SclI: An Abundant Chromosome Scaffold Protein
Is a Member of a Family of Putative ATPases with an Unusual Predicted Tertiary Structure

Noriko Saitoh, Ilya G. Goldberg, Edgar R. Wood, and William C. Earnshaw
Department of Cell Biology and Anatomy, Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Abstract. Here, we describe the cloning and characterization of SclI, the second most abundant protein after topoisomerase II, of the chromosome scaffold fraction to be identified. SclI is structurally related to a protein, Smclp, previously found to be required for accurate chromosome segregation in Saccharomyces cerevisiae. SclI and the other members of the emerging family of SMCl-like proteins are likely to be novel ATPases, with NTP-binding A and B sites separated by two lengthy regions predicted to form an α-helical coiled-coil. Analysis of the SclI B site predicted that SclI might use ATP by a mechanism similar to the bacterial recN DNA repair and recombination enzyme. SclI is a mitosis-specific scaffold protein that colocalizes with topoisomerase II in mitotic chromosomes. However, SclI appears not to be associated with the interphase nuclear matrix. SclI might thus play a role in mitotic processes such as chromosome condensation or sister chromatid disjunction, both of which have been previously shown to involve topoisomerase II.

In interphase human cells, chromosomal DNA molecules totaling ~2 m long are packaged into nuclei that are only ~10 μm in diameter. At mitosis, the chromosomes become further condensed by about fourfold. This overall 10,000-fold compaction of the DNA is accomplished by a hierarchy of DNA and chromatin packaging (Earnshaw, 1991). At the lowest level, the DNA fiber is compacted six- to sevenfold by winding around the histone core of the nucleosome, generating fibers of ~10 nm diameter (Kornberg, 1974). At the second level, association of histone H1 with the 10-nm fiber causes the fiber to shorten and thicken to ~30 nm in diameter, bringing the overall compaction of the DNA to ~40-fold (Finch and Klug, 1976; Thoma et al., 1979; Horowitz et al., 1994). How the remaining 250-fold compaction of the 30-nm fiber is accomplished remains a matter of active investigation and considerable controversy (Earnshaw, 1991).

At present, the most widely accepted model for higher order chromosome structure proposes that the 30-nm fiber is gathered into loops, each containing ~50-100 kb of DNA, and tethered to nonhistone proteins of the nuclear scaffold or matrix (Laemmli et al., 1978). This model proposes that at the onset of mitosis, the scaffold proteins at the base of the loops associate with one another, thus pulling the chromosomal loop domains closer together. The aggregates of chromosomal scaffolding with their associated loops are thought to form either rosettes that coil along a helical path (Comings and Okada, 1971; Rattner and Lin, 1985; Boy de la Tour and Laemmli, 1988) or stack above one another to form minibands (Pienta and Coffey, 1984). Recent microscopy analysis using DNA fluorochromes under conditions where they bind preferentially to AT-rich or GC-rich DNA has suggested that mitotic chromosome arms consist of a more or less tightly coiled axial region of AT-rich DNA with loops of GC-rich DNA protruding from it (Saitoh and Laemmli, 1994).

The loop models of chromosome organization all suggest, however, that the chromatin fiber is packed into the final chromosome structure, and special molecules must exist that bind to the chromatin and define the base of each loop domain. At present, both the DNA sequences and polypeptide components that comprise this putative loop-fastener complex are unknown, although candidates for both have been suggested. The polypeptide components have been suggested to be components of the mitotic chromosome scaffold (or nuclear matrix) Adolph et al., 1977a, 1977b; Izaurralde et al., 1989; Zhao et al., 1993). The DNA sequences are known variously as MARs or SARs (matrix or scaffold attachment regions) (Mirkovitch et al., 1984; Gasser et al., 1989).

Chromosome scaffold proteins comprise the 5-10% of nonhistone chromosomal proteins that remain insoluble after treatment of isolated metaphase chromosomes with nuclease and subsequent extraction under a variety of conditions, including high salt (2 M NaCl), low ionic strength (dextran sulfate/heparin), or chaotropes (lithium diiodosalicylate) (Lewis and Laemmli, 1982). Although the chromo-
some scaffold is in reality a biochemical fraction, the term has been widely interpreted as describing a structural network within mitotic chromosomes. This, in part, results from the observation that isolated chromosome scaffolds retain the overall chromosomal morphology, with paired sister chromatids and condensed centromeres (Adolph et al., 1977b; Earnshaw and Laemmli, 1983). However, the role, if any, played by chromosome scaffold proteins in chromosome structure and function remains an important unsolved question.

The first chromosome scaffold protein to be conclusively identified was DNA topoisomerase II (Earnshaw et al., 1985; Berrios et al., 1985; Gasser et al., 1986) (initially termed Scl [Lewis and Laemmli, 1982]). This protein turns out to be the major component of the chromosome scaffold fraction (Gasser et al., 1986; Heck and Earnshaw, 1986). Several independent mapping techniques revealed that topoisomerase II is concentrated in the axial region of expanded mitotic chromosomes, and that it is largely absent from the expanded chromosomal loop domains (Earnshaw and Heck, 1985; Gasser et al., 1986). Functional studies support the notion that topoisomerase II plays an essential role in mitotic chromosome structure and function both early and late in mitosis. The protein is required for normal chromosome condensation in fission yeast (Uemura et al., 1987), and also for chromosome condensation in vitro when interphase nuclei or naked DNA are added to mitotic extracts prepared from Xenopus eggs (Adachi et al., 1991; Hirano and Mitchison, 1993). The role of topoisomerase II during chromosome condensation is not known. On the one hand, it is possible that the enzyme is simply required to sort out DNA entanglements that impede orderly chromosome condensation. For example, the mitotic condensation process may serve as a rectification mechanism, whereby neighboring chromosomes are untangled from one another so that they can assort independently during mitosis (Holm, 1994). On the other hand, topoisomerase II may actually make a structural contribution to the condensed chromosome (Earnshaw et al., 1985; Gasser et al., 1986; Adachi et al., 1991). This could occur through interactions of the protein with the SAR/MAR sequences that have been proposed to form the base of chromosomal loop domains (Adachi et al., 1989). The notion that topoisomerase II plays a structural role in chromosomes is controversial, even when results obtained with the same experimental system are compared (Adachi et al., 1989; Hirano and Mitchison, 1993).

Genetic analysis in the yeasts has revealed that topoisomerase II is required for disjunction of sister chromatids at anaphase (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1986). This function is also conserved in vertebrates, as shown both by drug treatments of cultured cells (Downes et al., 1991; Clarke et al., 1993) and by analysis of sister chromatid disjunction in Xenopus cell cycle extracts (Shamu and Murray, 1992). It has been speculated that assembly of topoisomerase II into the chromosomal structure might be important for regulation of its action during disjunction of sister chromatids (Earnshaw et al., 1985).

These studies of topoisomerase II provided the first concrete evidence that members of the chromosome scaffold fraction actually do play an important role in mitotic chromosome structure and function. However, with the exception of CENP-B (Earnshaw et al., 1984; Earnshaw and Rothfield, 1985), CENP-C (Earnshaw et al., 1984; Earnshaw and Rothfield, 1985), CENP-E (Yen et al., 1991), and the IN-CENPs (Cooke et al., 1987), all of which are concentrated in and around the centromere, topoisomerase II has remained the only member of this fraction to be characterized. Other abundant members of the fraction, including ScII (135 kD) (Lewis and Laemmli, 1982) and ScIII (140 kD), have remained unstudied.

In this paper, we provide the first characterization of ScII. We prepared antibodies to chicken ScII and used them to obtain cDNA clones encoding the chicken polypeptide. Like topoisomerase II, ScII is concentrated in the axial region of swollen chromosomes throughout the entire length of the chromosome arms. Biochemical fractionation confirms that ScII is a prominent component of the mitotic chromosome scaffold fraction. However, the protein associates only very loosely with interphase nuclei, with ≳95% leaking out into the cytoplasm during Dounce homogenization. Thus, ScII is not a component of the nuclear matrix. DNA sequence analysis reveals that ScII is a member of an emerging family of proteins with two internal regions of coiled-coil and highly conserved NTP-binding motifs at the amino and carboxy termini. The best characterized member of this family, Smclp, is required for accurate chromosome segregation in the budding yeast Saccharomyces cerevisiae (Strunnikov et al., 1993). Analysis of the deduced polypeptide sequence, together with previous results, suggests that ScII may be a chromosomal enzyme that may function in a complex with topoisomerase II.

Materials and Methods

Isolation of Chicken Chromosome Scaffold Proteins and Production of Guinea Pig Antibody

Mitotic chromosomes were isolated from chicken lymphoblastoid cell line MSB-1 as previously described (Earnshaw et al., 1985). Scaffolds were prepared by subjecting chromosomes to nuclease digestion and 2 M NaCl extraction (Adolph et al., 1977b). The pelleted scaffold fraction was applied onto preparative SDS-PAGE, the gels were stained with Coomassie blue, and a 135-kD band was excised and used to immunize a guinea pig to produce antisera 5B2 (Earnshaw et al., 1985).

cDNA cloning

Library Screening. Antiserum 5B2 was used to screen an oligo-dT-primed λgt11 library prepared from chick embryo fibroblast mRNA (gift of B. Vennstrom, Karolinska Institute, Stockholm, Sweden). Expression screening (Ausubel et al., 1991) yielded three positive clones. These clones were used to screen a λZAP library made from MSB-1 mRNA (Mackay et al., 1993). The eight clones recovered by high stringency nucleic acid hybridization screening were characterized by restriction enzyme mapping and by partial sequencing. The largest clone, pBSc20 (insert size = 3.8 kb), was used in further experiments.

Rapid Amplification of cDNA Ends (RACE). To recover cDNA sequences 5' to the beginning of the clone pBSc20, RACE-PCR (Frohman et al., 1988) was used. First, ScII-specific antisense primers (5'CCAAAGAGATCTTGGACATCCCA... [nt 545-507 in Fig. 2] or 5'TTGCCTAGTTAGATGAGATGAT[3' [nt 189-1866 in Fig. 2A]) were annealed to 1 μg of poly (A)+ RNA isolated from logarithmically growing MSB-1 cells with the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). Next, the first strand of cDNA was synthesized with 10 U of AMV reverse transcriptase (Life Technologies, Inc., Bethesda, MD). A poly(A)+ tail was attached to the 3' end of the first strand with 25 U of terminal deoxynucleotidyl transferase (Boehringer-Mannheim Biochemicals Corp., Indianapolis, IN). The second strand was synthesized with 2.5 U of AmpliTaq enzyme (Perkin-Elmer Corp., Norwalk, CT) and RACE-1 primer...
Bacterial Expression and Antibody Production

Fluorescence Microscopy

Chromosomes were spread and dried using an aquarium air pump (Earnshaw et al., 1989) (Hagen 800), and swollen by immersion in nol-acetic acid (3:1 vol/vol) for 2 min. This was followed by immersion of three changes (2 min each) of TEEN (1 mM triethanolamine/HCl, pH 8.5, 150 mM NaCl, and 0.1% BSA) at 37 ° for 1 h. After washing in D-PBS (8.06 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, 2.7 mM KCI, 0.68 mM CaCl2, and 0.492 mM MgCl2) for 5 min, and were then fixed for 5 min in 3% paraformaldehyde in D-PBS at room temperature. Then, coverslips were washed with KB* (10 mM Tris/HCl, pH 7.7, 150 mM KCl, 0.6 mM K2PO4, 0.1% Triton X-100) three times, and were incubated with anti-SclII antibodies (1:200 dilution) in KB- (10 mM Tris/HCl, pH 7.7, 0.1% BSA, and 0.1% Triton X-100) had been added (Earnshaw et al., 1989). Next, cells were incubated with secondary and tertiary detection reagents were performed as described previously (Cooke et al., 1987; Earnshaw et al., 1989). The filters were then washed twice for 10 min with 2× SSC and 0.1% SDS, followed by a 1-h wash with 0.3× SSC and 0.1% SDS at 65 ° overnight. The filters were then processed as described previously (Cooke et al., 1987; Earnshaw et al., 1989).

Subcellular Fractionation

Dounce Homogenization. Exponentially growing cells (“interphase cells”) or cells grown in the presence of 0.1 μg/ml colcemid (“mitotic cells”) were collected by centrifugation at room temperature. Cells were resuspended and swollen in 1× RSB (Lewis and Laemmli, 1982) at room temperature for 5 min, and centrifuged for 3 min at 800 g. Subsequent steps were performed on ice. The pellet was vigorously resuspended in 1× buffer A (15 mM Tris/HCl, pH 7.4, 80 mM KCl, 2 mM KErDA, pH 7.4, 0.75 mM spermidine, 0.3 mM spermine, and 0.1% digitonin Sigma Immunocoul) (Lewis and Laemmli, 1982), and were lysed with 10 strokes of a Dounce homogenizer. The lysate was centrifuged at 3,200 g for 20 min to separate the supernatant (“cytoplast”) from the pellet ("nuclei" or "chromosomes"). To reduce cross-contamination, the supernatant was centrifuged again at 3,200 g for 20 min, and the pellet was discarded. A small portion of the supernatant was observed under the microscope to confirm that nuclei and chromosomal components were absent. The pellet was washed with solution 3 (5 mM Tris/HCl, pH 7.4, 2 mM KCl, 2 mM KErDA, pH 7.4, 0.375 mM spermidine). Further fractionation of chromosomes to scaffold and non-scaffold fractions was performed as described previously (Cooke et al., 1987; Earnshaw et al., 1985). Total (lysate without centrifugation), cytoplast, and nuclei or chromosome fractions were then mixed with SDS-PAGE sample buffer, boiled, sonicated, and applied to SDS-PAGE. Electrophoresis and immunoblotting were performed as described previously (Earnshaw et al., 1984).

Mass Enucleation. Preparation of cytoplasts and karyoplasts was performed as described in (Prescott and Kirkpatrick, 1973) with slight modifications. Briefly, cells were grown on round plastic coverslips (PGC Scientific, Gaithersburg, MD), and were pre-centrifuged in warm RPMI 1640 ( Gibco Laboratories, Grand Island, NY) cell side down at 37 ° for 10 min at 5,000 g to remove dead cells. The coverslips were then transferred to centrifuged tubes containing warm RPMI plus 10 μg/ml cytochalasin B (Sigma Immunocoul), and centrifuged for 10 min at 17,000 g at 37 °. Karyoplasts in the pellet and cytoplasts still attached to the coverslip were dissolved in SDS-PAGE sample buffer and used for gel analysis. The cytoplasts were also observed in the microscope after fixation with 3% paraformaldehyde and stained with DAPI. More than 95% of the cells remaining attached to the coverslips had been enucleated.

Phylogenetic Analysis

Assembling the Walker B Profile. The region at the COOH terminus of SclII

Abbreviations used in this paper: DAPI, 4',6' diamino phenylindole; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

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Saitoh et al. Molecular Analysis of Chromosome Scaffold Protein SclII
that is most conserved between ScII, Snc2, Snc1, and P115 was used to construct a position-specific scoring table (profile) using the PILEUP program from the GCG package of sequence alignment programs (Devereux et al., 1984). This profile was used to search the entire coding region of representatives from the ABC transporter family and from the RNA helicase family using PROFILESEARCH. In addition, using PROFILESEARCH, we searched the entire SwissProt database to find B motifs more closely related to the ScII group. The highest scoring match for a known ATPase was for UvrA, with the recombination gene RecN being the most closely related protein overall. In each case, the best match to the profile was a conserved region homologous to the Walker B motif, which is well characterized for ABC transporters (Higgins et al., 1986) and for RNA helicases (Linder et al., 1989). Representative sequences from each group were added to the ScII profile using PILEUP to generate a profile with more divergent B motifs, the Walker B profile. To gain some understanding of the possible role of the Walker B motif in ATP binding or hydrolysis, the Walker B profile was aligned to the sequence for rabbit muscle adenylate kinase, an ATPase for which detailed structural information is available. The best match to the Walker B profile contained the entire so-called segment 3 (Fry et al., 1986), which is conserved in other ATPases and contains what has been implicated as the catalytic residue, Asp-119 (Asp-120 in SelII). The Walker B profile was aligned to the sequence for rabbit muscle adenylate kinase, an ATPase for which detailed structural information is available. The best match to the Walker B profile contained the entire so-called segment 3 (Fry et al., 1986), which is conserved in other ATPases and contains what has been implicated as the catalytic residue, Asp-119 (Asp-120 in SelII).

Constructing the Phylogenetic Tree. Distances between sequences in the Walker B profile were computed using the DISTANCES program from the GCG package. The resultant matrix of distances was used to construct a phylogenetic tree using the Fitch-Margoliash algorithm as implemented in the program FITCH from the PHYLIP phylogeny inference package (version 3.52c) (Felsenstein, 1989). The order in which sequences were added to the tree was randomized five times, and the best tree of 7,028 examined is reported. The tree as presented was rooted at its centroid. The sequences of the Walker B sites used in the phylogenetic tree are given in Table 1, together with the appropriate references.

### Table I. Sequences Examined as NTP-binding B Sites and Used to Establish Phylogenetic Tree in Fig. 13

| Protein | Amino acid sequences including B site | Notes | Reference |
|---------|-------------------------------------|-------|-----------|
| ScII    | LSGGQRSLA LSLLAIILLF KPAPIY1LDE VDAALD | Members of SMC family | This study |
| Smc2p   | LSGGQRSLA LSLLMALQPF RPAPMYLDE VDAALD | Strunnikov (unpublished) |
| Smc1p   | LSGGKTVAA LALLFAINSY QPSPFLVDLE VDAALD | Strunnikov et al. (1993) |
| P115    | LSGGKAI A SSLFLAIKA R1P1C1LDE VEAALD | Notarnicola et al. (1991) |
| B.s-RecN| A8SGGELSRVM LAIKSIFSQ QDVTSS1FDE VDGVS | DNA repair-recognition proteins | Van Hoy and Hoch (1990) |
| Ec-RecN | A8SGGELSRMV LAIQVTARK METPALF1DE VDGVS | Rostas et al. (1987) |
| UvTa B2 | LSGGEOAR1R LASSQA-GA VGV-MYVLDE PSL1G1H | Husain et al. (1986) |
| UvTa B3 | LSGGEOAVRK LARESLLGRT GQT-LYI1DE PTTG1H | Husain et al. (1986) |
| Nodl    | LSGGMKRLT LAGALIND-- -PQ-LIL1DE PTT1GLD | ABC transporters | Evans and Downie (1986) |
| OppD    | LSGGMRQVRM IAMALLCR-- -PK-LIL1ADE PTT1ALD | Higgins et al. (1985) |
| HlyB    | LSGGQQR1A IARLAVNN-- -PK-L1F1DE ATSDALD | Felmlie et al. (1985) |
| OppP    | LSGGQQOR1G IARAIL1E-- -PK-L1CDC AVSALD | Hiles et al. (1987) |
| HisP    | LSGGQQQVRYS IARALAME-- -PD-VLFLDE PTSDALD | Higgins et al. (1982) |
| PmB     | LSGGQQQRLC IARG1AIR-- -PE-VOLL1DE PSCALD | Surin et al. (1985) |
| FtsE    | LSGGEOQVRG IARRVVNV-- -PA-VLALDE PTG1NL | Gill et al. (1986) |
| RbsA    | LS1GDQVMVE IAKVL5F-- -ESKV1M1DE PT1ALT | Bell et al. (1986) |
| MalK    | LSGGQOQRVA IGR1TVLAE-- -PS-VF1LLE PLSNL1D | Gilson et al. (1982) |
| eIF-4A1 | IVGTPGVRVF MNLN-RLYSP KY1KMFVLDE ADEML5 | RNA helicases | Nielsen et al. (1985) |
| eIF-4AII| VVVTGPPUFD MLNR-RYLSL KY1KMFVLDE ADEML5 | Nielsen and Trachsel (1988) |
| Tif1    | VVGGPGRVRVF MRNL-RLYLSL DK1KMFVLDE ADEML5 | Linder and Slonimski (1989) |
| p65    | CIAPTGLR1D FLEC-GKTNL RRTTYL1DE ADRMLD | Ford et al. (1988) |
| PL10    | LVATPGLYLD MMER-GK1GL DCYKL1DE ADRMLD | Leroy et al. (1989) |
| vasa   | V1ATPGR1LDD FDVR-11TF DEDT1VFLDE ADRMLD | Lasko and Ashburner (1988) |
| MSS116  | V1ATPGR1LDD VLEY1SKFF RFVYKVLDE ADLRLDE | Seraphin et al. (1989) |
| SrmB    | VVATTGRLQY I1YE-1NFD RAVET1L1DE ADRMLD | Nishi et al. (1988) |
| Adn kin Sg3 | LIDGYPQVQQ QGEEFFERRA OP3LLYYVDA GPETMQ | Kinase | Kuby et al. (1984) |

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open reading frame of ScII was carried out in three stages. First, a chicken cDNA λgt11 expression library (Young and Davis, 1983) was screened with guinea pig anti-ScII. Several positive clones were characterized, and were found to cover a portion of the ScII cDNA. In the second round of screening, the inserts from these phage were used for further DNA screening of a chicken MSB-1 AZAP library (Mackay et al., 1993) by plaque hybridization. Eight positive clones were obtained, the longest of which was designated pBSc20 (insert size = 3.8 kb) (Fig. 1 A). Primer extension analysis (data not shown) indicated that pBSc20 was lacking ~400 bp from its 5' end relative to the 5' end of the ScII mRNA. Therefore, in a third round of cloning, these missing sequences were recovered by the RACE-PCR method (Frohman et al., 1988). Four clones from two independent RACE reactions with ScII mRNA were sequenced and turned out to be identical to each other and contiguous with the 5' end of pBSc20. These were used to complete the molecular characterization of the 5' end of the ScII cDNA. pBSc20 was fully sequenced on both strands, subcloned for bacterial expression, and used for a probe in Southern (DNA) and Northern (RNA) hybridization analysis.

The 4,134-bp ScII cDNA encodes a single undisrupted ORF encoding a predicted polypeptide of 1,189 amino acids, with a calculated molecular mass of 134,900 D (Fig. 2 A). This corresponds to the apparent molecular mass of ScII previously determined from SDS-PAGE (135 kD) (Lewis and Laemmli, 1982). The ScII polypeptide is predicted to be slightly basic (calculated pI = 8.6) and highly hydrophilic.

**Validation of the Clones**

We obtained evidence that pBSc20 encodes bona fide ScII by demonstrating that the cloned and chromosomal polypeptides share at least three independent epitopes. Several subcloned regions of pBSc20 (shown in Fig. 1 C) were expressed in *E. coli* using a T7 RNA polymerase-based expression system (Tabor and Richardson, 1985). Peptides N, M, and C were designed to cover the NH2-terminal, middle and COOH-terminal regions, respectively. These polypeptides do not overlap one another. Between them, these molecules cover the entire ScII molecule, except for 120 amino acids at the NH2 terminus. One further peptide, A, covers a region equivalent to the sum of N and M.

Bacterially expressed peptides N, M, and C produced prominent bands in SDS-PAGE of induced bacterial extracts, all of which migrated with approximately the expected molecular weights. Peptide A was somewhat degraded in vivo. Peptides A, N, and M reacted with the original guinea pig anti-ScII antibody in immunoblots; however, peptide C did not (data not shown). All four expressed proteins were isolated by SDS-PAGE and injected into rabbits for production of polyclonal antisera.

Despite the fact that antibodies N, M, and C are directed against epitopes on nonoverlapping peptides, all of them recognized a 135-kD band in the chromosome protein fraction by immunoblot. This immunoreactive polypeptide comigrated with ScII as detected with the original guinea pig antibody, 5B2 (Fig. 3). Thus, our cDNA clones encode multiple independent epitopes present on ScII, suggesting that pBSc20 is a bona fide cDNA clone for ScII.

**ScII is Encoded by a Single Gene and Transcribed into a 4.2 kb mRNA**

When chicken genomic DNA was digested with restriction endonucleases, electrophoresed and transferred to nitrocellulose, and hybridized with various subclones derived from pBSc20, only single or double bands were observed. This suggests that ScII is encoded by a single gene (Fig. 4 A). Northern (RNA) blots of poly(A)+ mRNA revealed a single 4.2-kb transcript with probes derived from either the 5' or 3' portion of pBSc20 (Fig. 4 B). This mRNA appears to be reasonably abundant, at least in the chicken MSB-1 lymphoblastoid cell line. Interestingly, lower levels of transcript were detected in a parallel analysis of mRNA from chick embryonic fibroblasts (Fig. 4 C).

**ScII is a Member of an Emerging Family of Putative Nucleotide-binding Proteins Defined by Yeast Protein Smclp**

A computer database search with the ScII cDNA sequence revealed limited sequence similarities to several other proteins, including Smclp. This yeast protein (stability of minichromosomes) was recently shown to be required for proper chromosomal segregation in budding yeast. The *SMC1* gene product is the founder member of a group of proteins that
Chromosomal proteins from MSB-1 cells were subjected to SDS-PAGE, blotted to nitrocellulose, cut into strips, and probed with antisera. P, preimmune serum; I, immune serum. 5B2, Guinea pig antisera raised against the 135-kD band of the chicken chromosomal scaffold fraction. A, N, M, and C, Rabbit antisera raised against bacterially expressed peptides described in Fig. 1 C. As shown in Fig. 5 A, ScII and Smc2p share 65% identity over a 210-amino acid stretch near the NH2 terminus, and 66% identity over a 136-amino acid stretch near the COOH terminus. As shown in Fig. 6, the amino-terminal sequence of all SMC1 family members contains a consensus NTP-binding motif (GXXXXGKS, the so-called Walker A site [Walker et al., 1982]). The carboxy-terminal sequence of SMC1 was suggested to be a novel motif, and was termed the DA box (Strunnikov et al., 1993). As described below, our analysis suggests that this is one version of the NTP-binding B site (the so-called Walker B site [Walker et al., 1982]). The B site is implicated in ATP hydrolysis, where structural evidence is available (Serpersu et al., 1986), or thought to be required in the coupling of ATP hydrolysis to ATPase function (Pause and Sonenberg, 1992). A third characteristic motif found in GTP-binding proteins is not present in ScII.

All SMC1 family members have two internal regions that are predicted to form α-helical coiled coil. These regions (of 256 and 352 amino acids, respectively, in ScII) do not show sequence similarity between ScII and Smc2, but the region between coils is 47% identical over 122 amino acids (Fig. 5, B and C).

**Figure 4.** Chicken ScII is encoded by single gene and transcribed into a 4.2-kb mRNA. (A) Genomic southern blot. Total DNA from MSB-1 cells was digested with EcoRI (E), BamHI (B), and PstI (P), separated on an agarose gel, transferred to nitrocellulose, and probed with 32P-labeled HPs fragment as described in Fig. 1 B. (B) RNA blot. 1 μg of poly(A)+ RNA from MSB-1 cells was loaded in each lane. The blot was probed with either the 5' (EC) or 3' probe (PoX) as described in Fig. 1 B. (C) 1 μg of poly(A)+ RNA from MSB-1 cells (MSB-1) or chicken embryo fibroblasts (CF) was separated on a denaturing agarose gel and stained with ethidium bromide (left panel), or blotted and probed with the 3' probe (right panel).
in the nuclei, possibly as a component of the insoluble nuclear matrix. The result could not be explained by disruption of the nuclei during fractionation, since when the same preparations were probed with a control antibody recognizing topoisomerase II, this molecule was detected solely in nuclei (Fig. 7 A, right panel). A similar analysis of mitotic cells revealed that \( \sim 50\% \) of the ScII is associated with the chromosomes (Fig. 8 A). Of this, \( \sim 70\% \) remains as a component of the chromosome scaffolds after nuclease digestion and extraction of the chromosomes with 2 M NaCl (Fig. 8 B).

We considered two possible explanations for this result. (a) ScII might be a cytoplasmic protein in interphase cells that associates with chromosomes after nuclear envelope breakdown in mitosis. In this case, the different localizations of ScII during interphase and mitosis might reflect different molecular associations and possibly different functional roles. At one extreme, ScII could be a cytoplasmic protein that sticks to chromosomes at mitosis as a result of nonspecific interactions. (b) A second possibility was that ScII is actually a nuclear protein, but readily leaks out from the nuclei during Dounce homogenization. A number of well-characterized nuclear proteins such as DNA polymerase \( \alpha \) and RNA polymerase I have been found to leak out from nuclei during mechanical cell lysis (Herrick et al., 1976).

To decide between these possibilities, we used another method of cell fractionation that eliminates leakage of proteins from the nucleus (Krek et al., 1992). This involves mass enucleation of adherent cells after exposure to cytochalasin B. In this method, coverslips with adherent cell monolayers were centrifuged cell side down at 37°C in medium containing cytochalasin B. Under these conditions, the cell cortex becomes much less robust, so that karyoplasts (nuclei surrounded by plasma membrane containing a thin
Figure 6. Probable NTP binding A and B sites were found in conserved regions of ScII near the NH₂ and COOH termini, respectively. Amino acid sequence alignments are shown for Smc family members ScII, Smc2p, Smc1p, and P115. (Upper part) Sequence alignment for the NH₂ terminus, including the NTP binding A site. (Lower part) Sequence alignment for the COOH terminus, including the putative NTP-binding B site. Numbers above the sequence correspond to amino acid numbering of ScII (Fig. 2A). Identical amino acids are boxed. The consensus sequence derived from the Smc family ("consensus") is shown together with consensus sequence for NTP-binding A and B sites of the ABC transporter family (NTP-A and NTP-B) (Fath and Kolter, 1993).

Figure 7. Determination of subcellular localization of ScII in interphase cells. (A) The majority of ScII is found in the cytoplasmic fraction when cells are fractionated by Dounce homogenization followed by centrifugation. Total (T), nuclear (N), and cytoplasmic (C) proteins of chicken cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-ScII or anti-topoisomerase II antibodies. The amount of protein in each lane was normalized for the number of starting cells (~10⁴). Anti-topoisomerase II was used as a nuclear protein marker. (B) The majority of ScII is found in nuclei (karyoplasts) when cells are fractionated by mass enucleation. Total (T), karyoplast (K), and cytoplast (Cy) proteins of chicken cells were prepared as described in Materials and Methods. They were separated on SDS-PAGE, stained with Coomassie blue (left panel) or transferred to nitrocellulose, and probed with anti-ScII, anti-topoisomerase II, or antitubulin antibodies (a cytoplasmic protein marker).
SclI Is Distributed along the Arms of Mitotic Chromosomes

SclI was localized in mitotic chromosomes using two different fixation and spreading protocols.

In the first, cells were hypotonically swollen, placed on coverslips whose surface had been rendered hydrophilic by glow discharging (Earnshaw and Migeon, 1985), and centrifuged briefly to burst them open. The coverslips were then transferred immediately to a buffer at physiological ionic strength, and were fixed with 3% paraformaldehyde. Chromosomes prepared in this way are frequently expanded, presumably as a result of shear forces during lysis of the cells. However, it is important to note that they have not been subjected to any chemical extraction. It is also important to note that these preparations were made with cells that had not been previously blocked with colcemid, thus minimizing the possibility of artifactual redistribution of the antigens in vivo (Compton et al., 1991).

Under these conditions, SclI was found to be distributed along the axis of the chromosome arms over their entire length, including the centromere (Fig. 9). The DNA was often considerably more diffuse in aspect, suggesting that chromatin loops were extended outward from the axial region containing SclI. These images look virtually identical to earlier images obtained using this technique in conjunction with antibodies to topoisomerase II (Earnshaw and Heck, 1985).

Condensed staining of centromere regions by anti-SclI was reproducibly observed by this technique. This may reflect a concentration of SclI in the heterochromatin of the centromere in vivo. Alternatively, it could simply reflect a differential tendency of the centromeric heterochromatin and chromosome arms to expand during centrifugation.

These images should not be interpreted as showing that SclI forms an axial core up the center of the chromosome arms, as in some versions of the chromosome scaffold model (Earnshaw, 1991). Instead, we suggest that SclI is associated with an insoluble network that is distributed throughout the chromatic arms. When the chromatin loops splay outwards during cell lysis, this network more or less retains its original shape. Thus it is unlikely that SclI is tightly associated with the bulk chromatin of the radial loops. Instead, it is apparently associated either with the bases of these loops, or with some other nonchromatin component of the chromosomes.

Our second procedure for visualizing SclI in mitotic chromosomes involves hypotonic swelling of cells, followed by fixation with methanol–acetic acid and spreading induced by blowing with a stream of air from an aquarium pump. This is a standard procedure for obtaining cytological spreads of chromosomes, which we have previously modified to preserve the reactivity of antigens with anticientromere antibodies (Earnshaw et al., 1989).

This method again reveals the presence of SclI along the entire length of the chromosome arms (Fig. 10). As in the formaldehyde-fixed cells, SclI appears to occupy a somewhat more restricted domain than the bulk chromatin, even though the latter is less swollen than in the case of spreading by centrifugation. Interestingly, HeLa chromosomes, which are larger than their chicken counterparts, often show a locally coiled morphology of SclI staining (Fig. 10 D) similar to that observed for topoisomerase II by Laemmli and co-workers (Boy de la Tour and Laemmli, 1988; Saitoh and Laemmli, 1994). Similar staining patterns were obtained using antibodies against antigens N, M, and C, or affinity-purified antibody against antigen A, suggesting strongly that the staining results from specific recognition of SclI. Thus, two independent methods of fixation and spreading confirm...
that ScII can be visualized along the entire length of the chromosome arms, with a distribution reminiscent of that seen previously with antibodies to topoisomerase II. To confirm colocalization of ScII and topoII, we tried double staining of chicken cells and indeed, we obtained an indistinguishable staining pattern of ScII and topoII on the mitotic chromosome (Fig. 11).

The localization of ScII to mitotic chromosomes was confirmed by immunofluorescence of cells processed in situ without hypotonic swelling or spreading. Unfortunately, with our present antibody reagents, we have been unable to demonstrate a convincing cellular localization of ScII during interphase under a variety of fixation conditions.

**Discussion**

**ScII, the Second Most Abundant Component of the Mitotic Chromosome Scaffold, Is Not a Component of the Interphase Nuclear Matrix**

ScII was originally identified as a component of the mitotic chromosome scaffold fraction (Lewis and Laemmli, 1982). [35S]methionine autoradiography of HeLa cells suggests that ScII is second in abundance in this fraction only to topoisomerase II (Lewis and Laemmli, 1982). ScII is thus an excellent candidate for a protein that plays a major role in mitotic chromosome structure or function. As we will discuss below, ScII exists in at least one characterized complex with topoisomerase II, and it is therefore tempting to speculate that the protein might function in either chromosome condensation or disjunction, since both of these processes require topoisomerase II.

Unlike topoisomerase II, ScII is not a component of the interphase nuclear matrix fraction. Rather, the protein appears to leak out of nuclei during subcellular fractionation. We were only able to convincingly demonstrate the presence of ScII in nuclei when cells were fractionated into karyoplasts and cytoplasts by centrifugation in the presence of cytochalasin B.

A different situation is observed during mitosis, when standard fractionation procedures reveal that ~50% of the ScII is associated with isolated chromosomes. When these
Figure 10. Localization of SclI in cytological spreads of chicken (MSB-1, A and B) and human (HeLa, C and D) cells. Cells were fixed with methanol-acetic acid, and cytological spreads were prepared by gentle blowing with an aquarium pump. Cells were stained with DAPI (A and C) or anti-SclI antibodies (B and D).

Figure 11. Colocalization of SclI and topoisomerase II throughout the axial region of mitotic chromosome arms. MSB-1 cells were fixed with methanol-acetic acid, the chromosomes were spread with an aquarium air pump, and stained with anti-SclI antisera (A), anti-topoisomerase II (B), or DAPI (C). We note that SclI appears to occupy a slightly more condensed distribution along the chromatid arms than does topoisomerase II.
chromosomes are subjected to DNase digestion and extraction to reveal the insoluble chromosome scaffold, ~70% of the chromosomal SclI remains in this fraction. In similar experiments, we previously observed that 72% of chromosomal topoisomerase II is retained in the chromosome scaffold fraction (Earnshaw et al., 1985).

Thus, SclI interacts very differently with chromosomes during interphase and in mitosis. The basis for this change is not understood. It is possible that phosphorylation of the SclI polypeptide during mitosis alters its interactions with one or more chromosomal components.

**SclI Belongs to an Emerging Family of Polypeptides that have Two Internal Coiled-Coil Domains Flanked by NTP-binding A and B Sites at Either End**

Our analysis of the SclI cDNA clones predicts a polypeptide with a molecular weight of 134,900 and a pI of 8.6. The deduced amino acid sequence shows limited similarity to a protein, Smclp, which is involved in chromosome segregation in the budding yeast. The Smcl mutation was first identified in a screen for mutations that resulted in increased frequencies of selective loss of a plasmid minichromosome (Strunnikov et al., 1993). Subsequent disruption of the gene resulted in a perturbation of the segregation of bona fide yeast chromosomes (Strunnikov et al., 1993). Thus, the genetic analysis of the SMCI gene and our biochemical analysis of SclI are consistent with the notion that these proteins play some role in chromosome structure or function during mitosis.

The SMCI protein is the founder member of an emerging family of related polypeptides in organisms from myco-

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**Figure 12.** Proposed model for the structure of SclI. The circled A and B represent NTP-binding A and B sites. Those sites are brought close together by bending of the spacer between the coiled coils (left) or forming an antiparallel homodimer (right). Thick boxed regions represent the predicted coiled-coils, and the shaded regions represent the interactions between the coils that hold the structure together.

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plasma to man, which share three common features (Strunnikov et al., 1993). These polypeptides, which range from 115 to 165 kD in size, all have a nucleotide-binding pocket (A site) very close to their amino terminus, and a second conserved region very close to their carboxy terminus. As described below, this second site resembles the NTP-binding B site found in several classes of ATPases. These two sites, potentially involved in nucleotide utilization, are widely separated by two regions predicted to form α-helical coiled coil (~250 and 350 amino acids in length, respectively, in SclI), and a central spacer. Overall comparison of the primary amino acid sequences for the entire molecules does not show a particularly high level of similarity among SMCI family members. This is partly because the regions of coiled-coil are divergent in sequence, even between the most closely related family members, SclI and Smc2p. If the coiled-coils serve primarily as spacers to position the Walker A and B sites with respect to one another, then the relative lengths of the coils may be more important than their detailed amino acid sequences.

It is unusual to have the NTP-binding A and B sites so far apart in an ATPase, since both must interact with the nucleoside triphosphate, a small molecule. This leads us to predict one of two conformations for members of the SMCI family. First, the proteins could function as monomers if the central region is a hinge (Fig. 12, right). In this case, the two regions of coiled-coil could fold back on one another in an antiparallel conformation, thus giving the molecule a hairpin shape and bringing the two parts of the NTP binding fold together. Alternatively, the proteins could function as extended antiparallel dimers, with the NTP-binding A site of one monomer associating with the B site from its partner (Fig. 12, left). Of course all of this speculation rests on the validity of the assumption that these proteins are, in fact, ATPases. It will be essential to test this hypothesis as purified proteins become available.

When the sequences of the SMCI family members are compared in detail, some similarity is also noted in the re-
region of the central spacer that separates the two regions of coiled-coil. As suggested above, this region might act as a hinge, creating a characteristic fold so that two A and B sites are brought together. Alternatively, it may form a site for interaction with other proteins such as topoisomerase II (see below) or with DNA.

**SMC1 Family Members Resemble a Bacterial DNA Repair and Recombination Enzyme**

NTP-binding A and B sites were defined by Walker and colleagues by using amino acid sequences from several different ATPases, including adenylate kinase, RecA protein, and myosin (Walker et al., 1982). A variety of studies, including site-directed mutagenesis and structural analysis by nuclear magnetic resonance, have led to the thinking that the positively charged lysine residue in the A site binds to the negatively charged phosphate, while the negatively charged aspartate in the B site coordinates the positively charged Mg²⁺. This aspartate in the B site may be involved in hydrolysis of the phosphodiester bond (Serpersu et al., 1986; Pause and Sonenberg, 1992).

Although the core sequence of the A site is fairly well conserved, the B site is considerably more divergent. However, functionally related proteins tend to have B site sequences that are more closely related than the corresponding regions of functionally distinct proteins. To gain some insight about the possible function of ScII, we assembled a consensus sequence for the conserved COOH-terminal region common to the proteins whose ATPase activities were well known. The fact that the similarity of this conserved sequence to the Walker family members, contained the above mentioned aspartate as the only absolutely conserved residue. Next, taking advantage of the alignments between the B sites of respective ABC transporters, RNA helicases, and adenylate kinase, we established a phylogenetic tree (Fig. 13) based on the distance between the sequences in the alignments. The *SMC1* family members was positioned in the middle of this tree. Similarities between ABC transporters, UvrA, and recN (Doolittle et al., 1986; Gorbalenya and Koonin, 1990) that have previously been found by comparing the entire coding sequences were also found by our strategy.

We were very interested to find that the protein whose NTP-binding B site most closely resembles the *SMC1* family consensus also shares a second significant structural feature with the *SMC1* family members. In this protein, bacterial recN (a DNA repair and recombination enzyme), the two NTP-binding sites are also separated by a relatively long stretch of ~500 amino acids that has a significant probability of forming a coiled-coil. We emphasize that the phylogenetic tree of Fig. 13 only concerns the sequence of the NTP-binding B site, and therefore, probably only indicates proteins that hydrolyse ATP in a similar manner. However, this additional similarity between recN and the *SMC1* family members may indicate further similarities of function, and it serves as a starting point for future functional analysis of the role of ScII in the chromosome.

**The Function of ScII is Unknown, but the Protein Has Been Shown To Be in at Least One Complex with Topoisomerase II**

Although the function of ScII is unknown, evidence obtained in an earlier study indicated that the protein can exist in a complex with topoisomerase II. In that study, a protein complex called UB2 was purified from undifferentiated murine erythroleukemia cells based on its binding to a specific sequence motif found in the 3′ flanking region of the β-globin gene (Ma et al., 1993). This binding site also occurs near the carboxy anhydrase I and c-myb genes, as well as in the immunoglobulin heavy chain enhancer region (Ma et al., 1991). These regions shared two discernible sites that are important for UB2 binding. The UB2 DNA-binding activity was not present in MEL cells that had been induced to differentiate and had initiated transcription of the β-globin gene. Thus, it was postulated that the UB2 complex might be involved in regulation of β-globin expression.

The UB2 activity passed over DEAE sepharose and DNA affinity columns was found to copurify with three major polypeptides of 170, 116, and 48 kD, and a minor polypeptide of 135 kD (Ma et al., 1993). The 170-kD component was shown by immunoblotting to be topoisomerase II, and addition of anti-topoisomerase II to the nucleoprotein gels produced a super shift of the UB2 complex. The 135-kD component of this complex was found to be ScII. Addition of affinity-purified anti-ScII antibodies caused a specific increase in the mobility of the UB2 complex, consistent with dissociation of one or more components.

In addition to the presence of topoisomerase II and ScII in the UB2 complex, two other observations also support the notion that the two molecules might interact. First, we have noted that the distribution of ScII in spread mitotic chromosomes is essentially identical to that seen for topoisomerase II. Second, in an independent method of purifying topoisomerase II, nuclear extracts from a chicken lymphoblastoid cell line were passed sequentially through hydroxyapatite and phosphocellulose columns (Wood, E. R., unpublished data). The final fraction contained roughly equimolar amounts of topo II, ScII, and a third (unidentified) species of 116 kD. Although we have not yet shown that these proteins are actually associated in a complex, the similarity of this fraction to the UB2 complex is striking.

It will be very important in future studies to determine whether ScII is, as its sequence suggests, an ATPase, and whether the protein has an essential role (possibly enzymatic) in chromosome condensation or sister chromatid disjunction. It will also be interesting to confirm whether ScII and topoisomerase II do exist in specific complexes in chromosomes, and if so to assess the relevance of such complexes in vivo for chromosome structure and function.

The authors gratefully acknowledge Dr. Bjorn Vennstrom's gift of his chicken α-globin expression library and Dr. Don Cleveland's gift of his anti-tubulin antibody. We thank Dr. Yuri Lazebnik, Dr. Alastair Mackay, Dr. Ann Piata, Dr. John Tomkiel, and Dr. Charles Yang for many insightful.
suggestions during the course of this work. We also thank Victor Chua for help in sequencing.

These experiments were supported by National Institutes of Health grant GM30983 to W. C. Earnshaw.

Received for publication 29 June 1994 and in revised form 29 July 1994.

Note Added in Proof. We have recently learned of two further SMC1 family members that resemble ScII. Fission yeast cut 14p is 34% identical to ScII, and it is required for chromosome condensation and disjunction in mitosis (Saka, Y., T. Sutani, Y. Yamashita, S. Saitoh, M. Takeuchi, Y. Nakaseko, and M. Yanagida. EMBO J. 13:3997-4006).

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