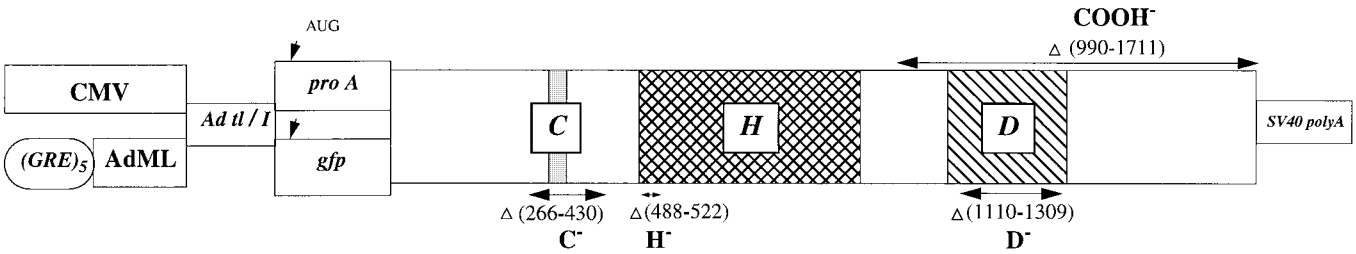

Fig. 1. Schematic diagram of the constructs used for transfection experiments. Either the cytomegalovirus promoter (CMV) or a synthetic promoter consisting of five tandem glucocorticoid response elements fused to the –34 to +33 region of the adenovirus major late promoter [$(GRE)_5$AdML] was linked to a segment containing the adenovirus tripartite leader and a spliceable intron (*Ad tl/I*). This segment was linked to a fragment encoding either protein A (*pro A*) or green fluorescent protein (*gfp*), which, in turn, was linked to cDNA encoding wild-type or mutant CHD1. The C, H, and D domains are indicated by *stippled*, *cross-hatched* and *hatched bars*, respectively. The deletions in various mutant constructs are indicated by *horizontal arrows* with the amino acid positions given in *parentheses*. Note that the C^- deletion eliminates both the typical chromodomain (amino acids 294–345) and a second motif (amino acids 385–433), which also has chromodomain characteristics (Aasland and Stewart 1995; Koonin et al. 1995). *SV40 polyA* is a segment containing the polyadenylation site from simian virus 40. The lengths of the various segments are not drawn strictly to scale; the exact sizes in base pairs are given in Materials and methods

the H^- or C^- deletion and the D^- deletion (H^-D^- and C^-D^-), and a 700 amino acid carboxy-terminal truncation ($COOH^-$).

In an initial series of transfection experiments with the CMV-driven constructs and either HeLa or U2OS osteosarcoma cells, we observed a striking difference in the intranuclear distribution of pro A-tagged wild-type and mutant CHD1 proteins (data not shown). To investigate this phenomena in greater detail under more controlled conditions, we carried out similar experiments with the glucocorticoid-inducible constructs. In these experiments, the expression of CHD1 proteins in transfected U2OS cells was induced with 250 nM dexamethasone and after various intervals, the cells were examined by fluorescence and phase-contrast microscopy (Fig. 2). The intranuclear distribution of wild-type CHD1, tagged with either pro A (panel a) or gfp (panel c), was indistinguishable from that of endogenous CHD1 (Stokes and Perry 1995), i.e., it was finely granular and homogeneously distributed throughout the nucleus except for the regions occupied by nucleoli. In contrast, CHD1 protein bearing the C^- or H^- deletion was concentrated in markedly punctate bodies, which could be visualized with the pro A or gfp tags (Fig. 2a, c), with anti-CHD1 antibody staining (Fig. 8h, k and data not shown), and with phase-contrast microscopy (Fig. 2a). The phase-contrast images of nuclei containing gfp-tagged proteins (data not shown) were essentially the same as those of nuclei containing the corresponding pro A-tagged proteins. The punctate bodies were not evident with DNA stains such as 4', 6-diamidino-2-phenylindole (DAPI) or Hoechst 33258 (Fig. 2a). This observation might be interpreted to mean that these mutant forms of CHD1 do not associate with DNA. However, since only a small fraction of genomic DNA is normally associated with CHD1 [a few percent, based on the abundance of CHD1 relative to that of nucleosomes (Stokes and Perry 1995)], it is doubtful whether one would be able to detect changes in the intranuclear distribution of this fraction when examining total nuclear DNA. The formation of the punctate bodies was very rapid: for the H^- mutant, they were readily observable and widespread within 2 h after induced expression and for the C^- mutant, they were clearly evident after 4 to 10 h. (Fig. 2c). The average size of the punctate bodies was similar in H^- and C^- transfectants. For the most part the bodies were small (<1 μm in diameter) although after 10 h of induction, we observed a few nuclei with larger bodies (Fig. 2c).

CHD1 protein bearing the D^- deletion was distributed similarly to wild-type CHD1 (Fig. 2b, c), and protein bearing both the H^- and D^- deletions was distributed similarly to the H^- mutant (data not shown). Interestingly, protein bearing both the C^- and D^- deletions was often concentrated in exceptionally large punctate bodies (Fig. 2a, also see Fig. 9). These large bodies were evident in some nuclei after only 4 h of induction, and by 9–10 h they were widespread. CHD1 protein bearing the large carboxy-terminal truncation was less efficiently localized to the nucleus. Nevertheless, the nuclear portion was evenly distributed (Fig. 2b).

In each field of cells examined, there were cells that did not express the transfected genes (compare the DNA and phase panels with the CHD1 panels in Fig. 2a and 2b). The nuclear morphology of these nonexpressing cells was indistinguishable from that of cells that were not exposed to the transfection/induction protocol, indicating that the protocol, per se, did not contribute to the observed phenotypes of the transfected cells. A similar set of transfection experiments with HeLa cells gave results that were virtually identical to those obtained with the U2OS cells (data not shown).

In previous studies, we observed that CHD1 is progressively extracted from nuclei together with bulk chromatin by incubation with 0.2–0.6 M NaCl (Stokes and Perry 1995). When nuclear extracts from transfected cells were immunoblotted with anti-CHD1 antibody, we observed that the relative proportions of exogenous and endogenous CHD1 extracted at each salt concentration were virtually identical for both wild-type and mutant proteins (Fig. 3). Thus, despite their markedly different intranuclear distribution, the H^- and C^-D^- mutant proteins have solubility properties that are indistinguishable from those of wild-type and endogenous CHD1.

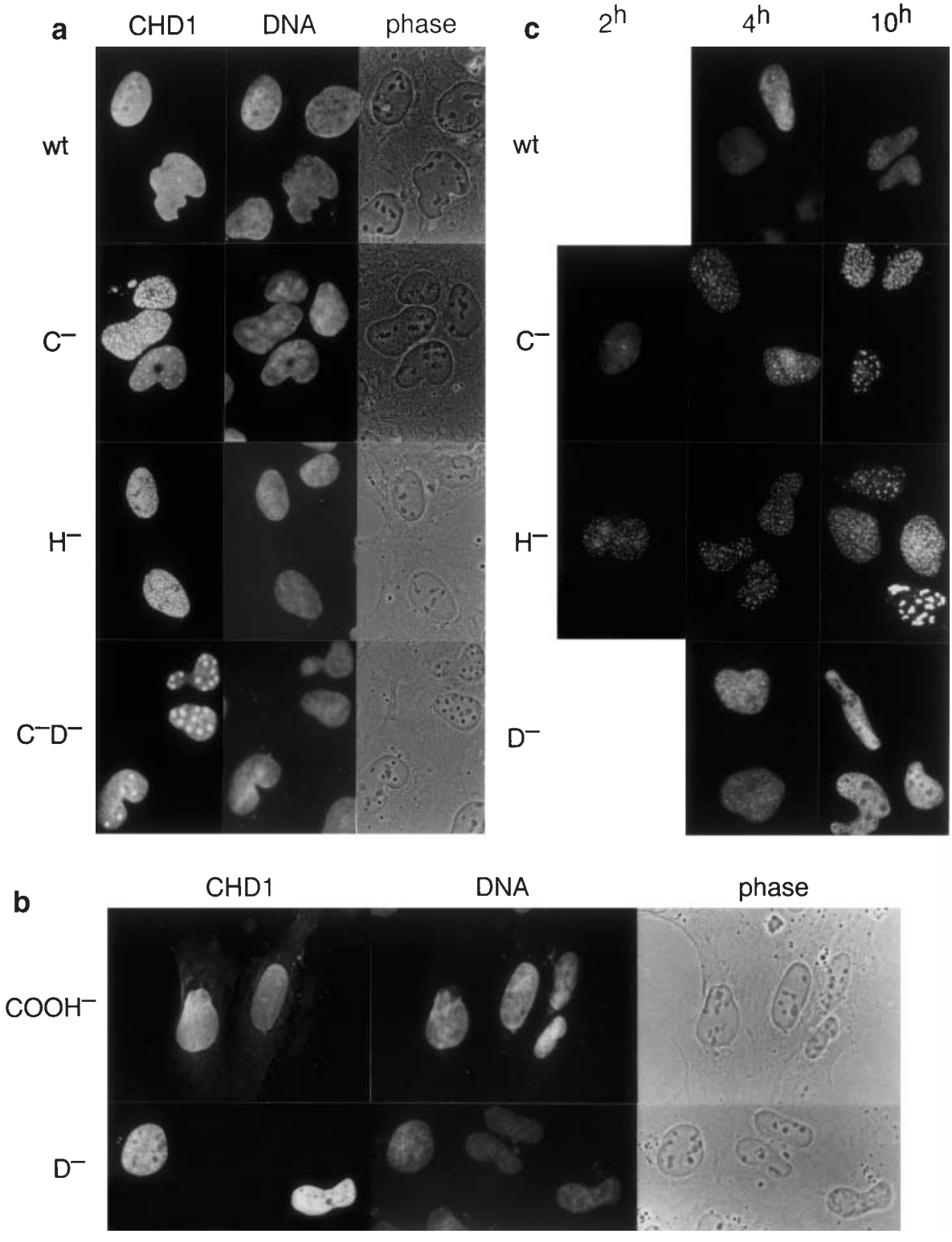

Fig. 2a–c. Distribution of wild-type (*wt*) and mutant forms of CHD1 in U2OS cells transfected with constructs directed by the $(GRE)_5$AdML promoter. Expression was induced with 250 nM dexamethasone for 10 h (**a**) , 4 h (**b**) or 2, 4 and 10 h (**c**). CHD1 was monitored by immunostaining of pro A-tagged proteins with Texas Red-coupled IgG (**a–b**) or direct fluorescent emission of gfp-tagged proteins (**c**). Cells were counterstained with the Hoechst 33258 fluorochrome to monitor DNA, and were also observed by phase-contrast microscopy

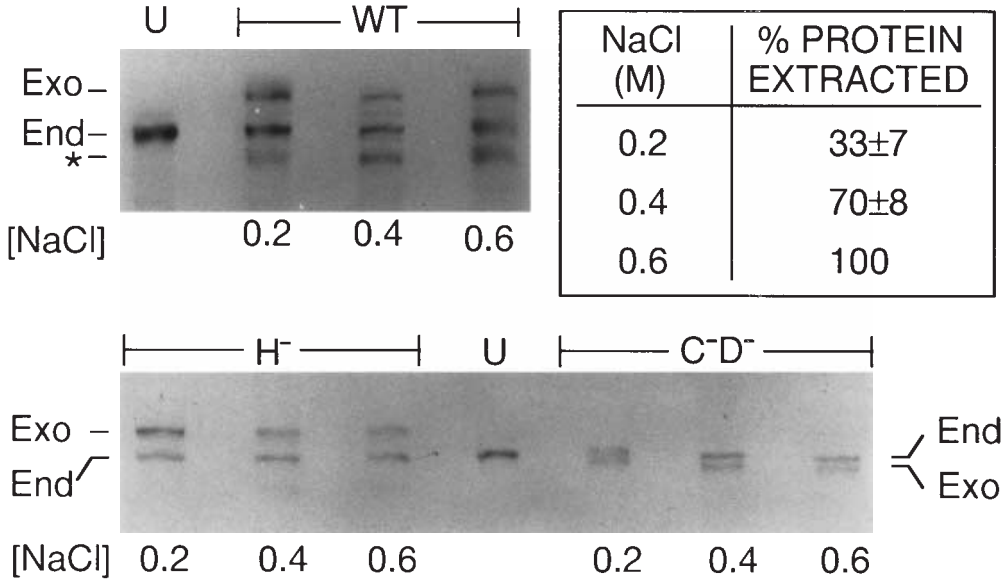

Fig. 3. Comparison of the salt-extractibility of CHD1 produced from transfected (exogenous) genes with that of endogenous CHD1. HeLa cells transfected with gfp-tagged wild-type CHD1 (*upper panel*) or U2OS cells transfected with pro A-tagged mutant CHD1 (*lower panel*) were harvested approximately 20 h after induction with 250 nM dexamethasone, and their nuclei extracted with buffer containing the indicated concentrations of NaCl. The extracted protein was submitted to immunoblot analysis with anti-CHD1 antibody. 15 µg of HeLa nuclear extract or 8 µg of U2OS nuclear extract was loaded in each lane. The exogenous and endogenous proteins can be distinguished on the basis of size. The lanes labeled U are 0.6 M NaCl extracts from untransfected control cells. The band marked by an *asterisk* is a derivative of the exogenous protein. The proportion of total nuclear protein extracted at each salt concentration, normalized to the amount extracted by 0.6 M NaCl and averaged for the three transfection experiments, is shown in the *box at the upper right*. These proportions represent the relative amounts of CHD1 extracted at each salt concentration since the amounts of both exogenous and endogenous CHD1 per microgram of extract were essentially the same within each series. Additional immunoblot analyses of the 0.6 M residual pellets demonstrated that the extraction was complete at 0.6 M NaCl for both exogenous and endogenous CHD1

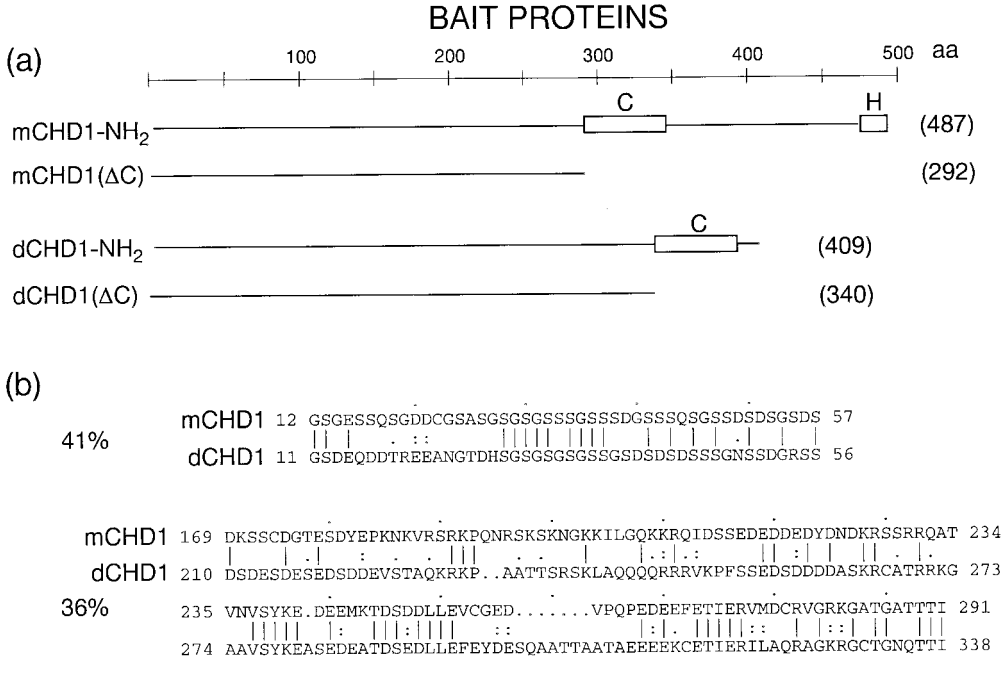

Fig. 4a, b. The CHD1 bait proteins that were fused to lexA for the two-hybrid interaction trap assays. **a** Segments of mouse (*m*) or *Drosophila* (*d*) CHD1 extending from amino acid 2 to the position indicated in *parentheses*. The chromodomain and a portion of the ATPase/helicase domain are indicated by *boxes*. **b** A best-fit comparison of the amino acid sequences near the amino-terminal ends of mouse and *Drosophila* CHD1. The percent identity is shown on the left

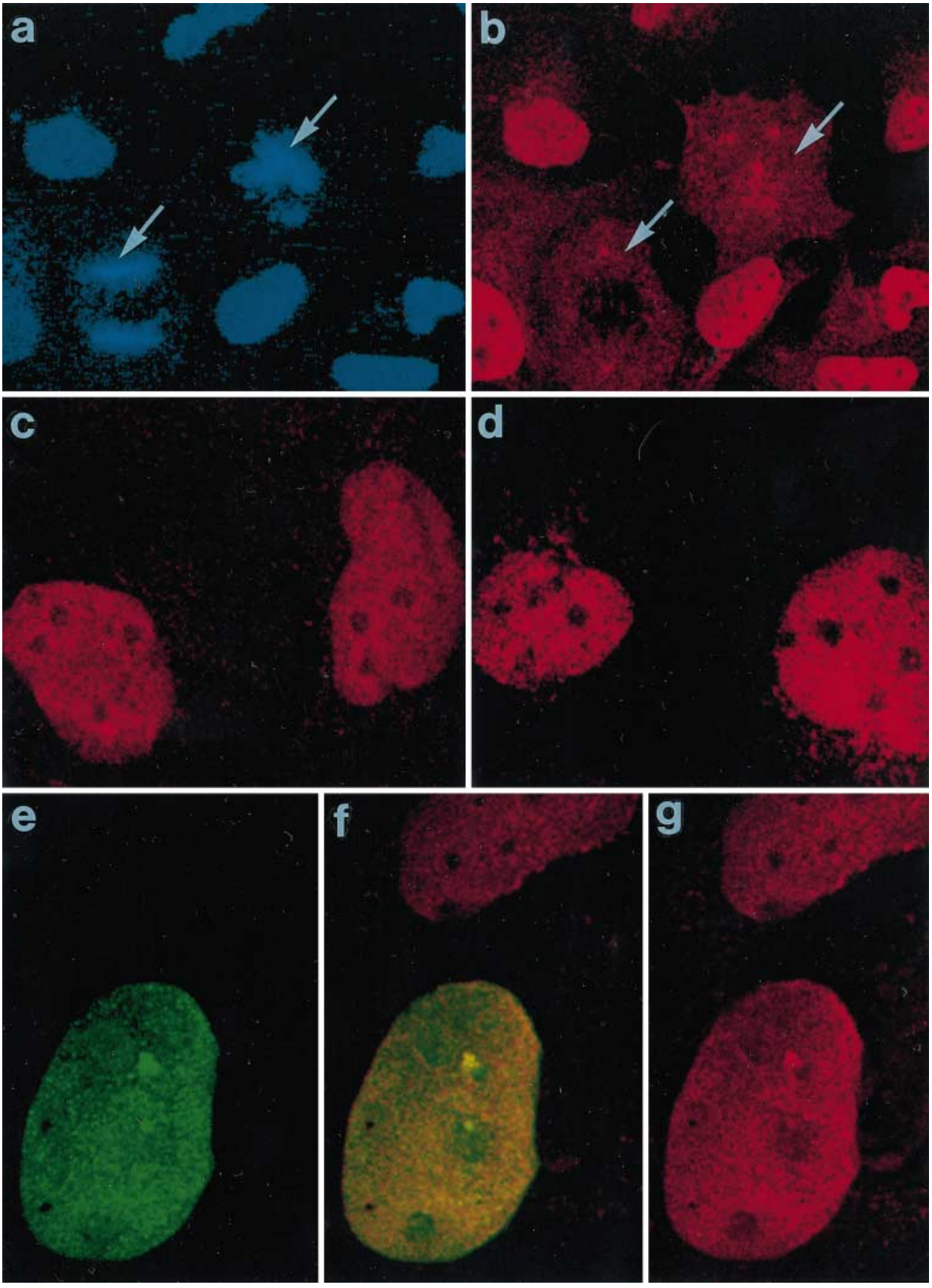

Fig. 5a–g. Intranuclear distribution of SSRP1 in U2OS cells is indistinguishable from that of CHD1. The same group of cells stained with Hoechst 33258 (**a**) and anti-SSRP1 antibody (**b**); note the intense staining of SSRP1 in interphase nuclei and the cytoplasmic staining of mitotic cells (*arrows*). Interphase nuclei stained with anti-CHD1 (**c**) or anti-SSRP1 (**d**) antibodies; note the finely granular staining in non-nucleolar regions. Interphase nucleus 9 h after induction of gfp-tagged wild-type CHD1, visualized by gfp fluorescence (**e**) or anti-SSRP1 staining (**g**). Panel **f** shows a merged image of **e** and **g**. The antibody staining was carried out by sequential incubations with primary rabbit antibodies, biotinylated goat anti-rabbit antibody and Texas Red-coupled streptavidin

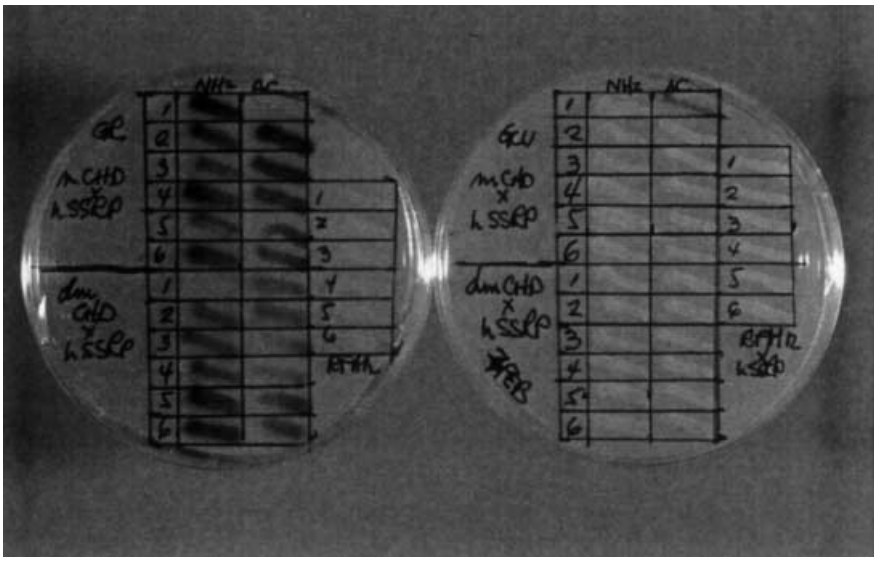

Fig. 6. Two-hybrid specificity tests. Yeast cells containing a plasmid encoding one of the four LexA-fused bait proteins described in Fig. 4 and a LacZ reporter plasmid with two LexA operators were transformed with the SSRP1-encoding plasmid that was isolated in the initial two-hybrid screen. Transformants were plated in sextuplicate streaks on minimal medium plates containing 5-bromo-4-chloro-3-indolyl-galactopyranoside (Xgal) and either galactose/raffinose (*left plate*) or glucose (*right plate*). *Top sectors*: mouse CHD1 amino-terminal segment, *left column*; ΔC segment, *right column* and SSRP1. *Bottom sectors*: *Drosophila* CHD1 amino-terminal segment, *left column*; ΔC segment, *right column* and SSRP1. At the *far right* is a negative control in which the plasmid encoding the CHD1 bait protein was replaced by a plasmid encoding a nonactivating fusion of LexA and the homeodomain of the bicoid protein

These data also enabled us to estimate the ratio of exogenous to endogenous CHD1 in transfected cells. The overall transfection efficiency in these experiments, judged from the fraction of pro A- or gfp-positive nuclei, was about 10%–20%. Since the anti-CHD1 immunoblots of nuclear extracts, which represent a mixture of protein from transfected and nontransfected cells, indicate roughly equivalent amounts of exogenous and endogenous protein, we estimate that the average level of exogenous protein is about five- to tenfold higher than that of endogenous CHD1 in transfected cells.

The foregoing results indicate that the C and H domains have important roles in determining the proper association of CHD1 with interphase chromatin. Although the role of the D domain is more subtle, its importance is accentuated in the absence of the C domain. As will be shown below, the aberrant localization of mutant CHD1 proteins is accompanied by the mislocalization of a putative interaction partner.

A search for proteins that interact with CHD1

Most of the chromatin-associated proteins that contain H-like domains are constituents of large multiprotein complexes (Cairns et al. 1996; Kingston et al. 1996; Wang et al. 1996; Pazin and Kadonaga 1997; Tsukiyama and Wu 1997). Moreover, a preliminary analysis of fractionated HeLa cell nuclear extract (Owen-Hughes and Stokes, unpublished data) suggested that CHD1 might also be part of a multiprotein complex. Therefore, we decided to use the yeast two-hybrid system to search for proteins that interact with CHD1, and which could conceivably be constituents of such a complex. In our initial experiments, the bait protein consisted of the DNA-binding domain of LexA fused to a 487 residue amino-terminal segment of mouse CHD1 (Fig. 4a). Potential prey proteins were generated by a library of plasmids in which a segment encoding an acidic transcriptional activation domain was linked to random fragments of human cDNA. A sequence analysis of cDNAs yielded five candidates that were found in multiple isolates. Three of these cDNAs encoded carboxy-terminal regions of known proteins that have been entered into sequence databases; the other two encoded portions of proteins that have yet to be identified.

The three known proteins are SSRP1, an M_r 81,000 nuclear protein, which contains a high mobility group (HMG) domain, and which specifically recognizes structurally modified DNA (Bruhn et al. 1992); p54, an arginine-rich nuclear protein, which is believed to be involved in RNA splicing (Chaudhary et al. 1991; Zhang and Wu 1996); and hNop56, the human ortholog of a yeast nucleolar protein involved in ribosome biogenesis (Gautier et al. 1997). Since CHD1 is conspicuously absent from nucleoli, we consider hNop56 to be a false-positive of the two-hybrid screen. Similarly, p54 seemed like an unlikely candidate because it is preferentially located in the speckled bodies that are known to contain proteins involved in RNA processing (Chaudhary et al. 1991), whereas CHD1 is uniformly distributed throughout the nucleus. Furthermore, as will be shown below (see Fig. 10d–f), p54 also fails to colocalize with mutant forms of CHD1.

In contrast to hNop56 and p54, the intranuclear distribution of SSRP1 is indistinguishable from that of CHD1: both proteins exhibit uniform, finely granular staining in the non-nucleolar portions of interphase nuclei (Fig. 5c, d) and both are released into the cytoplasm when cells enter mitosis (Fig. 5a, b) and Stokes and Perry (1995). Based on these observations we carried out further studies on the relationship between CHD1 and SSRP1.

The chromodomain of CHD1 is not required for interactions with SSRP1

Our initial choice of the amino-terminal segment of CHD1 as bait was based on an assumption that the chromodomain, which has been implicated in protein–

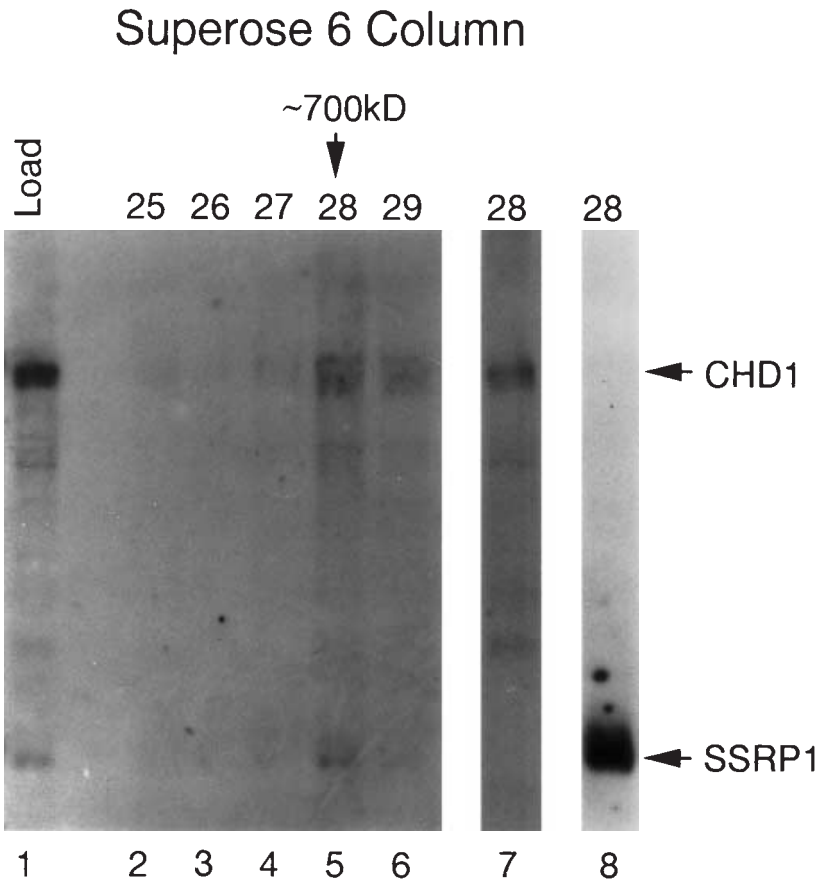

Fig. 7. Copurification of SSRP1 and CHD1. HeLa cell nuclear extract was applied to a phosphocellulose P11 column and stepwise eluted with increasing concentrations of KCl. The fraction eluting at 0.7 M KCl was applied to a Superose 6 sizing column and eluted with a solution containing 350 mM KCl. Aliquots of the 0.7 M fraction (*lane 1*) and Superose 6 fractions (*lanes 2–8*) were precipitated with 10% trichloroacetic acid, redissolved and submitted to immunoblot analysis with anti-CHD1 antibody (*lane 7*), anti-SSRP1 antibody (*lane 8*) or a mixture of both antibodies (*lanes 1–6*). The size of fraction 28 was estimated by comparison with a set of calibration markers (dextran blue, 2,000 kDa, fraction 19; thyroglobulin, 669 kDa, fraction 28; ferritin, 440 kDa, fraction 32)

protein interactions of repressor proteins such as HP1 and Polycomb (Platero et al. 1995), might play a similar role for CHD1. To test this assumption, we examined SSRP1 interactions with a second bait segment, mCHD1(ΔC), which lacks the chromodomain, and with a pair of *Drosophila* CHD1 amino-terminal segments containing and lacking the chromodomain (Fig. 4a). Contrary to our initial assumption, we found that the chromodomain was not required for interactions with SSRP1 (Fig. 6). Interestingly, the *Drosophila* amino-terminal and ΔC segments also interacted with human SSRP1, albeit somewhat less strongly. An independent experiment in which a liquid β-galactosidase assay was used to measure the LacZ reporter activity indicated that the strength of the SSRP1 interaction with *Drosophila* CHD1 is about one-fourth that of the SSRP1 interaction with mouse CHD1 (data not shown). An amino acid sequence comparison of the interactive regions of mouse and *Drosophila* CHD1 indicates significant similarity, particularly in the placement of serine, glycine and charged residues (Fig. 4b).

SSRP1 and CHD1 copurify in fractionated nuclear extracts

Evidence indicating that CHD1 and SSRP1 are part of the same multiprotein complex was provided by an analysis of fractionated HeLa cell nuclear extract. The relevant samples of fractionated extract were generously supplied by Tom Owen-Hughes and Jerry Workman, who had prepared them for studies of nucleosome disruption activity (Steger et al. 1997). In the preparation of these samples, HeLa cell nuclei were extracted with 0.3 M KCl in HEPES buffer and the extract applied to a phosphocellulose P11 column, which was then stepwise eluted with 0.3, 0.5, 0.7 and 1 M KCl. Monitoring of CHD1 by immunoblot analysis indicated that essentially all of it was recovered in the 0.7 M fraction. Whether this fraction also contained all of the nuclear SSRP1 was not ascertained. When the 0.7 M fraction was run over a Superose 6 sizing column, both CHD1 and SSRP1 eluted in fractions corresponding to a complex of about M_r 700,000 (Fig. 7). The copurification was indicated by a blot simultaneously probed with CHD1 and SSRP1 antibodies (lanes 1–6). Identification was verified by blots probed with the individual antibodies (lanes 7 and 8).

Our attempts to purify these complexes by immunoprecipitation with anti-CHD1 antibody were not successful. Although the available antibody preparations were very reliable for immunoblot and immunocytochemical analyses, they were not effective for the immunprecipitation of CHD1 from either the 0.7 M KCl P11 eluate or nuclear extracts, most likely because of epitope masking and/or inadequate avidity.

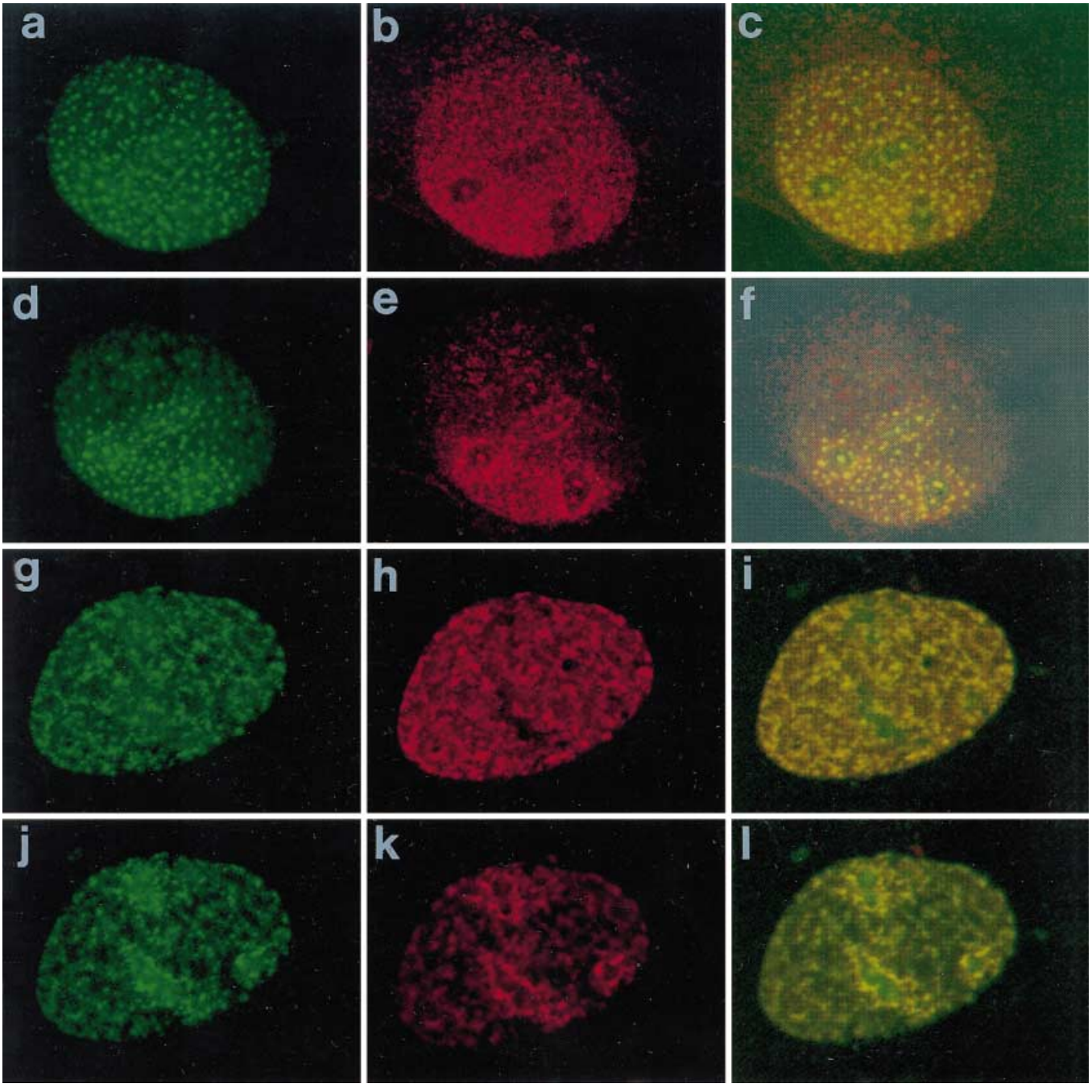

Fig. 8a–l. Colocalization of H^- CHD1 and SSRP1 in punctate bodies. Expression of gfp-tagged H^- CHD1 in U2OS cells was induced for 9 h with 250 nM dexamethasone, after which the cells were fixed and stained with anti-SSRP1 or anti-CHD1 antibody, as described in Fig. 5. **a–c** and **d–f** are successive confocal images of the same nucleus stained with SSRP1 antibody; **g–i** and **j–l** are successive confocal images of another nucleus stained with CHD1 antibody. **a, d, g** and **j** show gfp fluorescence of H^- CHD1; **b** and **e** show SSRP1 antibody stain; **h** and **k** show CHD1 antibody stain; **c, f, i** and **l** are merged images showing the coincidence of H^- CHD1 and the antibody-reactive proteins

SSRP1 colocalizes with wild-type and mutant CHD1 in transfected cells

Additional evidence for an association of CHD1 and SSRP1 in mammalian nuclei was obtained by an immunocytochemical analysis of transfected U2OS cells. The intranuclear distribution of SSRP1 in cells transfected with gfp-tagged wild-type CHD1 is indistinguishable from that of SSRP1 and CHD1 in untransfected cells, i.e., it is finely granular and homogenous (Fig. 5e, g). Indeed, as seen in the merged image (Fig. 5f), the texture of the granularity pattern is very similar, indicating a significant degree of colocalization. Moreover, in cells transfected with the gfp-tagged H^- mutant of CHD1, SSRP1

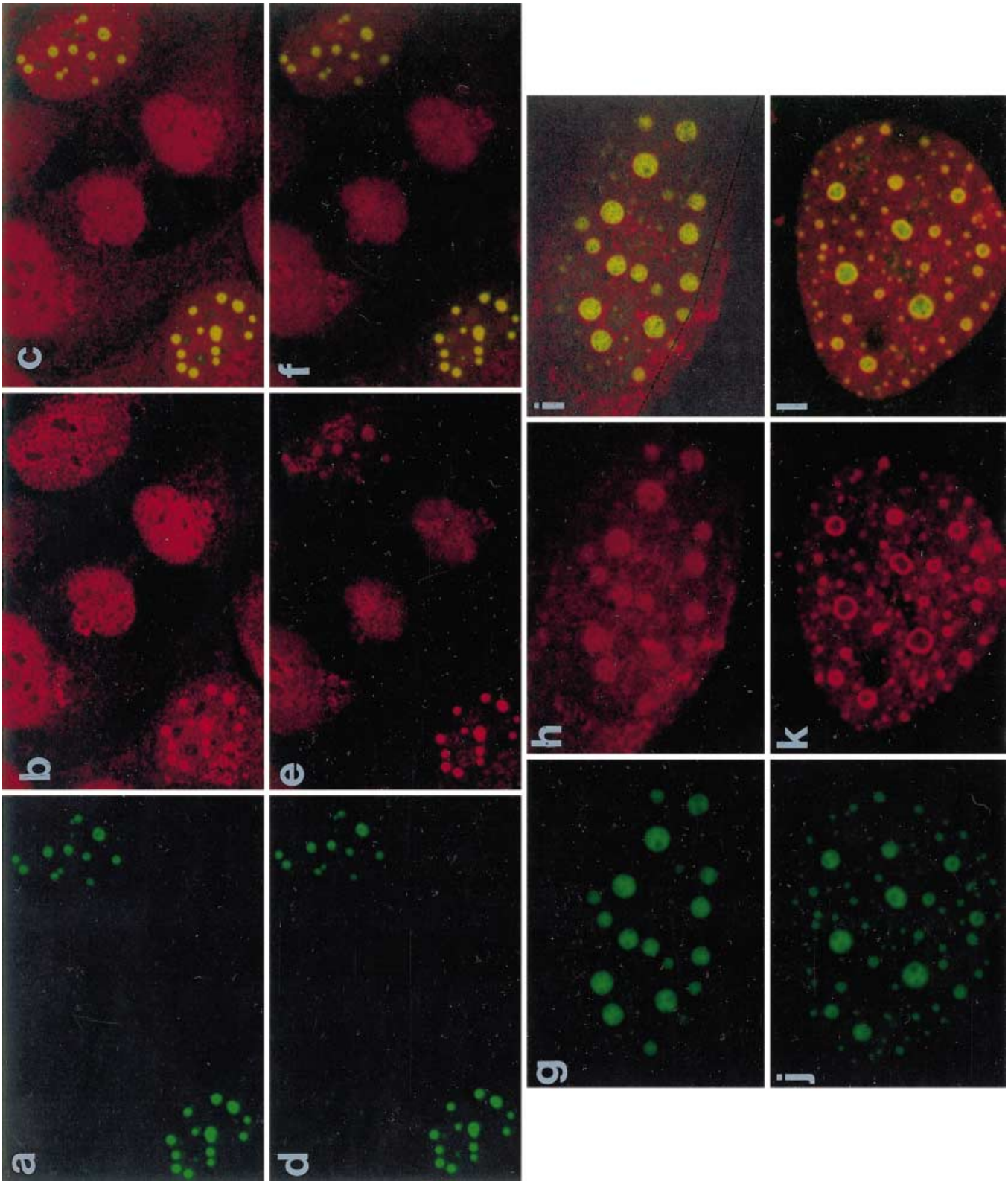

Fig. 9a–l. Colocalization of $C^{-}D^{-}$ CHD1 and SSRP1 in large punctate bodies. Expression of gfp-tagged $C^{-}D^{-}$ CHD1 in U2OS cells was induced for 9 h with 250 nM dexamethasone, after which the cells were fixed and stained with anti-SSRP1 or anti-CHD1 antibody, as described in Fig. 5. **a–c** and **d–f** are successive confocal images through the same field of cells; **g–i** and **j–l** are composite confocal images (at higher magnification) of two different nuclei. **a, d, g** and **j** show gfp fluorescence; **b, e** and **h** show anti-SSRP1 stain; **k** shows anti-CHD1 stain; **c, f, i** and **l** are merged images showing coincidence of $C^{-}D^{-}$ CHD1 and antibody-reactive proteins. Some of the large punctate bodies are highly vacuolated, which may contribute to the annular appearance of the antibody stain at certain focal planes. In some cases, inadequate access of antibody to the interior of these bodies may also be a contributing factor

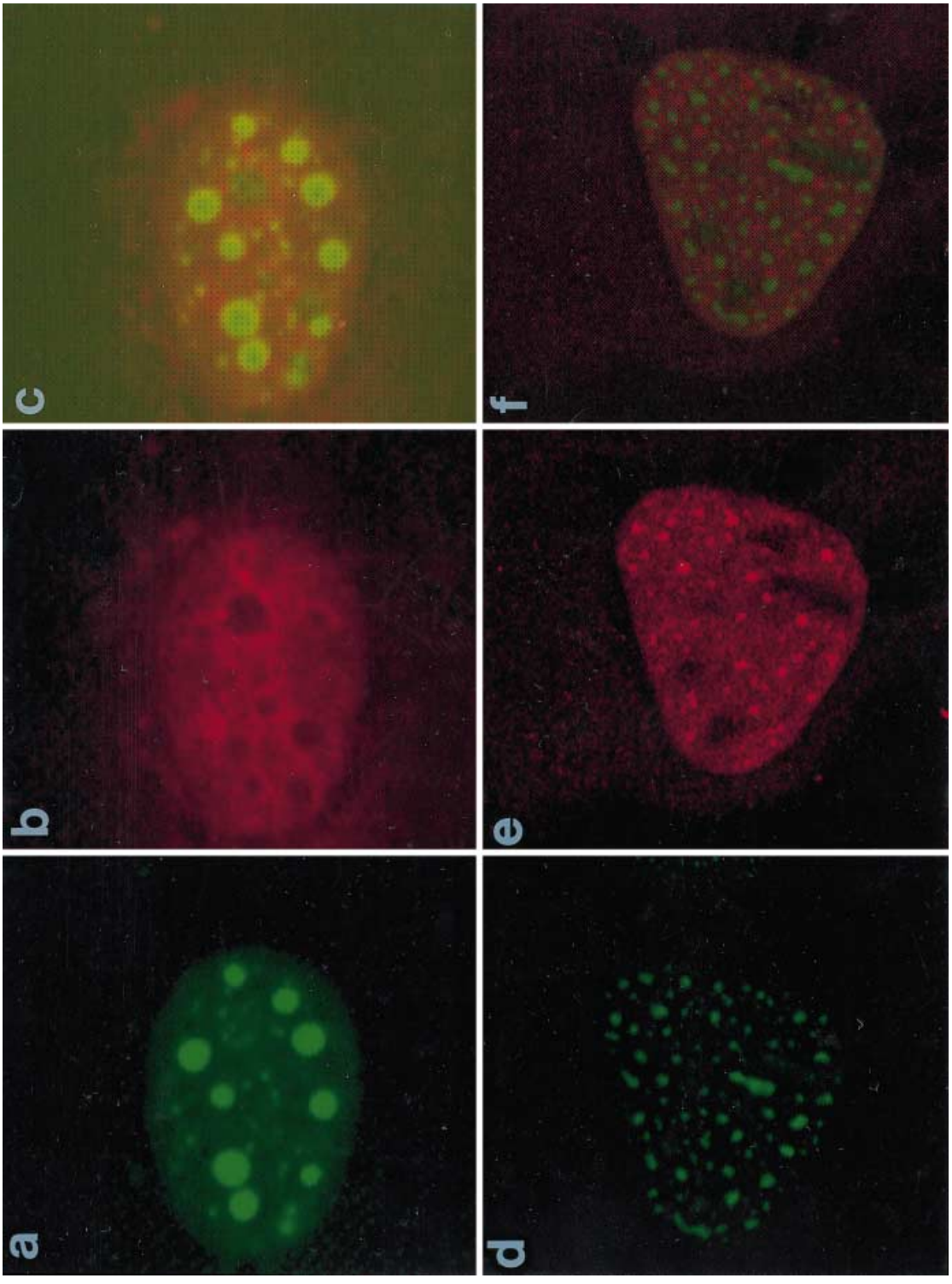

Fig. 10a–f. RNA polymerase II (pol II) and the arginine-rich protein p54 are not localized in the punctate bodies. Expression of gfp-tagged $C^{-}D^{-}$ CHD1 (**a–c**) or gfp-tagged H^{-} CHD1 (**d–f**) was induced for 9 h with 250 nM dexamethasone, after which the cells were fixed and stained with anti-pol II (S7) antibody (**b**) or anti-p54 antibody (**e**), as described in Fig. 5, and visualized with the confocal microscope. **a** and **d** show gfp fluorescence; **b** and **e** show antibody stain; **c** and **f** are merged images. Note that the punctuate bodies seen in **d** are entirely distinct from the speckles seen in **e**

is nonuniformly distributed in punctate bodies, which are clearly revealed in successive confocal images with the gfp (Fig. 8a, d, g, j) and either the anti-SSRP1 (Fig. 8b, e) or anti-CHD1 (Fig. 8h, k) fluorescent probes. As is apparent in Fig. 8, punctate bodies containing overexpressed H^- CHD1 protein sometimes penetrate the nucleolar space or are concentrated in regions surrounding the nucleoli. Strikingly, the colocalization of CHD1 and SSRP1 is strictly maintained in these aberrantly located bodies.

Even more dramatic, is the colocalization of CHD1 and SSRP1 in cells transfected with the gfp-tagged C^-D^- mutant. The large punctate bodies produced by this mutant (Fig. 9a, d, g, j) were stained similarly with SSRP1 (Fig. 9b, e, h) and CHD1 (Fig. 9k) antibodies. The precise colocalization was evident in both successive single focal-plane images (Fig. 9c, f) and enlarged composite images (Fig. 9i, l). As expected, there was no punctate SSRP1 staining in adjacent cells that did not express C^-D^- CHD1 (Fig. 9b, c, e, f).

The specificity of the gfp-SSRP1 colocalization result was verified by control experiments with antibodies directed against other nuclear proteins that are involved in transcription (the S7 subunit of RNA polymerase II and the general transcription factor TFIIEα) or RNA processing (the arginine-rich protein p54 and the spliceosome component SC-35). In cells transfected with the C^-D^- mutant, polymerase II continues to be uniformly distributed in the non-nucleolar regions of the nucleus (Fig. 10a–c), as it is in untransfected cells (Khazak et al. 1998). An identical result was obtained with the TFIIEα antibody (data not shown). Similarly, in cells transfected with the H^- or C^-D^- mutants, the p54 and SC-35 proteins continue to be preferentially localized in speckles, which are clearly distinct from the punctate bodies containing the CHD1 mutant proteins (Fig. 10d–f and data not shown).

CHD1 and SSRP1 are similarly distributed on Drosophila polytene chromosomes

The foregoing cytological and biochemical experiments indicate a close association of CHD1 and SSRP1 in mammalian interphase chromatin. Both of these mammalian proteins have well-conserved orthologs in the fruit fly *Drosophila melanogaster*. Overall, mouse and fly CHD1 are 50% identical (Stokes et al. 1996) while human and fly SSRP1 are 48% identical (Bruhn et al. 1993). We therefore sought to determine whether SSRP1, like CHD1, is preferentially localized in the puffs and interbands of *Drosophila* polytene chromosomes. We suspected that the human SSRP1 antibody might cross-react with the fly SSRP1 since it was raised to a region of the protein that included several stretches of exceptionally high conservation, e.g. ≥75% identity in blocks of 50 amino acids. In accordance with this expectation, when this antibody was used in an immunoblot of a nuclear extract from *Drosophila* embryos, we observed a single prominent band with an electrophoretic mobility that was slightly slower than that of human SSRP1 and entirely distinct from that of *Drosophila* CHD1 (Fig. 11).

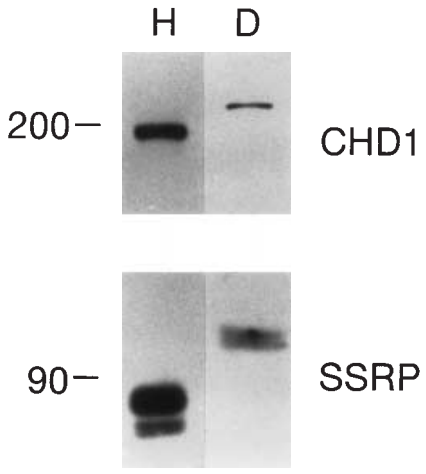

Fig. 11. The antibody raised against human SSRP1 recognizes a protein of similar size in human and *Drosophila* nuclear extracts. Immunoblot analysis of HeLa cell (H) and *Drosophila* embryo (D) nuclear extracts with anti-mouse CHD1 antibody (*left upper lane*), anti-*Drosophila* CHD1 antibody (*right upper lane*) and anti-human SSRP1 (*lower lanes*). The *numbers on the left* are the estimated sizes in kilodaltons, based on a set of calibration markers. The estimated size of the *Drosophila* protein is about 10% larger than that expected from the known molecular weight of dSSRP1 (Bruhn et al. 1993)

Polytene chromosomes from the salivary glands of third instar larvae gave very similar patterns when stained with SSRP1 and CHD1 antibodies (Fig. 12). In both cases, the staining was confined to puffs (arrowheads) and interband regions. Although a precise locus by locus colocalization study has not been carried out, it is clear that several of the readily recognizable puffs, such as 2B (Fig. 12 g) or 71DE, 72D, 74EF, 75B and 78D (Fig. 12c) were as intensely stained by anti-SSRP1, as with anti-CHD1 (Fig. 12f and Stokes et al. 1996). These results are consistent with the idea that CHD1 and SSRP1 are part of an evolutionarily conserved multiprotein complex.

Discussion

Importance of C, H and D domains for CHD1-chromatin association

Our initial interest in CHD1 stemmed from its novel combination of structural features, which made it a member of two protein families with antipodal functions. The C domain links it to proteins such as HP1 and Polycomb, which have important roles in chromatin compaction and transcriptional silencing (Paro 1993), whereas the H domain links it to proteins such as SNF2/SWI2, Brahma and ISWI, which have been implicated in chromatin decompaction and transcriptional activation (Pazin and Kadonaga 1997). Given this curious novelty, it became important to establish whether or not the C and H domains of CHD1 actually have functional significance.

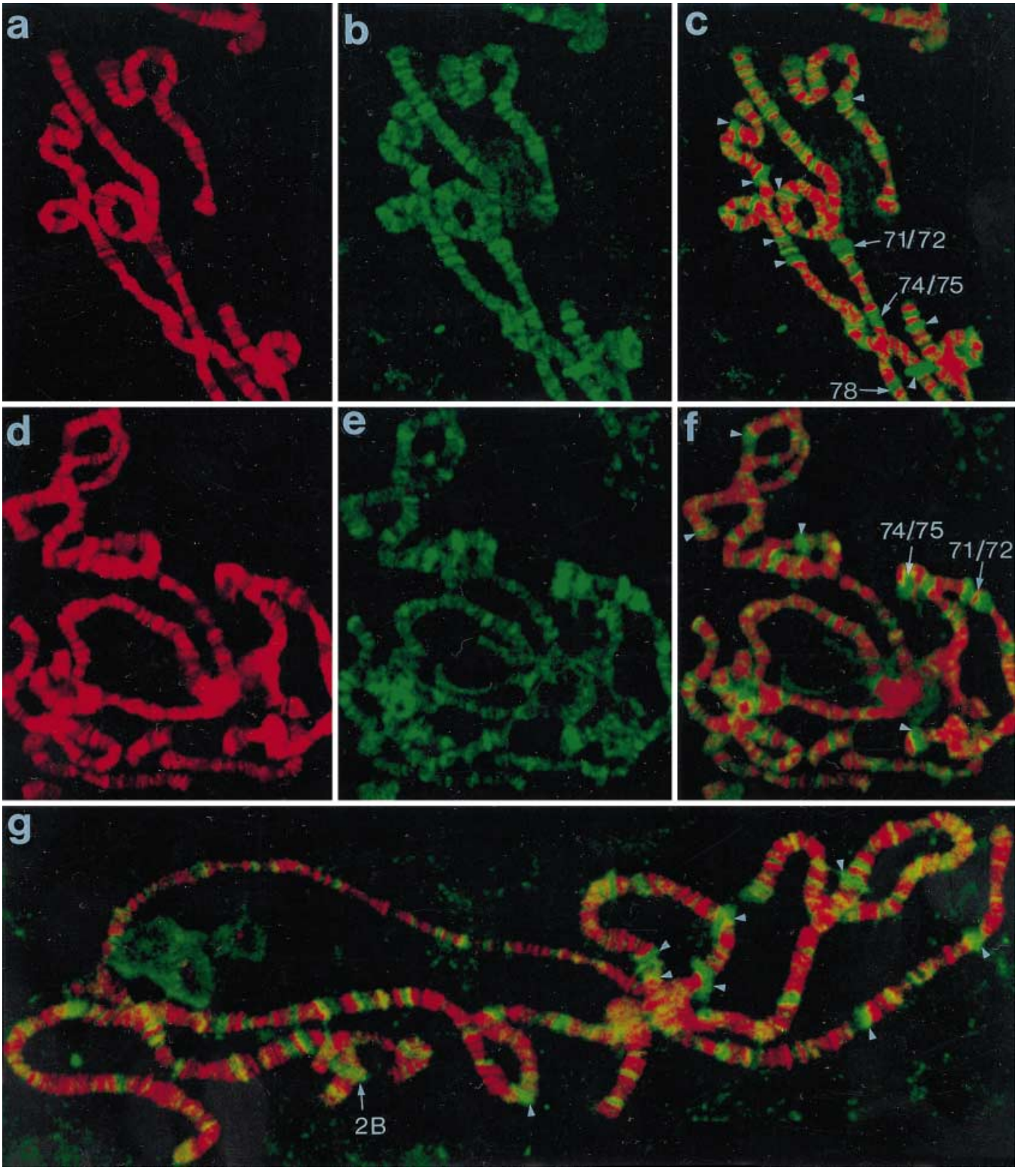

Fig. 12a–g. Staining of *Drosophila* polytene chromosomes with anti-SSRP1 and anti-CHD1 antibodies. **a** and **d** show propidium iodide staining of regions of compacted DNA. Antibody stain is largely confined to the decompacted interband and puff regions of the chromosomes for both anti-SSRP1 antibody (**b, c, g**) and anti-CHD1 antibody (**e, f**). The similarity in staining patterns is clearly seen by comparing the merged images (**c** and **g** vs. **f**). Representative puffs are indicated by *arrowheads*. Selected loci on chromosomes *3L* and *X* are identified with *arrows*. Antibody staining was as described in Fig. 5, except that the biotinylated secondary antibody was revealed with fluorescein isothiocyanate-coupled streptavidin

The transfection experiments with mutant forms of CHD1 have demonstrated that both the C and H domains are essential for its proper association with chromatin. In contrast to endogenous CHD1, which is evenly distributed in the non-nucleolar regions of interphase nuclei, CHD1 lacking the chromodomain or a critical portion of the ATPase/helicase-like domain rapidly becomes localized in markedly punctate bodies. This aberrant localization cannot simply be due to the overexpression of exogenous protein because neither wild-type CHD1 nor CHD1 bearing a deletion of the DNA-binding domain is detectably mislocalized. The normal appearance of the D^- transfectants also argues against the possibility that the punctate bodies are the result of protein misfolding. If the punctate phenotype were simply the result of protein misfolding, one might expect that the D^- deletion or the large carboxy-terminal truncation, both of which are substantially larger than either the C^- or H^- deletion, would also engender an aberrant punctate phenotype. Although we cannot rigorously exclude the possibility that the various deletion mutants are differentially misfolded, we consider this to be an unlikely explanation for our observations.

It is not clear whether the punctate bodies also contain endogenous CHD1. Although they are vividly stained with anti-CHD1 antibody, the preponderance of staining (80%–90%) is due to the exogenous protein. However, as discussed below, endogenous SSRP1, a protein that interacts with CHD1, is demonstrably present in the punctate bodies. Since the salt-solubilization properties of the CHD1 in punctate bodies are indistinguishable from those of endogenous CHD1 and bulk chromatin, these bodies could conceivably represent coalescences of disorganized chromatin. Alternatively, they might represent repositories of proteins that cannot be properly incorporated into chromatin, but which happen to have similar solubility properties. In either case, it seems reasonable to conclude from these experiments that both the C and H domains have important roles in CHD1 function.

Several studies of HP1, Polycomb and their orthologs have indicated that the chromodomain, via its participation in protein-protein interactions, helps direct these proteins to appropriate chromosomal loci (Messmer et al. 1992; Paro 1993; Platero et al. 1995; Strutt and Paro 1997; Lehming et al. 1998; Seller et al. 1998). In the case of Polycomb, the chromodomain is essential for this targeting function (Messmer et al. 1992; Paro 1993; Strutt and Paro 1997), whereas with HP1, targeting can also be mediated by another, related motif termed the chromo shadow domain (Aasland and Stewart 1995; Platero et al. 1995; Lehming et al. 1998; Seller et al. 1998). Neither of these proteins can bind DNA directly and, therefore, their locus specificity is believed to occur via other members of the multiprotein complexes of which they are constituents (Strutt and Paro 1997; Lehming et al. 1998; Seller et al. 1998). In contrast, CHD1 has DNA-binding capability by virtue of its D domain, which selectively recognizes the minor-groove of AT-rich stretches, including those in the matrix-attachment regions of chromosomes (Stokes and Perry 1995). Interestingly, CHD1 lacking both the C and D domains is localized in exceptionally large and prominent punctate bodies. Thus, in CHD1, the particular role of the D domain may be manifested only when the major targeting function, i.e., that of the chromodomain, is inoperative.

The mislocalization of CHD1 protein lacking the nucleotide-binding pocket of the H domain is noteworthy because of the rapidity with which it occurs. Within 2 h of induced expression, when the presence of exogenous protein is first readily detectable, one can observe widely dispersed punctate bodies containing H^- CHD1 (Fig. 2c). In all of the H domain-containing proteins that have so far been implicated in the remodeling of nucleosomal arrays, ATP hydrolysis, and hence the nucleotide-binding pocket, is essential for this function (Kingston et al. 1996; Pazin and Kadonaga 1997; Tsukiyama and Wu 1997). Thus, it seems reasonable to suppose that ATP hydrolysis is required for the association of CHD1 with decompacted, transcriptionally competent chromatin.

From structural considerations, one might expect that the C, H and D domains would perform distinct roles in the overall function of CHD1. The fact that we can discriminate the effects of the C^-, H^- and C^-D^- mutations on the basis of kinetic and/or morphological parameters seems to confirm this expectation. The importance of newly identified domains, such as the amino-terminal segment that interacts with SSRP1, can eventually be evaluated by mutational analyses similar to the one used here.

CHD1 and SSRP1 are interaction partners

A yeast two-hybrid/interaction trap screen with a 487 amino acid amino-terminal segment of mouse CHD1 yielded several possible binding partners, some of which corresponded to previously characterized proteins. Among these was the protein SSRP1, which appears to be an authentic binding partner of CHD1, based on its intranuclear colocalization with both wild-type and mutant CHD1 and its cofractionation as a complex of about M_r 700,000 on Superose 6 sizing columns. Particularly compelling evidence was provided by the precise colocalization of endogenous SSRP1 with the H^- and C^-D^- mutant forms of CHD1 in punctate bodies (Figs. 8, 9). The colocalization with C^-D^- CHD1 indicated that the chromodomain is not required for this interaction, and, indeed, additional two-hybrid assays demonstrated that a 292 amino acid amino-terminal segment lacking the chromodomain was sufficient. Interestingly, a similar amino-terminal segment of *Drosophila* CHD1 also interacted with human SSRP1 in the two-hybrid assay, albeit somewhat less strongly. The mouse and *Drosophila* amino-terminal segments contain similarly spaced blocks that are particularly rich in serine, glycine and charged amino acids. Presumably, these blocks could collectively form an SSRP1 recognition interface.

The portion of hSSRP1 sufficient to serve as a prey protein corresponded to a 202 amino acid carboxy-terminal segment encoded by the cDNA that was isolated in the original two-hybrid screen. Although this segment,

which contains an HMG1-like domain and flanking highly charged regions (Bruhn et al. 1992), is clearly adequate for interactions with CHD1, additional interaction interfaces might be provided by other portions of SSRP1, some of which are remarkably well conserved among animal and plant species (Dyer et al. 1998).

An interaction between CHD1 and SSRP1 in *Drosophila* was also suggested by the similar immunostaining of polytene chromosomes with anti-CHD1 and anti-SSRP1 antibodies. In both cases, staining was largely confined to puffs and interbands and excluded from regions containing compacted chromatin.

Human SSRP1 was originally identified on the basis of its ability to recognize and bind specifically to cisplatin-modified DNA (Bruhn et al 1992). Orthologs in several other animal and plant species have been identified, mostly on the basis of selective binding to DNA fragments containing specific sequences and/or structural features (Shirakata et al. 1991; Yamaguchi-Shinozaki and Shinozaki 1992; Wang et al. 1993). Similarly to CHD1, SSRP1 is expressed in a wide variety of tissues and cell types (Bruhn et al. 1992; Stokes and Perry 1995). The salient characteristics of SSRP1 proteins are a highly charged carboxy-terminal portion consisting of an HMG1/2-like domain, flanked by regions that are very rich in basic and acidic residues, and an amino-terminal portion that is remarkably well conserved over a broad range of plant and animal species. Recently, hSSRP1 was found to bind specifically to an element of the epsilon-globin promoter and positively regulate its transcriptional activity via its ability to bend DNA (Dyer et al. 1998). Interestingly, the ε-globin promoter element that binds SSRP1 is relatively AT-rich (70%), a feature shared by the sequences that are recognized by CHD1. Moreover, it is also noteworthy that an AT-rich sequence in the chicken δ1-crystallin enhancer core (ACTTTTTCTGAAATATTCAT) can be recognized by the chicken orthologs of both CHD1 (clone JF 11) and SSRP1 (clone JF 10) (Funahashi et al. 1993). The relationship between the sequence-specific and structure-specific recognition properties of HMG1 domain-containing proteins like SSRP1 is still unclear (Dyer et al. 1998), but both properties might be required if chromatin remodeling must accommodate DNA distortions.

Other evidence linking proteins with HMG1/2 domains to chromatin remodeling has recently been presented. On the side of transcriptional activation, it was found that BAF 57, a core component of a mammalian SWI/SNF-related complex, contains an HMG1/2 domain (Wang et al. 1998). The importance of this domain for BAF57 function is presently unclear, however, since complexes containing a BAF57 component that lacks the HMG domain still exhibit nucleosome disruption activity and an ability to bind four-way junction DNA. On the side of transcriptional repression, human HP1 proteins were found to interact indirectly with hHMG2 via an intermediary protein termed SP100B (Lehming et al. 1998) and to interact directly with a splice variant of SP100 that possesses an HMG1/2 domain (Seller et al. 1998). The HP1 proteins may also be capable of interacting with BAF proteins (Le Douarin et al. 1996), suggesting a complex network in which different combinations of interacting components exert diverse effects on chromatin structure.

A niche for CHD complexes?

At present, it is impossible to assign a particular niche in the overall remodeling of chromatin to any of the known activation/repression complexes. It is not clear whether different complexes operate at different levels of chromatin structure or as components of different regulatory circuits. Even within the CHD family, there may be members with divergent functions. We have previously posited an activation function for CHD1 based on its association with transcriptionally active puffs and interbands in polytene chromosomes (Stokes et al. 1996). The fact that SSRP1, which interacts with CHD1, can bind to and help activate the ε-globin promoter (Dyer et al. 1998), is in agreement with this idea. On the other hand, the *Xenopus* ortholog of CHD4, a distantly related variant of CHD1, has recently been found to be associated with a histone deacetylase complex, suggesting its possible involvement in gene repression (Wade et al. 1998). It is worth noting that although all members of the CHD family contain C and H domains, CHD1 and CHD2 differ considerably from CHD3 and CHD4 at both ends. In particular, CHD3/4 does not contain a recognizable D domain and lacks the conserved sequence features of the SSRP1-interactive amino-terminal region.

In yeast, deletion or overexpression of the solitary CHD gene does not significantly affect cell growth or viability except under suboptimal conditions, e.g., in the presence of 6-azauracil, which causes nucleotide pool depletion and slows down transcriptional elongation, or in low-density pre-exponential cultures (Woodage et al. 1997; Jin et al. 1998). In these circumstances, a deficiency of CHD is associated with an acceleration in cell growth, leading to the idea that it might normally have a repressive function. However, the relationship between these effects on growth and the transcriptional activities of specific genes remains to be established. Clearly, much additional information will be required to sort out the particular function(s) of the CHD proteins and to establish their niche among the various chromatin remodeling complexes. The power of current genetic and biochemical methodology makes this a plausible goal for future research.

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