that Rb, $p16^{Ink4a}$ and cyclin D1/Cdk4 can form a quaternary complex *in vitro* (data not shown). These results suggest that $p16^{Ink4a}$ binds to a site on Cdk4/6 that is different from that for ATP, the substrate Rb and cyclin D1. Similarly, $p19^{Ink4d}$ binds to the cyclin D/Cdk4 complex without disrupting the interaction between the cyclin and Cdk4[4]. In addition, an R24C mutant of Cdk4, which inhibits binding of $p16^{Ink4a}$ and is targeted by cytolytic T lymphocytes in a human melanoma, has been identified[4]. This mutation affects the interaction of $p16^{Ink4a}$, but not that of p21 or p27, suggesting that the two classes of CDKI bind to different sites. Another mutation that affects $p16^{Ink4a}$ binding is found at Lys 22, and replacement of residues 95–97 in human Cdk4 with those from Cdk2 also abolishes binding to $p16^{Ink4a}$ (M. Serrano and D. Beach, unpublished results). If it is assumed that Cdk4 and Cdk6 have structures similar to Cdk2 (Cdk4 and Cdk6 share 39 and 43% identity with Cdk2, respectively), then all these mutations are likely to cluster together near the junction between the N- and C-terminal lobes of the CDK, next to the active site. The results suggest that the Ink4 class of CDK inhibitors bind in this cleft (Fig. 3b).

The structure of $p19^{Ink4d}$ allows us to interpret and analyse the large amount of data now available on mutants of $p16^{Ink4a}$. In particular, it indicates which of these mutants might have altered function, as opposed to altered structure, and which would thus qualify for further investigation. Our analysis of $p16^{Ink4a}$ and Cdk4 mutants and of $p16^{Ink4a}$ peptides provides a model for the interaction of the Ink4 class CDKIs with Cdk4 and Cdk6 which can now be tested using site-directed mutagenesis and biochemical analysis. □

Methods

Protein purification. Mouse $p19^{Ink4d}$ as a fusion to the glutathione-*S*-transferase (GST) protein[4] was coexpressed with GroEL and GroES[11] and uniformly $^{13}C/^{15}N$- and ^{15}N-labelled proteins were prepared for the NMR experiments by growing *E. coli* BL21 (DE3) (Novagen), harbouring the expression plasmids, in a minimal medium containing $(^{15}NH_4)_2SO_4$ either with or without $^{13}C_6$-glucose. Deuterated proteins were prepared as described[23]. The protein was affinity-purified on glutathione-Sepharose beads (Pharmacia). $p19^{Ink4d}$ was then cleaved from the fusion protein while it was still attached to the resin, using thrombin (Boehringer-Mannheim), which was subsequently removed from the supernatant with an anti-thrombin resin (Sigma). The protein was exchanged into phosphate buffer (20 mM, pH 7.5) containing 100 mM NaCl, 1 mM EDTA and 5 mM DTT.

NMR spectroscopy. All NMR spectra were acquired at 30 °C on Bruker AMX600 NMR spectrometers. The ^1H, ^{13}C and ^{15}N resonances of the backbone of the protein were assigned using a combination of 3-D CBCA(CO)NNH and CBCANNH experiments. The side-chain signals were assigned using 4D HCC(CO)NNH and $^{13}C/^{15}N$-separated NOESY experiments, 3D HCCH-COSY and HCCH-TOCSY experiments, and 2D ^1H-^{15}N HMBC, ^1H-^{13}C HSQC, CβHδ and CβHϵ experiments[24].

Structure calculations. The structures were calculated using the program X-PLOR[25]. Initial structures were based on 1,967 completely unambiguous ^1H–^1H distance restraints obtained from 4D $^{13}C/^{15}N$- or $^{15}N/^{15}N$- and 3D ^{15}N- or ^{13}C-separated NOESY spectra[26] of either uniformly ^{15}N-, $^{13}C/^{15}N$- and $^2H/^{13}C/^{15}N$-labelled or selectively protonated proteins[23]. In addition, 82 ϕ dihedral angle restraints were obtained from a 3D HNHA experiment[27]. A total of 2,212 ambiguous NOE restraints were then included in the structure calculations. The initial structures were iteratively refined using ARIA methodology[28], in which the previous set of structures is used to identify restraint violations and to reduce the level of ambiguity in the ambiguous restraint tables by discarding restraints that contribute little to the intensity.

Received 27 May; accepted 29 July 1997.

1. Sherr, C. J. Cancer cell cycles. *Science* **274,** 1672–1677 (1996).
2. Chan, F. K. M., Zhang, J., Cheng, L., Shapiro, D. N. & Winoto, D. A. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to $p16^{INK4}$. *Mol. Cell. Biol.* **15,** 2682–2688 (1995).
3. Guan, K. *et al*. Isolation and characterization of $p19^{INK4d}$, a p16 related inhibitor specific to CDK6 and CDK4. *Mol. Biol. Cell* **7,** 57–70 (1996).
4. Hirai, H., Roussel, M. F., Kato, H.-Y., Ashmun, R. A. & Sherr, C. J. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol. Cell. Biol.* **15,** 2672–2681 (1995).
5. Zhang, B. & Peng, Z.-y. Defective folding of mutant $p16^{INK4a}$ proteins encoded by tumor-derived alleles. *J. Biol. Chem.* **271,** 28734–28737 (1996).
6. Serrano, M., Hannon, G. J. & Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/Cdk4. *Nature* **366,** 704–707 (1993).
7. Bork, P. Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally. *Prot. Struct. Funct. Genet.* **17,** 363–374 (1993).
8. Hannon, G. J. & Beach, D. $p15^{INK4B}$ is a potential effector of TGF-β-induced cell-cycle arrest. *Nature* **371,** 257–261 (1994).
9. Foulkes, W. D., Flanders, T. Y., Pollock, P. M. & Hayward, N. K. The *CDKN2A* (p16) gene and human cancer. *Mol. Med.* **3,** 5–20 (1997).
10. Serrano, M. *et al*. Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85,** 27–37 (1996).
11. Merlo, A. *et al*. 5′ CPG island methylation is associated with transcriptional silencing of the tumor-suppressor $p16^{CDKN2/MTS1}$ in human cancers. *Nature Med.* **1,** 686–692 (1995).
12. Yasukawa, T. *et al*. Increase of solubility of foreign proteins in *Escherichia coli* by co-production of the bacterial thioredoxin. *J. Biol. Chem.* **270,** 25328–25331 (1995).
13. Wick, S. T., Dubay, M. M., Imanil, I. & Brizuela, L. Biochemical and mutagenic analysis of the melanoma tumor-suppressor gene-product p16. *Oncogene* **11,** 2013–2019 (1995).
14. Wölfel, T. *et al*. A $p16^{INK4A}$-insensitive CDK4 mutant targeted by cytolytic T-lymphocytes in a human melanoma. *Science* **269,** 1281–1284 (1995).
15. Kalus, W. *et al*. NMR structural characterization of the CDK inhibitor $p19^{INK4d}$. *FEBS Lett.* **401,** 127–132 (1997).
16. Gorina, S. & Pavletich, N. P. Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. *Science* **274,** 1001–1005 (1996).
17. Tevelev, A. *et al*. Tumor suppressor $p16^{INK4A}$: Structural characterization of wild-type and mutant proteins by NMR and circular dichroism. *Biochemistry* **35,** 9475–9487 (1996).
18. Yang, R., Gombart, A. F., Serrano, M. & Koeffler, H. P. Mutational effects on the $p16^{INK4a}$ tumor suppressor protein. *Cancer Res.* **55,** 2503–2506 (1995).
19. Ranade, K. *et al*. Mutations associated with familial melanoma impair $p16^{INK4}$ function. *Nature Genet.* **10,** 114–116 (1995).
20. Koh, J., Enders, G. H., Dynlacht, B. D. & Harlow, E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375,** 506–510 (1995).
21. Parry, D. & Peters, G. Temperature-sensitive mutants of $p16^{CDKN2}$ associated with familial melanoma. *Mol. Cell. Biol.* **16,** 3844–3852 (1996).
22. Fåhraeus, R., Paramio, J. M., Ball, K. L., Lain, S. & Lane, D. P. Inhibition of pRB phosphorylation and cell-cycle progression by a 20-residue peptide derived from $p16^{CDKN2/INK4A}$. *Curr. Biol.* **6,** 84–91 (1996).
23. Smith, B. O. *et al*. An approach to global fold determination using limited NMR data from larger proteins selectively protonated at specific residue types. *J. Biomol. NMR* **8,** 360–368 (1996).
24. Clowes, R. T., Crawford, A., Raine, A. R. C., Smith, B. O. & Laue, E. D. Structural studies of proteins using NMR spectroscopy. *Curr. Opin. Biotechnol.* **6,** 81–88 (1995).
25. Brünger, A. T. *X-PLOR, Version 3.1: A System for X-ray Crystallography and NMR* (Yale Univ. Press, New Haven and London, 1992).
26. Clore, G. M. & Gronenborn, A. M. Structures of larger proteins in solution—3-dimensional and 4-dimensional heteronuclear NMR spectroscopy. *Science* **252,** 1390–1399 (1991).
27. Vuister, G. W. & Bax, A. Quantitative *J* correlation: A new approach for measuring homonuclear three-bond $J(H^NH^\alpha)$ coupling constants in ^{15}N-enriched proteins. *J. Am. Chem. Soc.* **115,** 7772–7777 (1993).
28. Nilges, M., Marcias, M. J., O'Donoghue, S. I. & Oschkinat, H. Automated NOESY interpretation with ambiguous distance restraints: the refined NMR solution structure of the pleckstrin homology domain from β-spectrin. *J. Mol. Biol.* **269,** 408–422 (1997).
29. Kraulis, P. J. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24,** 946–950 (1991).
30. Gilson, M. K., Sharp, K. A. & Honig, B. Calculation of the total electrostatic energy of a macromolecular system—solvation energies, binding energies and conformational analysis. *J. Comput. Chem.* **9,** 327–335 (1988).

Acknowledgements. We thank C. J. Sherr for the plasmid expressing GST-$p19^{Ink4d}$; S. Ishii for plasmids expressing GroEL and GroES; W. Boucher for computer programming; M. Nilges for protocols for the structure calculations; J. Krywko for the model of Cdk4; M. Serrano and D. Beach for assaying $p19^{Ink4d}$ and for communicating unpublished results; N. Pavletich for the coordinates of 53BP2; and A. Murzin for helpful discussion. This work was supported by a grant from the BBSRC. The Cambridge Centre for Molecular Recognition is supported by the BBSRC and the Wellcome Trust.

Correspondence should be addressed to E.D.L. (e-mail: e.d.laue@bioc.cam.ac.uk). The co-ordinates have been deposited in the Brookhaven PDB (accession number lap7).

erratum

Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II

Patrick D. Varga-Weisz, Matthias Wilm, Edgar Bonte, Katia Dumas, Matthias Mann & Peter B. Becker

Nature **388,** 598–602 (1997)

The meaning of the sentence that describes Fig. 5 of this Letter was distorted by erroneous duplication of text and should read: "Comparison of the ATPase activities of CHRAC with that of purified *Drosophila* topoisomerase II (Fig. 5) showed that topoisomerase II was only stimulated by free DNA, whereas CHRAC was stimulated by free DNA as well as by nucleosomes." □

resulted in the protein family shown in Fig. 1c. The highest-scoring non-related sequences after those shown consisted of *trithorax* family members that reached error probabilities of $P > 0.5$ in all of the profile iterations.

Immunocytochemistry. Antibodies were raised in rabbits against the purified GST fusion proteins encoded by pGEX-RIM-52 and pGEX-Rim-PDZ. Double and single immunofluorescence labelling of cryostat sections from rat spinal cord and bovine retinae was performed[27] with two independent polyclonal Rim antibodies and multiple monoclonal antibodies to synaptic vesicle proteins. Staining was visualized by Cy2- and Cy3-conjugated secondary antibodies and viewed in a BioRad MRC1024 confocal microscope. Immuno-electron microscopy was performed by a pre-embedding protocol with silver enhancement[27]. Controls for all immunocytochemistry experiments included the use of two independent antibodies, control stains with other antibodies, and experiments in which the first antibody was omitted.

PC12 cell transfections. PC12 cells (ATCC) were plated in collagen-coated 6-well dishes with 10^6 cells per well. Cells were transfected on day 1 with 0.2 μg of Qiagen-purified pCMV5-hGH encoding human growth hormone and 1 μg of the indicated expression plasmids using Lipofectamine (Life Technologies). Expression plasmids encoded the light chains of wild-type and mutant tetanus toxin in pCMV5 (ref. 28), LDL receptor and Rab3A in pCMV5, and residues 1–399 of Rim in pME18sf(−). On day 3, cells were collected, washed, and split into two portions, one of which was incubated for 20 min at 37 °C in control buffer (in mM) (145 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose, 0.5 ascorbate, 20 HEPES-NaOH, pH 7.4) and the other was incubated in the same buffer containing 56 KCl and 95 NaCl. After incubation, growth hormone in the medium and the cells was determined by radioimmunoassay (Nichols Institute). Secretion was calculated as the percentage growth hormone released as a function of stimulation. All experiments were done in triplicate at least three times.

Rim affinity chromatography. Glutathione–agarose columns without protein, with GST–Rim fusion protein encoded by pGEX-Rim-52, or with various control GST fusion proteins, were reacted with total rat brain homogenate prepared in 0.5% Triton X-100, 1 mM EDTA, 0.1 M NaCl, 0.1 g l^{-1} PMSF, and 50 mM HEPES-NaOH, pH 7.4, with either 0.5 mM GDP-βS or GTP-γS at 4 °C overnight. Samples were washed 5 times in the same buffer without nucleotides before analysis by SDS–PAGE and immunoblotting.

Other procedures. RNA blotting experiments were done using multiple tissue blots (Clontech). SDS–PAGE and immunoblotting were performed using standard procedures and antibodies described previously[1,12,28,29]. Signals were detected by ECL and quantified by ^{125}I-labelled secondary antibodies. Protein assays were performed with the BioRad kit. Subcellular fractionations were performed as described[29] and verified by immunoblotting against a series of synaptic-vesicle, plasma-membrane and cytosolic antigens.

Received 19 February; accepted 22 May 1997.

1. Geppert, M. *et al.* The role of Rab3A in neurotransmitter release. *Nature* **369,** 493–497 (1994).
2. Holz, R. W., Brondyk, W. H., Senter, R. A., Kuizon, L. & Macara, I. G. Evidence for the involvement of Rab3A in Ca^{2+}-dependent exocytosis from adrenal chromaffin cells. *J. Biol. Chem.* **269,** 10229–10234 (1994).
3. Johannes, L. *et al.* The GTPase Rab3a negatively controls calcium-dependent exocytosis in neuroendrocrine cels. *EMBO J.* **13,** 2029–2037 (1994).
4. Geppert, M., Goda, Y., Stevens, C. F. & Südhof, T. C. Rab3A regulates a late step in synaptic vesicle fusion. *Nature* **387,** 810–814 (1997).
5. Castillo, P. E., Janz, R., Südhof, T. C., Tzounopoulos, T., Malenka, R. C. & Nicoll, R. A. Rab3A is essential for mossy fibre long-term potentiation in the hippocampus. *Nature* **388,** 590–593 (1997).
6. Burstein, E. S., Brondyk, W. H., Macara, I. G., Kaibuchi, K. & Takai, Y. Regulation of the GTPase cycle of the neuronally expressed Ras-like GTP-bindign protein Rab3A. *J. Biol. Chem.* **268,** 22247–22250 (1993).
7. Fischer von Mollard, G., Stahl, B., Khokhlatchev, A., Südhof, T. C. & Jahn, R. Rab3C is a synaptic vesicle protein that dissociates from synaptic vesicles after stimulation of exocytosis. *J. Biol. Chem.* **269,** 10971–10974 (1994).
8. Südhof, T. C. Function of Rab3A GDP/GTP exchange. *Neuron* **18,** 519–522 (1997).
9. Bourne, H. R., Sanders, D. A. & McCormick, F. The GTPase superfamily: a conserved switch for diverse cel functions. *Nature* **348,** 125–132 (1990).
10. Nuoffer, C. & Balch, W. E. GTPases: multifunctional molecular switches regulating vesicular traffic. *Annu. Rev. Biochem.* **63,** 949–990 (1994).
11. Shirataki, H. *et al.* Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell. Biol.* **13,** 2061–2068 (1993).
12. Li, C. *et al.* Synaptic targeting of rabphilin-3A, a synaptic vesicle Ca^{2+}/phospholipid-binding protein, depends on rab3A/3C. *Neuron* **13,** 885–898 (1994).
13. Stahl, B., Chou, J. H., Li, C., Südhof, T. C. & Jahn, R. Rab3 reversibly recruits rabphilin to synaptic vesicles by a mechanism analogous to raf recruitment by ras. *EMBO J.* **15,** 1799–1809 (1996).
14. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. Mammalian ras interacts directly with the serin/threonine kinase raf. *Cell* **74,** 205–214 (1993).
15. Südhof, T. C. & Rizo, J. Synaptotagmins: C_2-domain proteins that regulate membrane traffic. *Neuron* **17,** 379–388 (1996).
16. Weisman, L. S. & Wickner, W. Molecular characterization of VAC1, a gene required for vacuole inheritance and vacuole protein sorting. *J. Biol. Chem.* **267,** 618–623 (1992).
17. Yamamoto, A. *et al.* Novel PI(4)P 5-kinase homologue, Fablp, essential for normal vacuole function and morphology in yeast. *Mol. Biol. Cell* **6,** 525–539 (1995).
18. Bean, A. J., Seifert, R., Chen, Y. A., Sacks, R. & Scheller, R. H. Hrs-2 is an ATPase implicated in Ca^{2+}-regulated secretion. *Nature* **385,** 826–829 (1997).
19. Sheng, M. PDZs and receptor/channel clustering: rounding up the latest suspects. *Neuron* **17,** 575–578 (1996).
20. Dowling, J. E. *The Retina. An Approachable Part of the Brain* (Belknap, Cambridge, MA, 1987).
21. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1989).
22. Hata, Y. & Südhof, T. C. A novel ubiquitous form of munc18 interacts with multiple syntaxins. *J. Biol. Chem.* **270,** 13022–13028 (1991).
23. Guan, K. L. & Dixon, J. E. Eukaryotic proteins expressed in Escherischia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192,** 262–267 (1991).
24. Bucher, P., Karplus, K., Moeri, N. & Hofmann, K. A flexible motif search technique based on generalized profiles. *Comput. Chem.* **20,** 3–23 (1996).
25. Henikoff, S. & Henikoff, J. G. Amino acid substitution matrices from protein blocks. *Proc. Natl Acad. USA* **89,** 10915–10919 (1992).
26. Hofmann, K. & Bucher, P. The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. *Trends Biochem. Sci.* **20,** 347–349 (1995).
27. Schmitz, F., Bechmann, M. & Drenckhahn, D. Purification of synaptic ribbons, structural components of the photoreceptor active zone complex. *J. Neurosci.* **15,** 7109–7116 (1996).
28. McMahon, H. *et al.* Cellubrevin: a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* **364,** 346–349 (1993).
29. Ichtchenko, K. *et al.* Neuroligin 1: A splice-site specific ligand for β-neurexins. *Cell* **81,** 435–443 (1995).
30. Matsui, Y. *et al.* Nucleotide and deduced amino acid sequences of a GTP-binding protein family with molecular weights of 25,000 from bovine brain. *J. Biol. Chem.* **263,** 11071–11074 (1988).

Acknowledgements. We thank R. Janz, R. Jahn, M. Zerial and S. Butz for reagents, and J. L. Goldstein and M. S. Brown for advice and support. This work was partially supported by a grant from the HFSP.

Correspondence and requests for materials should be addressed to T.C.S. (e-mail: tsudho@mednet.swmed.edu).

Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II

Patrick D. Varga-Weisz, Matthias Wilm, Edgar Bonte, Katia Dumas, Matthias Mann & Peter B. Becker

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

Repressive chromatin structures need to be unravelled to allow DNA-binding proteins access to their target sequences. This de-repression constitutes an important point at which transcription and presumably other nuclear processes can be regulated[1,2]. Energy-consuming enzyme complexes that facilitate the interaction of transcription factors with chromatin by modifying nucleosome structure are involved in this regulation[3–5]. One such factor, nucleosome-remodelling factor (NURF), has been isolated from *Drosophila* embryo extracts[4,6,7]. We have now identified a chromatin-accessibility complex (CHRAC) which uses energy to increase the general accessibility of DNA in chromatin. However, unlike other known chromatin remodelling factors, CHRAC can also function during chromatin assembly: it uses ATP to convert irregular chromatin into a regular array of nucleosomes with even spacing. CHRAC combines enzymes that modulate nucleosome structure and DNA topology. Using mass spectrometry, we identified two of the five CHRAC subunits as the ATPase ISWI, which is also part of NURF[6,8], and topoisomerase II. The presence of ISWI in different contexts suggests that chromatin remodelling machines have a modular nature and that ISWI has a central role in different chromatin remodelling reactions.

Chromatin reconstituted in the cell-free assembly system from *Drosophila* embryos[9,10] is maintained in a dynamic state characterized by increased nucleosome mobility and overall DNA accessibility in the presence of ATP[11]. Using ATP-dependent endonuclease cleavage of chromatin to assay for DNA accessibility[11], we purified and characterized a new type of chromatin remodelling

machine, CHRAC. CHRAC activity is sensitive to the detergent Sarkosyl (Fig. 1a). Addition of ATP to Sarkosyl-washed chromatin does not increase the accessibility of DNA (Fig. 1b, lanes 3–6); however, when CHRAC-containing fractions are added, DNA in chromatin becomes accessible to restriction enzymes (Fig. 1b, lane 7). Using this assay, we purified CHRAC from nuclear embryo extracts to apparent homogeneity (Fig. 2). On a gel filtration column, the purified activity ran as a complex with an apparent relative molecular mass of 670,000 (M_r 670K) (Fig. 2a). Consistently, proteins of 175K, 160K (which stains more strongly) and 130K, and two small proteins of M_r ~18K and 20K copurified with CHRAC activity with roughly equal stoichiometry (Fig. 2b).

CHRAC increased the accessibility of DNA in chromatin, but not of free DNA, in an energy-dependent manner. DNA in chromatin may be rendered accessible by, for example, alteration of the nucleosome core structure, loss of linker-DNA-binding proteins or of other chromatin-associated proteins, or by unfolding of the nucleosomal fibre. To test whether CHRAC activity required any factors other than nucleosomes, we reconstituted nucleosomes from pure histones on long linear DNA by salt-gradient dialysis. Addition of CHRAC and hydrolysable ATP was necessary and sufficient to increase the accessibility of the nucleosomal DNA to the restriction endonuclease *Dra*I (Fig. 3). As in the crude assembly system[11], only ATP and dATP supported CHRAC activity, whereas other nucleotides were inactive. This result identifies the nucleosome core particle as a target for CHRAC action.

The band corresponding to the 130K subunit was cut from a silver-stained SDS–polyacrylamide gel, cleaved in the gel by trypsin, and analysed by nano-electrospray mass spectrometry[12]. Tandem mass spectrometry of the tryptic cleavage products and computer-assisted sequence interpretation through database searches with peptide sequence tags[13] led to the unambiguous identification of the 130K subunit as the known ATPase, ISWI[6,8] (see Methods).

The 160K subunit was also analysed by mass spectrometry. Nine tryptic peptides were sequenced and the enzyme topoisomerase II (topo II; see Methods) was unambiguously identified. Both ISWI and topo II cofractionated precisely with CHRAC, as shown by western blotting, and topo II was active, as revealed by its ATP-dependent relaxation of negative supercoils (data not shown). By co-immunoprecipitation from crude CHRAC-containing fractions, we confirmed that topo II and ISWI were located in the same complex (Fig. 4). Topoisomerase II functions as a dimer[14], which is consistent with the staining of the 160K topo II band in CHRAC being stronger than the other subunits (Fig. 2). Assuming that two molecules of topo II are present, the relative molecular masses of the individual subunits add up to the observed M_r of 670K.

ISWI is also a subunit of NURF, another nucleosome-remodelling complex that facilitates the binding of transcription factors to chromatin. No other subunit is shared by the complexes[4,6]. Two hSNF2L(the human ISWI homologue)-containing complexes of ~700K and 450K have been identified[15], corresponding to the M_r determined here for CHRAC and with that of NURF[4], suggesting the existence of human counterparts for both ISWI-containing complexes.

The ATPase activity of NURF is stimulated by nucleosomal but not free DNA[6]. By contrast, the ATPase activity of topo II is stimulated by free DNA[16]. Comparison of the ATPase activities of CHRAC with that of purified *Drosophila* topoisomerase II (Fig. 5)

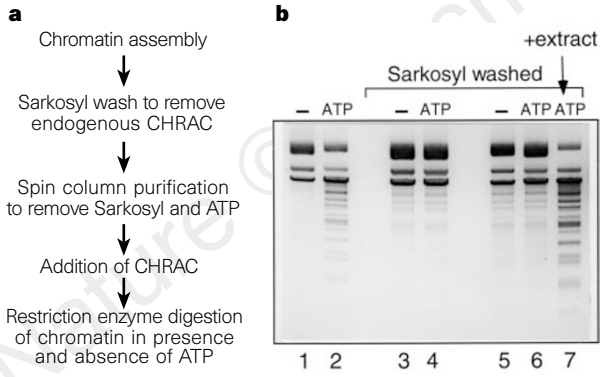

Figure 1 CHRAC assay. **a**, Scheme of the assay. **b**, Lanes: 1, 2, endogenous CHRAC activity revealed by enhanced *Dra*I cleavage of reconstituted chromatin in the presence of ATP; 3–6, Sarkosyl-stripped chromatin becomes more accessible in general, but lacks the ATP-dependent opening; 7, addition of 1 μl CHRAC-containing extract to stripped chromatin reconstitutes ATP-dependent endonuclease accessibility. Chromatin in lanes 3, 4 was washed with 0.075% Sarkosyl, chromatin in lanes 5–7 with 0.15% Sarkosyl. A negative image of the ethidium bromide-stained agarose gel is shown.

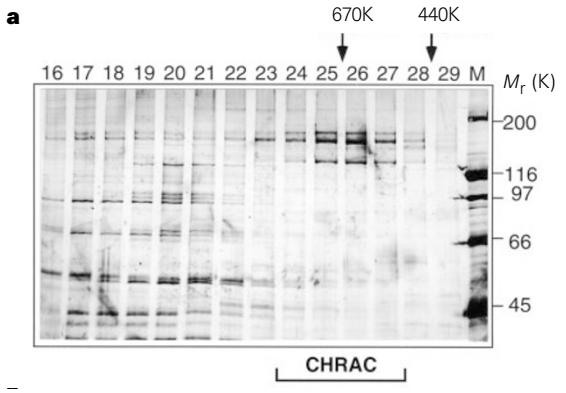

Figure 2 Purification of CHRAC. **a**, 8% SDS–PAGE of fractions from the Superose-6 gel filtration column, calibrated with thyroglobulin (M_r 670K) and ferritin (440K) size standards (Pharmacia). CHRAC activity (''CHRAC'') peaks in fractions 25 and 26. **b**, Purified CHRAC (CHR) was resolved by 8% and 15% SDS–PAGE and stained with silver. M: size markers (BioRad) with indicated relative molecular masses in thousands. Asterisks: small proteins that co-fractionate with CHRAC activity. These proteins run with histone markers on other gels.

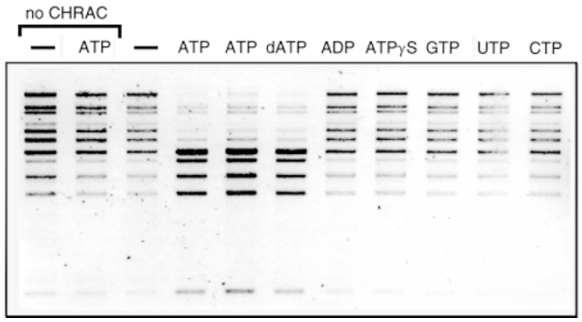

Figure 3 CHRAC targets nucleosomes. Nucleosomes were reconstituted on linear DNA from *Drosophila* histones by salt-gradient dialysis. 28 ng of fraction 25 (Fig. 2) or buffer (no CHRAC) were added to ~80 ng DNA reconstituted into chromatin in EX120 in the presence of 200 μg ml^{-1} BSA and the indicated nucleotides at 1 mM. After *Dra*I digestion, the purified DNA was separated on an agarose gel. A negative image of the ethidium bromide-stained gel is shown.

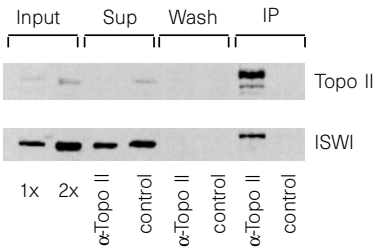

Figure 4 ISWI and topo II co-immunoprecipitate from the crude first CM-Sepharose eluate. Topo II was immunoprecipitated with a protein A–Sepharose-purified rabbit polyclonal antibody (α-topoII) or a nonspecific control antibody. 10% (1×) and 20% (2×) of the input material, 10% of the supernatant (sup), 20% of the final wash and the entire immunoprecipitate (IP) was subjected to consecutive western analysis with an antibody against ISWI and topo II.

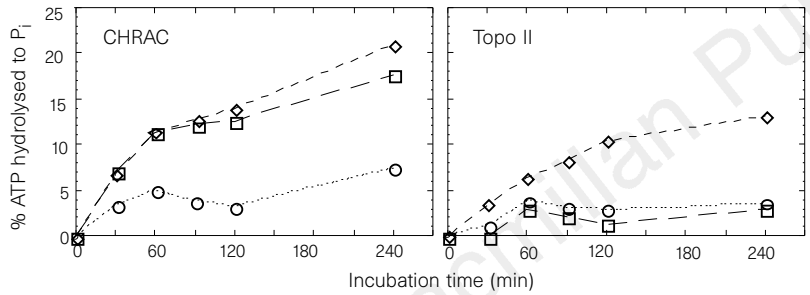

Figure 5 ATPase activities of purified CHRAC and topoisomerase II. The ATPase activity of roughly equivalent amounts (with respect to topoisomerase activity) of CHRAC (left) and purified topoisomerase II (right) were assayed in the presence of control buffer (circles), free DNA (diamonds) and nucleosomes (squares). The percentage of free phosphate was determined at the indicated reaction times.

showed that topoisomerase II was only stimulated by free DNA as well as by nucleosomes, whereas CHRAC was stimulated by free DNA as well as by nucleosomes. We assume that the nucleosome-stimulated ATPase activity in CHRAC is due to ISWI, which is common to both CHRAC and NURF.

CHRAC and NURF increase the accessibility of chromatin by different mechanisms. CHRAC and NURF preparations, standardized by their content of immunoreactive ISWI, had different activities when compared in accessibility assays: CHRAC was less effective in the GAGA-factor-dependent nucleosome remodelling assay than NURF; conversely, NURF did not stimulate the sensitivity to restriction endonucleases (data not shown). NURF perturbs histone–DNA interactions in mononucleosomes when added at a stoichiometry of one NURF per 20 nucleosome core particles[4]. The SWI/SNF and RSC complexes also perturb histone–DNA interactions, but require higher stoichiometries[1,5]. So far, we have not observed a similar effect when incubating equimolar amounts of CHRAC and mononucleosomes under comparable experimental conditions (unpublished observations).

The most striking difference between CHRAC and NURF, however, was in their effect on nucleosomal arrays. The assembly of nucleosomes is rapid in the *Drosophila* and related *Xenopus* systems in the absence of ATP, but correct nucleosome spacing requires time and energy[9,17]. The ATP-dependent spacing principle has so far remained elusive. Addition of CHRAC to chromatin assembled in the absence of ATP, and therefore lacking regular nucleosome spacing, resulted in an ATP-dependent alignment of nucleosomes which became evident from the new regularity of the micrococcal nuclease cleavage pattern (Fig. 6a). This result identifies CHRAC as an ATP-dependent nucleosome-spacing factor. NURF has the opposite effect: addition of large amounts of NURF to a nucleosomal array perturbs the regular repeat pattern[4]. The side-by-side comparison of NURF and CHRAC (standardized by their ISWI content) in our nucleosome-spacing assay demonstrated the difference between the two activities (Fig. 6b). Irregular chromatin obtained by only brief chromatin assembly was converted into a regular nucleosomal array in an ATP-dependent reaction by CHRAC, but not by NURF.

We propose two non-exclusive mechanisms by which CHRAC may mediate these two apparently opposing effects of increasing the accessibility of nucleosomal DNA and of imposing regularity on nucleosomal arrays. CHRAC might increase the mobility of nucleosomes with the expenditure of energy, a reaction that occurs in a cell-free system[11]. Enhanced nucleosome mobility may increase the chance of transient exposure of a particular sequence in accessible linker regions and at the same time facilitate the alignment of nucleosomes into a regular repeat structure. Alternatively, CHRAC may participate in nucleosome assembly and disassembly, like the histone chaperones which, depending on the circumstance, may function either as histone donors in nucleosome assembly or as histone acceptors during disassembly of nucleosome cores[18]. The identification of the ATPase ISWI in two structurally and functionally distinct chromatin remodelling complexes highlights the modular nature of these complexes and suggests that ISWI may drive diverse nucleosome remodelling reactions specified by different interaction partners. ISWI, being found in both NURF and CHRAC, may be directly involved in targeting and rearranging nucleosome structure.

The association of ISWI and topo II in one complex is interesting. The topoisomerase activity (DNA-strand breakage and religation) does not seem to be involved in CHRAC function: VP16, a specific inhibitor of topo II (ref. 19), did not prevent CHRAC from

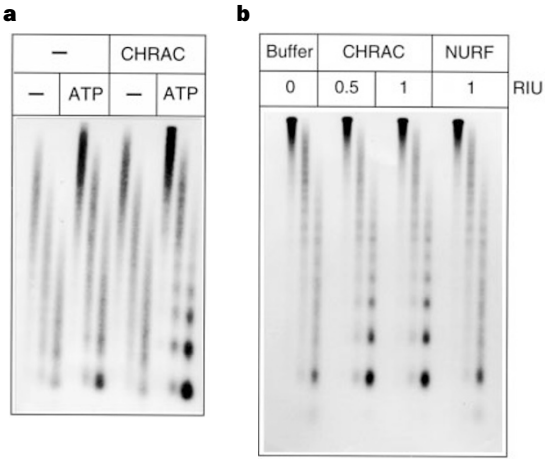

Figure 6 a, CHRAC is an ATP-dependent spacing factor. Chromatin was assembled without ATP, Sarkosyl-stripped to remove endogenous CHRAC, and the effect of addition of ATP and/or CHRAC on the regularity of the nucleosomal array was studied by micrococcal nuclease analysis as described in Methods. **b**, CHRAC and NURF are functionally distinct. Equivalent amounts of CHRAC and NURF (expressed as relative ISWI units, RIU) were compared for their ability to create a regular nucleosomal array.

increasing chromatin accessibility to restriction enzymes, and only slightly altered its effect on nucleosome spacing (data not shown). Purified topo II could not substitute for CHRAC in either of these reactions. The physiological importance of the association of ISWI and topoisomerase II in one complex will only become apparent once further CHRAC subunits have been identified. Nucleosome remodelling by ISWI may help the interaction of topo II with chromatin and thus enhance its function. Conversely, the association of topo II with chromosomes may localize CHRAC activity to specific chromosomal sites. Topoisomerase II is involved in many cellular processes such as the removal of catenates after replication, chromosome condensation and decondensation, kinetochore assembly and sister chromatid separation at mitosis (for reviews, see refs 16, 20–22); it is a major structure component of the mitotic chromosome axis[23,24] and is distributed widely over interphase chromosomes, but is also concentrated at specific heterochromatic sites[25]. Distinct populations of topo II with dynamic nuclear localization patterns during the cell cycle have been visualized[25]. The existence of topo II isoforms, cell-cycle-regulated post-translational modifications, and various associated proteins contribute to the structural and functional diversity of topo II[16]. The CHRAC-associated topo II represents only a fraction of the total topo II in a nucleus and may therefore correspond to a subpopulation dedicated to a specific function. We recently observed a stimulatory effect by CHRAC in a cell-free replication system: CHRAC, but not purified topo II, enhanced the initiation of replication from a nucleosomal SV40 origin (V. Alexiadis, P.D.V., P.B.B. and C. Gruss, manuscript in preparation). □

Methods

Nucleosome reconstitution and CHRAC assay. Chromatin was assembled in *Drosophila* embryo extracts as described[9–11]. After the assembly, 0.075% Sarkosyl (sodium lauroylsarcosine) was added to disrupt endogenous CHRAC activity. Sarkosyl and ATP were removed by gel filtration[11]. CHRAC assay: 10 μl Sarkosyl-washed chromatin (equivalent to ~40 ng DNA), 0.5–5 μl CHRAC-containing fractions, 10–50 units of *Dra*I or *Hinc*II and 1–2 mM ATP in 40 μl EX-120 buffer[11] were incubated for 30–60 min at 26 °C.

Nucleosome reconstitution by salt-gradient dialysis. 4 μg λ DNA, 2 μg *Drosophila* histones and 50 μg BSA (Fermentas) were mixed in 100 μl HSB (10 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 M NaCl) buffer. This mixture was dialysed in a microcollodion bag (Sartorius) against 600 ml HSB. During dialysis over 2 d at 4 °C, the salt was gradually removed by exchange of dialysis buffer with buffer lacking NaCl but containing 0.05% NP-40.

CHRAC purification. A comprehensive protocol is available upon request. CHRAC was purified from nuclear extracts of 12–16-h-old *Drosophila* embryos. Nuclei were extracted with ammonium sulphate[26] and extracts were dialysed into CB-150 buffer (CB: 10 mM HEPES-KOH, pH 7.6, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM EDTA, 0.5 mM sodium metabisulphite, 0.2 mM PMSF, 1 mM DTT; CB-150 contains 150 mM KCl) before precipitation with 20% methanol. All columns were equilibrated in CB-150 and loaded with fractions diluted to a conductivity equal to CB-150. Proteins were chromatographed on a CM–Sepharose FF column (all columns and resins from Pharmacia) and CHRAC activity was eluted with CB-400. Active fractions were rechromatographed on a smaller CM–Sepharose FF column from which the activity was eluted with CB-200. From the following Mono-Q HR 10/10 column, CHRAC eluted at a conductivity equal to CB-350. Successive concentrations involved a 1-ml CM–Sepharose FF column and a Filtron Microsep concentrator (M_r cutoff, 30K). The material was separated on a Superose-6 10/30 column in 20 mM HEPES-KOH, pH 7.6, 400 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 20% glycerol. If necessary, a final fractionation on a Mono-Q 1/1 column was added from which CHRAC was eluted by a salt gradient.

Estimations of purification factor and yield are complicated by the presence of CHRAC inhibitors in crude nuclear extracts. As the purification after the CM-Sepharose step leads to a 200–400 fold enrichment of CHRAC activity, we estimate the overall degree of purification to be at least 1,000-fold. Unless stated otherwise, the CHRAC concentration in the experiments was 250 μg ml^{-1}, as determined by a modified Bradford assay (Biorad) with BSA as standard.

Mass spectrometry. Details of the analysis are available upon request. Proteins were proteolytically trypsinized in the gel[12,27]. Extracted peptides were purified on a 100 nl R2 Poros microcolumn and step-eluted in 1 μl 60% methanol/5% formic acid into a nanoelectrospray capillary. Mass spectrometry was done on an API III triple quadrupole instrument (PE-Sciex, Ontario) equipped with an upgraded collision cell and the nanoelectrospray ion source[28]. For the 130K subunit, the sequence tag (776.6)(I/L)EQ(1146.4) from the fragment spectrum of a peptide (M_r 1,877) identified uniquely the tryptic peptide comprising amino acids 736–748 of the ISWI protein in a non-redundant database (data not shown). Four other ISWI peptides were sequenced from the same digest corresponding to amino acids 160–171, 394–402, 450–458 and 638–648. From the tryptic peptide mixture of the 160K protein, 9 peptides (T1–T9) were sequenced which identified unambiguously *Drosophila* topoisomerase II (SwissProt P15348; T_1: ITFSPDLAK, T_2: DLYGVFPLR, T_3: SLAVSGLGVIGR, T_4: SGIVESVLAWAK, T_5: YIFTIMSPLTR, T_6: IFDEILVNAADNK, T_7: ELMWVYDNSQNR, T_8: SQLEHILLRPDSYIGSV EFTK, T_9: GFQQVSFVNSIATYK; data not shown).

ATPase assay. Nucleosomes were reconstituted on a 146-bp DNA fragment by salt-gradient dialysis at high histone input to ensure that no free DNA contaminated the preparation. The reaction (9 μl) contained: 60 ng DNA or the equivalent amount of mononucleosomes, 6.6 mM HEPES, pH 7.6, 0.66 mM EDTA, 0.66 mM 2-mercaptoethanol, 0.033% N-P40, 1.1 mM $MgCl_2$, 33 μM ATP, 5 μCi [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$, Amersham) and 14 ng CHRAC or 11.5 units topo II (USB) or CHRAC400 buffer. Unreacted ATP and free phosphate were separated by thin-layer chromatography and quantified by using PhosphoImager and Imagequant software.

Co-immunoprecipitation. Equal amounts of protein A–Sepharose-purified rabbit polyclonal antibody against topoisomerase II (W4-250-1, provided by D. Arndt-Jovin) or a control protein A–purified rabbit IgG were added to 10 μl of the first CM–Sepharose eluate. 5 μl IP-150 (20 mM HEPES, pH 7.6, 0.5 mM EGTA, 10% glycerol, protease inhibitor, 150 mM KCl, 5 mM $MgCl_2$), 1 μl (20 μg) chicken ovalbumin and 2 μl of 3 M KCl were added. This mixture was kept for 1 h on ice, added to protein A–Sepharose beads (10 μl settled volume) in 50 μl IP-400 (as IP-150, but containing 400 mM KCl, 1 mM $MgCl_2$, 50 μg ovalbumin and 0.1% N-P40) and incubated further for 2 h at 4 °C. Beads were washed twice with 50 μl IP-400, once with IP-150, and resuspended in 25 μl IP-150. 5 μl 6 × SDS loading buffer were added and the sample was

analysed by SDS–PAGE and western blotting. Rabbit anti-ISWI antibody was biotinylated using the Clontech Biotin-X-NHS ester labelling kit. Detection was by streptavidin–peroxidase polymer (Sigma) with an ECL detection kit (Amersham).

Nucleosome spacing. *Drosophila* embryo assembly extract was dialysed against EX120, 0.2 mM PMSF, 0.5 mM sodium metabisulphite to remove ATP. 60 μl of extract, 66 μl EX50, 14 μl 30 mM $MgCl_2$, 10 mM DTT, 600 mM NaCl and 1 μg plasmid DNA were mixed and incubated for 3 h at 26 °C. Chromatin was Sarkosyl-stripped and purified by gel filtration[7,11]. 20 μl of this chromatin (~70 ng DNA) plus 30 μl EX120 were incubated for 90 min with either 1 μl CHRAC or buffer in presence or absence of 2 mM ATP. The nucleosome repeat was revealed with an oligonucleotide hybridizing to a GAGA element of the *hsp26* promoter as described[7,29]. 0.1 and 0.2 μl CHRAC and 1 μl NURF P11 (gift from T. Tsukiyama and C. Wu) were assayed in the experiment shown in Fig. 6b. The CHRAC preparation contained 5× more ISWI protein than the NURF preparation.

Received 29 January; accepted 28 May 1997.

1. Kingston, R., Bunker, C. & Imbalzano, A. N. Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev.* **10,** 905–920 (1996).
2. Felsenfeld, G. Chromatin unfolds. *Cell* **86,** 13–19 (1996).
3. Peterson, C. L. & Tamkun, J. W. The SWI–SNF complex: a chromatin remodelling machine? *Trends Biochem. Sci.* **20,** 143–146 (1995).
4. Tsukiyama, T. & Wu, C. Purification and properties of an ATP-dependent nucleosome remodelling factor. *Cell* **83,** 1011–1020 (1995).
5. Cairns, B. R. *et al.* RSC, an essential, abundant chromatin-remodelling complex. *Cell* **87,** 1249–1260 (1996).
6. Tsukiyama, T., Daniel, C., Tamkun, J. & Wu, C. ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kD subunit of the nucleosome remodelling factor. *Cell* **83,** 1021–1026 (1995).
7. Tsukiyama, T., Becker, P. B. & Wu, C. ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. *Nature* **367,** 525–532 (1994).
8. Elfring, L. K., Deuring, R., McCallum, C. M., Peterson, C. L. & Tamkun, J. W. Identification and characterization of *Drosophila* relatives of the yeast transcriptional activator SNF2/SWI2. *Mol. Cell. Biol.* **14,** 2225–2234 (1994).
9. Becker, P. B. & Wu, C. Cell-free system for assembly of transcriptionally repressed chromatin from *Drosophila* embryos. *Mol. Cell. Biol.* **12,** 2241–2249 (1992).
10. Becker, P. B., Tsukiyama, T. & Wu, C. Chromatin assembly extracts from *Drosophila* embryos. *Meth. Cell Biol.* **44,** 207–223 (1994).
11. Varga-Weisz, P. D., Blank, T. A. & Becker, P. B. Energy-dependent chromatin accessibility and nucleosome mobility in a cell-free system. *EMBO J.* **14,** 2209–2216 (1995).
12. Wilm, M. *et al.* Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379,** 466–469 (1996).
13. Mann, M. & Wilm, M. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Analyt. Chem.* **66,** 4390–4399 (1994).
14. Berger, J. M., Gamblin, S. J., Harrison, S. C. & Wang, J. C. Structure and mechanism of DNA topoisomerase II. *Nature* **379,** 225–232 (1996).
15. Wang, W. D. *et al.* Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.* **10,** 2117–2130 (1996).
16. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **65,** 635–692 (1996).
17. Almouzni, G. & Méchali, M. Assembly of spaced chromatin. Involvement of ATP and DNA topoisomerase activity. *EMBO J.* **7,** 4355–4365 (1988).
18. Walter, P. P., Owen-Hughes, T. A., Coté, J. & Workman, J. L. Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. *Mol. Cell. Biol.* **15,** 6178–6187 (1995).
19. Chen, G. L. *et al.* Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259,** 13560–13566 (1984).
20. Buchenau, P., Saumweber, H. & Arndt, J. D. Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by *in vivo* confocal laser scanning microscopy. *J. Cell Sci.* **104,** 1175–1185 (1993).
21. Poljak, L. & Käs, E. Resolving the role of topoisomerase II in chromatin structure and function. *Trends Cell Biol.* **5,** 348–354 (1995).
22. Wartburton, P. E. & Earnshaw, W. C. Untangling the role of DNA topoisomerase II in mitotic chromosome structure and function. *BioAssays* **19,** 97–99 (1997).
23. Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck, M. M. & Liu, L. F. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* **100,** 1706–1715 (1985).
24. Gasser, S. M., Laroche, T., Falquet, J., Boy de la Tour, E. & Laemmli, U. K. Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* **188,** 613–629 (1986).
25. Swedlow, J. R., Sedat, J. W. & Agard, D. A. Multiple chromosomal populations of topoisomerase II detected *in vivo* by time-lapse, three-dimensional wide-field microscopy. *Cell* **73,** 97–108 (1993).
26. Sandaltzopoulos, R., Mitchelmore, C., Bonte, E., Wall, G. & Becker, P. B. Dual regulation of the *Drosophila hsp26* promoter *in vitro*. *Nucleic Acids Res.* **23,** 2479–2487 (1995).
27. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analyt. Chem.* **68,** 850–858 (1996).
28. Wilm, M. & Mann, M. Analytical properties of the nanoelectrospray ion source. *Analyt. Chem.* **68,** 1–8 (1996).
29. Wall, G., Varga-Weisz, P. D., Sandaltzopoulos, R. & Becker, P. B. Chromatin remodelling by GAGA factor and heat shock factor at the hypersensitive *Drosophila hsp26* promoter *in vitro*. *EMBO J.* **14,** 1727–1736 (1995).

Acknowledgements. This research was supported by an EMBO fellowship to P.D.V. and by a grant for the DFG to P.B.B. We thank T. Tsukiyama and C. Wu for a generous gift of NURF fractions and ISWI antibodies, S. Beek and J. Tamkun for antibodies against ISWI, P. Fisher and D. Arndt-Jovin for antibodies against topo II; A. Shevchenko for technical help, H. Stunnenberg for important suggestions and discussions, T. Blank and C. Garcia-Jimenez for introduction to chromatography and P. Riedinger for artwork.

Correspondence and requests for materials should be addressed to P.B.B. (e-mail: becker@embl-heidelberg.de).

erratum

Modelling teleconnections between the North Atlantic and North Pacific during the Younger Dryas

Uwe Mikolajewicz, Thomas J. Crowley, Andreas Schiller & Reinhard Voss

Nature **387,** 384–387 (1997)

In Figs 1 and 3b, the numbering was very faint; also, shading was lost from Fig. 4. The corrected figures 1, 3b and 4 are shown here. □

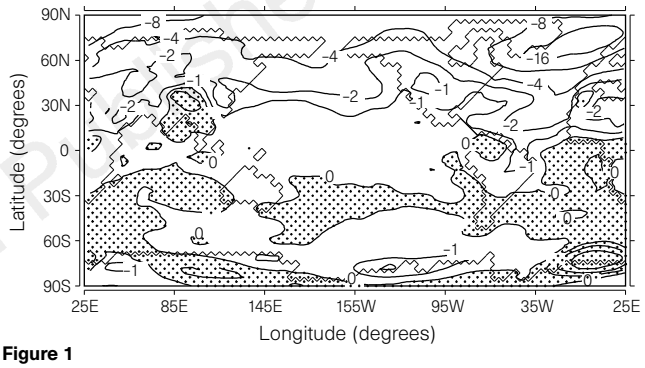

Figure 1

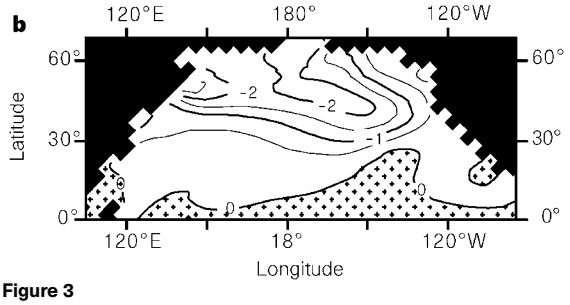

Figure 3

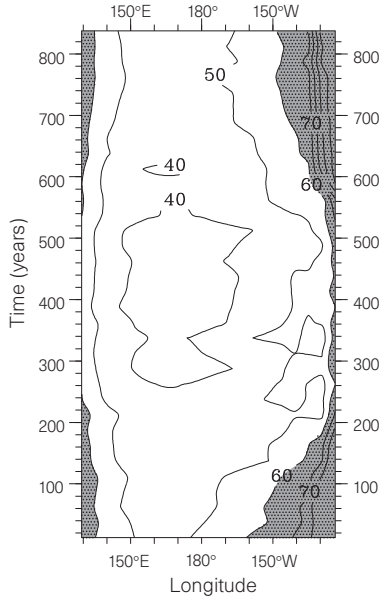

Figure 4

resulted in the protein family shown in Fig. 1c. The highest-scoring non-related sequences after those shown consisted of *trithorax* family members that reached error probabilities of $P > 0.5$ in all of the profile iterations.

Immunocytochemistry. Antibodies were raised in rabbits against the purified GST fusion proteins encoded by pGEX-RIM-52 and pGEX-Rim-PDZ. Double and single immunofluorescence labelling of cryostat sections from rat spinal cord and bovine retinae was performed[27] with two independent polyclonal Rim antibodies and multiple monoclonal antibodies to synaptic vesicle proteins. Staining was visualized by Cy2- and Cy3-conjugated secondary antibodies and viewed in a BioRad MRC1024 confocal microscope. Immuno-electron microscopy was performed by a pre-embedding protocol with silver enhancement[27]. Controls for all immunocytochemistry experiments included the use of two independent antibodies, control stains with other antibodies, and experiments in which the first antibody was omitted.

PC12 cell transfections. PC12 cells (ATCC) were plated in collagen-coated 6-well dishes with 10^6 cells per well. Cells were transfected on day 1 with 0.2 μg of Qiagen-purified pCMV5-hGH encoding human growth hormone and 1 μg of the indicated expression plasmids using Lipofectamine (Life Technologies). Expression plasmids encoded the light chains of wild-type and mutant tetanus toxin in pCMV5 (ref. 28), LDL receptor and Rab3A in pCMV5, and residues 1–399 of Rim in pME18sf(−). On day 3, cells were collected, washed, and split into two portions, one of which was incubated for 20 min at 37 °C in control buffer (in mM) (145 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose, 0.5 ascorbate, 20 HEPES-NaOH, pH 7.4) and the other was incubated in the same buffer containing 56 KCl and 95 NaCl. After incubation, growth hormone in the medium and the cells was determined by radioimmunoassay (Nichols Institute). Secretion was calculated as the percentage growth hormone released as a function of stimulation. All experiments were done in triplicate at least three times.

Rim affinity chromatography. Glutathione–agarose columns without protein, with GST–Rim fusion protein encoded by pGEX-Rim-52, or with various control GST fusion proteins, were reacted with total rat brain homogenate prepared in 0.5% Triton X-100, 1 mM EDTA, 0.1 M NaCl, 0.1 g l^{-1} PMSF, and 50 mM HEPES-NaOH, pH 7.4, with either 0.5 mM GDP-βS or GTP-γS at 4 °C overnight. Samples were washed 5 times in the same buffer without nucleotides before analysis by SDS–PAGE and immunoblotting.

Other procedures. RNA blotting experiments were done using multiple tissue blots (Clontech). SDS–PAGE and immunoblotting were performed using standard procedures and antibodies described previously[1,12,28,29]. Signals were detected by ECL and quantified by ^{125}I-labelled secondary antibodies. Protein assays were performed with the BioRad kit. Subcellular fractionations were performed as described[29] and verified by immunoblotting against a series of synaptic-vesicle, plasma-membrane and cytosolic antigens.

Received 19 February; accepted 22 May 1997.

1. Geppert, M. *et al.* The role of Rab3A in neurotransmitter release. *Nature* **369,** 493–497 (1994).
2. Holz, R. W., Brondyk, W. H., Senter, R. A., Kuizon, L. & Macara, I. G. Evidence for the involvement of Rab3A in Ca^{2+}-dependent exocytosis from adrenal chromaffin cells. *J. Biol. Chem.* **269,** 10229–10234 (1994).
3. Johannes, L. *et al.* The GTPase Rab3a negatively controls calcium-dependent exocytosis in neuroendrocrine cels. *EMBO J.* **13,** 2029–2037 (1994).
4. Geppert, M., Goda, Y., Stevens, C. F. & Südhof, T. C. Rab3A regulates a late step in synaptic vesicle fusion. *Nature* **387,** 810–814 (1997).
5. Castillo, P. E., Janz, R., Südhof, T. C., Tzounopoulos, T., Malenka, R. C. & Nicoll, R. A. Rab3A is essential for mossy fibre long-term potentiation in the hippocampus. *Nature* **388,** 590–593 (1997).
6. Burstein, E. S., Brondyk, W. H., Macara, I. G., Kaibuchi, K. & Takai, Y. Regulation of the GTPase cycle of the neuronally expressed Ras-like GTP-bindign protein Rab3A. *J. Biol. Chem.* **268,** 22247–22250 (1993).
7. Fischer von Mollard, G., Stahl, B., Khokhlatchev, A., Südhof, T. C. & Jahn, R. Rab3C is a synaptic vesicle protein that dissociates from synaptic vesicles after stimulation of exocytosis. *J. Biol. Chem.* **269,** 10971–10974 (1994).
8. Südhof, T. C. Function of Rab3A GDP/GTP exchange. *Neuron* **18,** 519–522 (1997).
9. Bourne, H. R., Sanders, D. A. & McCormick, F. The GTPase superfamily: a conserved switch for diverse cel functions. *Nature* **348,** 125–132 (1990).
10. Nuoffer, C. & Balch, W. E. GTPases: multifunctional molecular switches regulating vesicular traffic. *Annu. Rev. Biochem.* **63,** 949–990 (1994).
11. Shirataki, H. *et al.* Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell. Biol.* **13,** 2061–2068 (1993).
12. Li, C. *et al.* Synaptic targeting of rabphilin-3A, a synaptic vesicle Ca^{2+}/phospholipid-binding protein, depends on rab3A/3C. *Neuron* **13,** 885–898 (1994).
13. Stahl, B., Chou, J. H., Li, C., Südhof, T. C. & Jahn, R. Rab3 reversibly recruits rabphilin to synaptic vesicles by a mechanism analogous to raf recruitment by ras. *EMBO J.* **15,** 1799–1809 (1996).
14. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. Mammalian ras interacts directly with the serin/threonine kinase raf. *Cell* **74,** 205–214 (1993).
15. Südhof, T. C. & Rizo, J. Synaptotagmins: C_2-domain proteins that regulate membrane traffic. *Neuron* **17,** 379–388 (1996).
16. Weisman, L. S. & Wickner, W. Molecular characterization of VAC1, a gene required for vacuole inheritance and vacuole protein sorting. *J. Biol. Chem.* **267,** 618–623 (1992).
17. Yamamoto, A. *et al.* Novel PI(4)P 5-kinase homologue, Fablp, essential for normal vacuole function and morphology in yeast. *Mol. Biol. Cell* **6,** 525–539 (1995).
18. Bean, A. J., Seifert, R., Chen, Y. A., Sacks, R. & Scheller, R. H. Hrs-2 is an ATPase implicated in Ca^{2+}-regulated secretion. *Nature* **385,** 826–829 (1997).
19. Sheng, M. PDZs and receptor/channel clustering: rounding up the latest suspects. *Neuron* **17,** 575–578 (1996).
20. Dowling, J. E. *The Retina. An Approachable Part of the Brain* (Belknap, Cambridge, MA, 1987).
21. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1989).
22. Hata, Y. & Südhof, T. C. A novel ubiquitous form of munc18 interacts with multiple syntaxins. *J. Biol. Chem.* **270,** 13022–13028 (1991).
23. Guan, K. L. & Dixon, J. E. Eukaryotic proteins expressed in Escherischia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192,** 262–267 (1991).
24. Bucher, P., Karplus, K., Moeri, N. & Hofmann, K. A flexible motif search technique based on generalized profiles. *Comput. Chem.* **20,** 3–23 (1996).
25. Henikoff, S. & Henikoff, J. G. Amino acid substitution matrices from protein blocks. *Proc. Natl Acad. USA* **89,** 10915–10919 (1992).
26. Hofmann, K. & Bucher, P. The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. *Trends Biochem. Sci.* **20,** 347–349 (1995).
27. Schmitz, F., Bechmann, M. & Drenckhahn, D. Purification of synaptic ribbons, structural components of the photoreceptor active zone complex. *J. Neurosci.* **15,** 7109–7116 (1996).
28. McMahon, H. *et al.* Cellubrevin: a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* **364,** 346–349 (1993).
29. Ichtchenko, K. *et al.* Neuroligin 1: A splice-site specific ligand for β-neurexins. *Cell* **81,** 435–443 (1995).
30. Matsui, Y. *et al.* Nucleotide and deduced amino acid sequences of a GTP-binding protein family with molecular weights of 25,000 from bovine brain. *J. Biol. Chem.* **263,** 11071–11074 (1988).

Acknowledgements. We thank R. Janz, R. Jahn, M. Zerial and S. Butz for reagents, and J. L. Goldstein and M. S. Brown for advice and support. This work was partially supported by a grant from the HFSP.

Correspondence and requests for materials should be addressed to T.C.S. (e-mail: tsudho@mednet.swmed.edu).

Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II

Patrick D. Varga-Weisz, Matthias Wilm, Edgar Bonte, Katia Dumas, Matthias Mann & Peter B. Becker

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

Repressive chromatin structures need to be unravelled to allow DNA-binding proteins access to their target sequences. This de-repression constitutes an important point at which transcription and presumably other nuclear processes can be regulated[1,2]. Energy-consuming enzyme complexes that facilitate the interaction of transcription factors with chromatin by modifying nucleosome structure are involved in this regulation[3–5]. One such factor, nucleosome-remodelling factor (NURF), has been isolated from *Drosophila* embryo extracts[4,6,7]. We have now identified a chromatin-accessibility complex (CHRAC) which uses energy to increase the general accessibility of DNA in chromatin. However, unlike other known chromatin remodelling factors, CHRAC can also function during chromatin assembly: it uses ATP to convert irregular chromatin into a regular array of nucleosomes with even spacing. CHRAC combines enzymes that modulate nucleosome structure and DNA topology. Using mass spectrometry, we identified two of the five CHRAC subunits as the ATPase ISWI, which is also part of NURF[6,8], and topoisomerase II. The presence of ISWI in different contexts suggests that chromatin remodelling machines have a modular nature and that ISWI has a central role in different chromatin remodelling reactions.

Chromatin reconstituted in the cell-free assembly system from *Drosophila* embryos[9,10] is maintained in a dynamic state characterized by increased nucleosome mobility and overall DNA accessibility in the presence of ATP[11]. Using ATP-dependent endonuclease cleavage of chromatin to assay for DNA accessibility[11], we purified and characterized a new type of chromatin remodelling

machine, CHRAC. CHRAC activity is sensitive to the detergent Sarkosyl (Fig. 1a). Addition of ATP to Sarkosyl-washed chromatin does not increase the accessibility of DNA (Fig. 1b, lanes 3–6); however, when CHRAC-containing fractions are added, DNA in chromatin becomes accessible to restriction enzymes (Fig. 1b, lane 7). Using this assay, we purified CHRAC from nuclear embryo extracts to apparent homogeneity (Fig. 2). On a gel filtration column, the purified activity ran as a complex with an apparent relative molecular mass of 670,000 (M_r 670K) (Fig. 2a). Consistently, proteins of 175K, 160K (which stains more strongly) and 130K, and two small proteins of M_r ~18K and 20K copurified with CHRAC activity with roughly equal stoichiometry (Fig. 2b).

CHRAC increased the accessibility of DNA in chromatin, but not of free DNA, in an energy-dependent manner. DNA in chromatin may be rendered accessible by, for example, alteration of the nucleosome core structure, loss of linker-DNA-binding proteins or of other chromatin-associated proteins, or by unfolding of the nucleosomal fibre. To test whether CHRAC activity required any factors other than nucleosomes, we reconstituted nucleosomes from pure histones on long linear DNA by salt-gradient dialysis. Addition of CHRAC and hydrolysable ATP was necessary and sufficient to increase the accessibility of the nucleosomal DNA to the restriction endonuclease *Dra*I (Fig. 3). As in the crude assembly system[11], only ATP and dATP supported CHRAC activity, whereas other nucleotides were inactive. This result identifies the nucleosome core particle as a target for CHRAC action.

The band corresponding to the 130K subunit was cut from a silver-stained SDS–polyacrylamide gel, cleaved in the gel by trypsin, and analysed by nano-electrospray mass spectrometry[12]. Tandem mass spectrometry of the tryptic cleavage products and computer-assisted sequence interpretation through database searches with peptide sequence tags[13] led to the unambiguous identification of the 130K subunit as the known ATPase, ISWI[6,8] (see Methods).

The 160K subunit was also analysed by mass spectrometry. Nine tryptic peptides were sequenced and the enzyme topoisomerase II (topo II; see Methods) was unambiguously identified. Both ISWI and topo II cofractionated precisely with CHRAC, as shown by western blotting, and topo II was active, as revealed by its ATP-dependent relaxation of negative supercoils (data not shown). By co-immunoprecipitation from crude CHRAC-containing fractions, we confirmed that topo II and ISWI were located in the same complex (Fig. 4). Topoisomerase II functions as a dimer[14], which is consistent with the staining of the 160K topo II band in CHRAC being stronger than the other subunits (Fig. 2). Assuming that two molecules of topo II are present, the relative molecular masses of the individual subunits add up to the observed M_r of 670K.

ISWI is also a subunit of NURF, another nucleosome-remodelling complex that facilitates the binding of transcription factors to chromatin. No other subunit is shared by the complexes[4,6]. Two hSNF2L(the human ISWI homologue)-containing complexes of ~700K and 450K have been identified[15], corresponding to the M_r determined here for CHRAC and with that of NURF[4], suggesting the existence of human counterparts for both ISWI-containing complexes.

The ATPase activity of NURF is stimulated by nucleosomal but not free DNA[6]. By contrast, the ATPase activity of topo II is stimulated by free DNA[16]. Comparison of the ATPase activities of CHRAC with that of purified *Drosophila* topoisomerase II (Fig. 5)

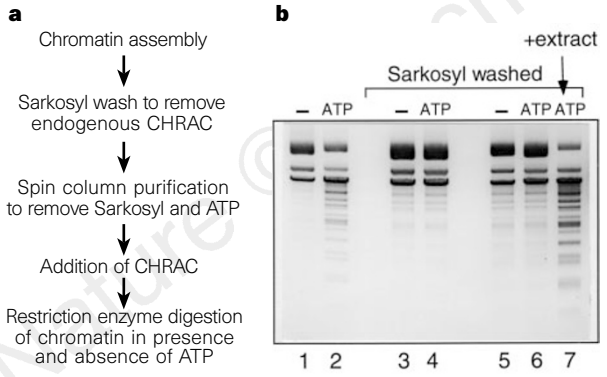

Figure 1 CHRAC assay. **a**, Scheme of the assay. **b**, Lanes: 1, 2, endogenous CHRAC activity revealed by enhanced *Dra*I cleavage of reconstituted chromatin in the presence of ATP; 3–6, Sarkosyl-stripped chromatin becomes more accessible in general, but lacks the ATP-dependent opening; 7, addition of 1 μl CHRAC-containing extract to stripped chromatin reconstitutes ATP-dependent endonuclease accessibility. Chromatin in lanes 3, 4 was washed with 0.075% Sarkosyl, chromatin in lanes 5–7 with 0.15% Sarkosyl. A negative image of the ethidium bromide-stained agarose gel is shown.

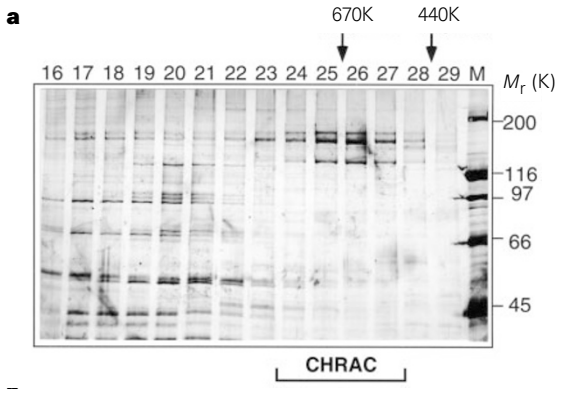

Figure 2 Purification of CHRAC. **a**, 8% SDS–PAGE of fractions from the Superose-6 gel filtration column, calibrated with thyroglobulin (M_r 670K) and ferritin (440K) size standards (Pharmacia). CHRAC activity (''CHRAC'') peaks in fractions 25 and 26. **b**, Purified CHRAC (CHR) was resolved by 8% and 15% SDS–PAGE and stained with silver. M: size markers (BioRad) with indicated relative molecular masses in thousands. Asterisks: small proteins that co-fractionate with CHRAC activity. These proteins run with histone markers on other gels.

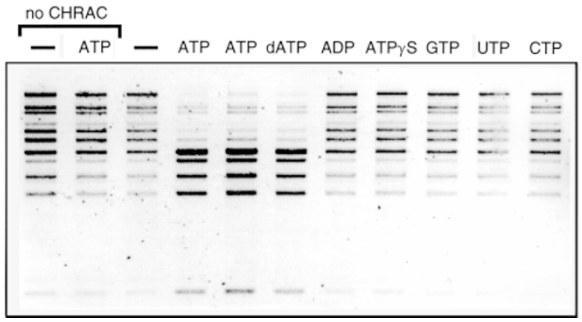

Figure 3 CHRAC targets nucleosomes. Nucleosomes were reconstituted on linear DNA from *Drosophila* histones by salt-gradient dialysis. 28 ng of fraction 25 (Fig. 2) or buffer (no CHRAC) were added to ~80 ng DNA reconstituted into chromatin in EX120 in the presence of 200 μg ml^{-1} BSA and the indicated nucleotides at 1 mM. After *Dra*I digestion, the purified DNA was separated on an agarose gel. A negative image of the ethidium bromide-stained gel is shown.

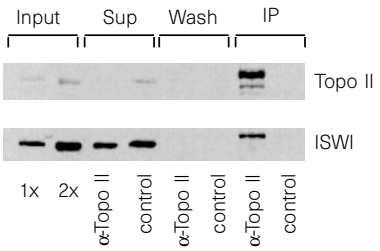

Figure 4 ISWI and topo II co-immunoprecipitate from the crude first CM-Sepharose eluate. Topo II was immunoprecipitated with a protein A–Sepharose-purified rabbit polyclonal antibody (α-topoII) or a nonspecific control antibody. 10% (1×) and 20% (2×) of the input material, 10% of the supernatant (sup), 20% of the final wash and the entire immunoprecipitate (IP) was subjected to consecutive western analysis with an antibody against ISWI and topo II.

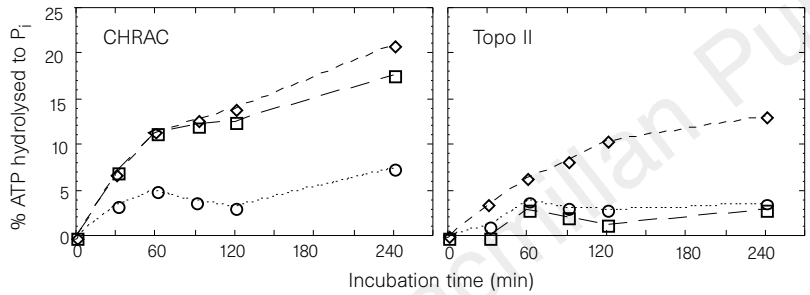

Figure 5 ATPase activities of purified CHRAC and topoisomerase II. The ATPase activity of roughly equivalent amounts (with respect to topoisomerase activity) of CHRAC (left) and purified topoisomerase II (right) were assayed in the presence of control buffer (circles), free DNA (diamonds) and nucleosomes (squares). The percentage of free phosphate was determined at the indicated reaction times.

showed that topoisomerase II was only stimulated by free DNA as well as by nucleosomes, whereas CHRAC was stimulated by free DNA as well as by nucleosomes. We assume that the nucleosome-stimulated ATPase activity in CHRAC is due to ISWI, which is common to both CHRAC and NURF.

CHRAC and NURF increase the accessibility of chromatin by different mechanisms. CHRAC and NURF preparations, standardized by their content of immunoreactive ISWI, had different activities when compared in accessibility assays: CHRAC was less effective in the GAGA-factor-dependent nucleosome remodelling assay than NURF; conversely, NURF did not stimulate the sensitivity to restriction endonucleases (data not shown). NURF perturbs histone–DNA interactions in mononucleosomes when added at a stoichiometry of one NURF per 20 nucleosome core particles[4]. The SWI/SNF and RSC complexes also perturb histone–DNA interactions, but require higher stoichiometries[1,5]. So far, we have not observed a similar effect when incubating equimolar amounts of CHRAC and mononucleosomes under comparable experimental conditions (unpublished observations).

The most striking difference between CHRAC and NURF, however, was in their effect on nucleosomal arrays. The assembly of nucleosomes is rapid in the *Drosophila* and related *Xenopus* systems in the absence of ATP, but correct nucleosome spacing requires time and energy[9,17]. The ATP-dependent spacing principle has so far remained elusive. Addition of CHRAC to chromatin assembled in the absence of ATP, and therefore lacking regular nucleosome spacing, resulted in an ATP-dependent alignment of nucleosomes which became evident from the new regularity of the micrococcal nuclease cleavage pattern (Fig. 6a). This result identifies CHRAC as an ATP-dependent nucleosome-spacing factor. NURF has the opposite effect: addition of large amounts of NURF to a nucleosomal array perturbs the regular repeat pattern[4]. The side-by-side comparison of NURF and CHRAC (standardized by their ISWI content) in our nucleosome-spacing assay demonstrated the difference between the two activities (Fig. 6b). Irregular chromatin obtained by only brief chromatin assembly was converted into a regular nucleosomal array in an ATP-dependent reaction by CHRAC, but not by NURF.

We propose two non-exclusive mechanisms by which CHRAC may mediate these two apparently opposing effects of increasing the accessibility of nucleosomal DNA and of imposing regularity on nucleosomal arrays. CHRAC might increase the mobility of nucleosomes with the expenditure of energy, a reaction that occurs in a cell-free system[11]. Enhanced nucleosome mobility may increase the chance of transient exposure of a particular sequence in accessible linker regions and at the same time facilitate the alignment of nucleosomes into a regular repeat structure. Alternatively, CHRAC may participate in nucleosome assembly and disassembly, like the histone chaperones which, depending on the circumstance, may function either as histone donors in nucleosome assembly or as histone acceptors during disassembly of nucleosome cores[18]. The identification of the ATPase ISWI in two structurally and functionally distinct chromatin remodelling complexes highlights the modular nature of these complexes and suggests that ISWI may drive diverse nucleosome remodelling reactions specified by different interaction partners. ISWI, being found in both NURF and CHRAC, may be directly involved in targeting and rearranging nucleosome structure.

The association of ISWI and topo II in one complex is interesting. The topoisomerase activity (DNA-strand breakage and religation) does not seem to be involved in CHRAC function: VP16, a specific inhibitor of topo II (ref. 19), did not prevent CHRAC from

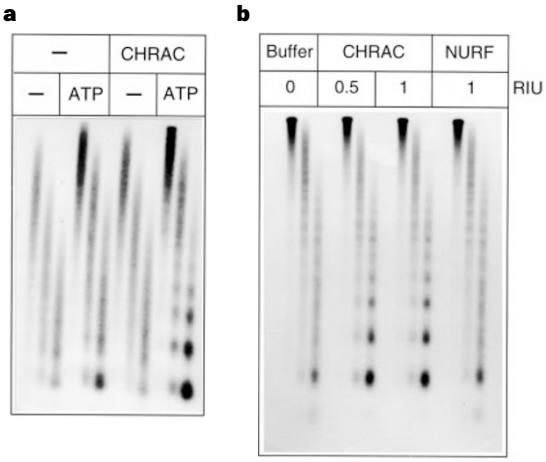

Figure 6 a, CHRAC is an ATP-dependent spacing factor. Chromatin was assembled without ATP, Sarkosyl-stripped to remove endogenous CHRAC, and the effect of addition of ATP and/or CHRAC on the regularity of the nucleosomal array was studied by micrococcal nuclease analysis as described in Methods. **b**, CHRAC and NURF are functionally distinct. Equivalent amounts of CHRAC and NURF (expressed as relative ISWI units, RIU) were compared for their ability to create a regular nucleosomal array.

increasing chromatin accessibility to restriction enzymes, and only slightly altered its effect on nucleosome spacing (data not shown). Purified topo II could not substitute for CHRAC in either of these reactions. The physiological importance of the association of ISWI and topoisomerase II in one complex will only become apparent once further CHRAC subunits have been identified. Nucleosome remodelling by ISWI may help the interaction of topo II with chromatin and thus enhance its function. Conversely, the association of topo II with chromosomes may localize CHRAC activity to specific chromosomal sites. Topoisomerase II is involved in many cellular processes such as the removal of catenates after replication, chromosome condensation and decondensation, kinetochore assembly and sister chromatid separation at mitosis (for reviews, see refs 16, 20–22); it is a major structure component of the mitotic chromosome axis[23,24] and is distributed widely over interphase chromosomes, but is also concentrated at specific heterochromatic sites[25]. Distinct populations of topo II with dynamic nuclear localization patterns during the cell cycle have been visualized[25]. The existence of topo II isoforms, cell-cycle-regulated post-translational modifications, and various associated proteins contribute to the structural and functional diversity of topo II[16]. The CHRAC-associated topo II represents only a fraction of the total topo II in a nucleus and may therefore correspond to a subpopulation dedicated to a specific function. We recently observed a stimulatory effect by CHRAC in a cell-free replication system: CHRAC, but not purified topo II, enhanced the initiation of replication from a nucleosomal SV40 origin (V. Alexiadis, P.D.V., P.B.B. and C. Gruss, manuscript in preparation). □

Methods

Nucleosome reconstitution and CHRAC assay. Chromatin was assembled in *Drosophila* embryo extracts as described[9–11]. After the assembly, 0.075% Sarkosyl (sodium lauroylsarcosine) was added to disrupt endogenous CHRAC activity. Sarkosyl and ATP were removed by gel filtration[11]. CHRAC assay: 10 μl Sarkosyl-washed chromatin (equivalent to ~40 ng DNA), 0.5–5 μl CHRAC-containing fractions, 10–50 units of *Dra*I or *Hinc*II and 1–2 mM ATP in 40 μl EX-120 buffer[11] were incubated for 30–60 min at 26 °C.

Nucleosome reconstitution by salt-gradient dialysis. 4 μg λ DNA, 2 μg *Drosophila* histones and 50 μg BSA (Fermentas) were mixed in 100 μl HSB (10 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 M NaCl) buffer. This mixture was dialysed in a microcollodion bag (Sartorius) against 600 ml HSB. During dialysis over 2 d at 4 °C, the salt was gradually removed by exchange of dialysis buffer with buffer lacking NaCl but containing 0.05% NP-40.

CHRAC purification. A comprehensive protocol is available upon request. CHRAC was purified from nuclear extracts of 12–16-h-old *Drosophila* embryos. Nuclei were extracted with ammonium sulphate[26] and extracts were dialysed into CB-150 buffer (CB: 10 mM HEPES-KOH, pH 7.6, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM EDTA, 0.5 mM sodium metabisulphite, 0.2 mM PMSF, 1 mM DTT; CB-150 contains 150 mM KCl) before precipitation with 20% methanol. All columns were equilibrated in CB-150 and loaded with fractions diluted to a conductivity equal to CB-150. Proteins were chromatographed on a CM–Sepharose FF column (all columns and resins from Pharmacia) and CHRAC activity was eluted with CB-400. Active fractions were rechromatographed on a smaller CM–Sepharose FF column from which the activity was eluted with CB-200. From the following Mono-Q HR 10/10 column, CHRAC eluted at a conductivity equal to CB-350. Successive concentrations involved a 1-ml CM–Sepharose FF column and a Filtron Microsep concentrator (M_r cutoff, 30K). The material was separated on a Superose-6 10/30 column in 20 mM HEPES-KOH, pH 7.6, 400 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 20% glycerol. If necessary, a final fractionation on a Mono-Q 1/1 column was added from which CHRAC was eluted by a salt gradient.

Estimations of purification factor and yield are complicated by the presence of CHRAC inhibitors in crude nuclear extracts. As the purification after the CM-Sepharose step leads to a 200–400 fold enrichment of CHRAC activity, we estimate the overall degree of purification to be at least 1,000-fold. Unless stated otherwise, the CHRAC concentration in the experiments was 250 μg ml^{-1}, as determined by a modified Bradford assay (Biorad) with BSA as standard.

Mass spectrometry. Details of the analysis are available upon request. Proteins were proteolytically trypsinized in the gel[12,27]. Extracted peptides were purified on a 100 nl R2 Poros microcolumn and step-eluted in 1 μl 60% methanol/5% formic acid into a nanoelectrospray capillary. Mass spectrometry was done on an API III triple quadrupole instrument (PE-Sciex, Ontario) equipped with an upgraded collision cell and the nanoelectrospray ion source[28]. For the 130K subunit, the sequence tag (776.6)(I/L)EQ(1146.4) from the fragment spectrum of a peptide (M_r 1,877) identified uniquely the tryptic peptide comprising amino acids 736–748 of the ISWI protein in a non-redundant database (data not shown). Four other ISWI peptides were sequenced from the same digest corresponding to amino acids 160–171, 394–402, 450–458 and 638–648. From the tryptic peptide mixture of the 160K protein, 9 peptides (T1–T9) were sequenced which identified unambiguously *Drosophila* topoisomerase II (SwissProt P15348; T_1: ITFSPDLAK, T_2: DLYGVFPLR, T_3: SLAVSGLGVIGR, T_4: SGIVESVLAWAK, T_5: YIFTIMSPLTR, T_6: IFDEILVNAADNK, T_7: ELMWVYDNSQNR, T_8: SQLEHILLRPDSYIGSV EFTK, T_9: GFQQVSFVNSIATYK; data not shown).

ATPase assay. Nucleosomes were reconstituted on a 146-bp DNA fragment by salt-gradient dialysis at high histone input to ensure that no free DNA contaminated the preparation. The reaction (9 μl) contained: 60 ng DNA or the equivalent amount of mononucleosomes, 6.6 mM HEPES, pH 7.6, 0.66 mM EDTA, 0.66 mM 2-mercaptoethanol, 0.033% N-P40, 1.1 mM $MgCl_2$, 33 μM ATP, 5 μCi [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$, Amersham) and 14 ng CHRAC or 11.5 units topo II (USB) or CHRAC400 buffer. Unreacted ATP and free phosphate were separated by thin-layer chromatography and quantified by using PhosphoImager and Imagequant software.

Co-immunoprecipitation. Equal amounts of protein A–Sepharose-purified rabbit polyclonal antibody against topoisomerase II (W4-250-1, provided by D. Arndt-Jovin) or a control protein A–purified rabbit IgG were added to 10 μl of the first CM–Sepharose eluate. 5 μl IP-150 (20 mM HEPES, pH 7.6, 0.5 mM EGTA, 10% glycerol, protease inhibitor, 150 mM KCl, 5 mM $MgCl_2$), 1 μl (20 μg) chicken ovalbumin and 2 μl of 3 M KCl were added. This mixture was kept for 1 h on ice, added to protein A–Sepharose beads (10 μl settled volume) in 50 μl IP-400 (as IP-150, but containing 400 mM KCl, 1 mM $MgCl_2$, 50 μg ovalbumin and 0.1% N-P40) and incubated further for 2 h at 4 °C. Beads were washed twice with 50 μl IP-400, once with IP-150, and resuspended in 25 μl IP-150. 5 μl 6 × SDS loading buffer were added and the sample was

analysed by SDS–PAGE and western blotting. Rabbit anti-ISWI antibody was biotinylated using the Clontech Biotin-X-NHS ester labelling kit. Detection was by streptavidin–peroxidase polymer (Sigma) with an ECL detection kit (Amersham).

Nucleosome spacing. *Drosophila* embryo assembly extract was dialysed against EX120, 0.2 mM PMSF, 0.5 mM sodium metabisulphite to remove ATP. 60 μl of extract, 66 μl EX50, 14 μl 30 mM $MgCl_2$, 10 mM DTT, 600 mM NaCl and 1 μg plasmid DNA were mixed and incubated for 3 h at 26 °C. Chromatin was Sarkosyl-stripped and purified by gel filtration[7,11]. 20 μl of this chromatin (~70 ng DNA) plus 30 μl EX120 were incubated for 90 min with either 1 μl CHRAC or buffer in presence or absence of 2 mM ATP. The nucleosome repeat was revealed with an oligonucleotide hybridizing to a GAGA element of the *hsp26* promoter as described[7,29]. 0.1 and 0.2 μl CHRAC and 1 μl NURF P11 (gift from T. Tsukiyama and C. Wu) were assayed in the experiment shown in Fig. 6b. The CHRAC preparation contained 5× more ISWI protein than the NURF preparation.

Received 29 January; accepted 28 May 1997.

1. Kingston, R., Bunker, C. & Imbalzano, A. N. Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev.* **10,** 905–920 (1996).
2. Felsenfeld, G. Chromatin unfolds. *Cell* **86,** 13–19 (1996).
3. Peterson, C. L. & Tamkun, J. W. The SWI–SNF complex: a chromatin remodelling machine? *Trends Biochem. Sci.* **20,** 143–146 (1995).
4. Tsukiyama, T. & Wu, C. Purification and properties of an ATP-dependent nucleosome remodelling factor. *Cell* **83,** 1011–1020 (1995).
5. Cairns, B. R. *et al.* RSC, an essential, abundant chromatin-remodelling complex. *Cell* **87,** 1249–1260 (1996).
6. Tsukiyama, T., Daniel, C., Tamkun, J. & Wu, C. ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kD subunit of the nucleosome remodelling factor. *Cell* **83,** 1021–1026 (1995).
7. Tsukiyama, T., Becker, P. B. & Wu, C. ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. *Nature* **367,** 525–532 (1994).
8. Elfring, L. K., Deuring, R., McCallum, C. M., Peterson, C. L. & Tamkun, J. W. Identification and characterization of *Drosophila* relatives of the yeast transcriptional activator SNF2/SWI2. *Mol. Cell. Biol.* **14,** 2225–2234 (1994).
9. Becker, P. B. & Wu, C. Cell-free system for assembly of transcriptionally repressed chromatin from *Drosophila* embryos. *Mol. Cell. Biol.* **12,** 2241–2249 (1992).
10. Becker, P. B., Tsukiyama, T. & Wu, C. Chromatin assembly extracts from *Drosophila* embryos. *Meth. Cell Biol.* **44,** 207–223 (1994).
11. Varga-Weisz, P. D., Blank, T. A. & Becker, P. B. Energy-dependent chromatin accessibility and nucleosome mobility in a cell-free system. *EMBO J.* **14,** 2209–2216 (1995).
12. Wilm, M. *et al.* Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379,** 466–469 (1996).
13. Mann, M. & Wilm, M. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Analyt. Chem.* **66,** 4390–4399 (1994).
14. Berger, J. M., Gamblin, S. J., Harrison, S. C. & Wang, J. C. Structure and mechanism of DNA topoisomerase II. *Nature* **379,** 225–232 (1996).
15. Wang, W. D. *et al.* Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.* **10,** 2117–2130 (1996).
16. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **65,** 635–692 (1996).
17. Almouzni, G. & Méchali, M. Assembly of spaced chromatin. Involvement of ATP and DNA topoisomerase activity. *EMBO J.* **7,** 4355–4365 (1988).
18. Walter, P. P., Owen-Hughes, T. A., Coté, J. & Workman, J. L. Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. *Mol. Cell. Biol.* **15,** 6178–6187 (1995).
19. Chen, G. L. *et al.* Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259,** 13560–13566 (1984).
20. Buchenau, P., Saumweber, H. & Arndt, J. D. Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by *in vivo* confocal laser scanning microscopy. *J. Cell Sci.* **104,** 1175–1185 (1993).
21. Poljak, L. & Käs, E. Resolving the role of topoisomerase II in chromatin structure and function. *Trends Cell Biol.* **5,** 348–354 (1995).
22. Wartburton, P. E. & Earnshaw, W. C. Untangling the role of DNA topoisomerase II in mitotic chromosome structure and function. *BioAssays* **19,** 97–99 (1997).
23. Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck, M. M. & Liu, L. F. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* **100,** 1706–1715 (1985).
24. Gasser, S. M., Laroche, T., Falquet, J., Boy de la Tour, E. & Laemmli, U. K. Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* **188,** 613–629 (1986).
25. Swedlow, J. R., Sedat, J. W. & Agard, D. A. Multiple chromosomal populations of topoisomerase II detected *in vivo* by time-lapse, three-dimensional wide-field microscopy. *Cell* **73,** 97–108 (1993).
26. Sandaltzopoulos, R., Mitchelmore, C., Bonte, E., Wall, G. & Becker, P. B. Dual regulation of the *Drosophila hsp26* promoter *in vitro*. *Nucleic Acids Res.* **23,** 2479–2487 (1995).
27. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analyt. Chem.* **68,** 850–858 (1996).
28. Wilm, M. & Mann, M. Analytical properties of the nanoelectrospray ion source. *Analyt. Chem.* **68,** 1–8 (1996).
29. Wall, G., Varga-Weisz, P. D., Sandaltzopoulos, R. & Becker, P. B. Chromatin remodelling by GAGA factor and heat shock factor at the hypersensitive *Drosophila hsp26* promoter *in vitro*. *EMBO J.* **14,** 1727–1736 (1995).

Acknowledgements. This research was supported by an EMBO fellowship to P.D.V. and by a grant for the DFG to P.B.B. We thank T. Tsukiyama and C. Wu for a generous gift of NURF fractions and ISWI antibodies, S. Beek and J. Tamkun for antibodies against ISWI, P. Fisher and D. Arndt-Jovin for antibodies against topo II; A. Shevchenko for technical help, H. Stunnenberg for important suggestions and discussions, T. Blank and C. Garcia-Jimenez for introduction to chromatography and P. Riedinger for artwork.

Correspondence and requests for materials should be addressed to P.B.B. (e-mail: becker@embl-heidelberg.de).

erratum

Modelling teleconnections between the North Atlantic and North Pacific during the Younger Dryas

Uwe Mikolajewicz, Thomas J. Crowley, Andreas Schiller & Reinhard Voss

Nature **387,** 384–387 (1997)

In Figs 1 and 3b, the numbering was very faint; also, shading was lost from Fig. 4. The corrected figures 1, 3b and 4 are shown here. □

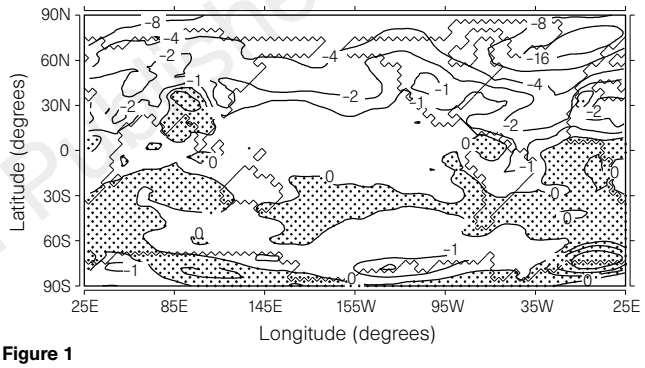

Figure 1

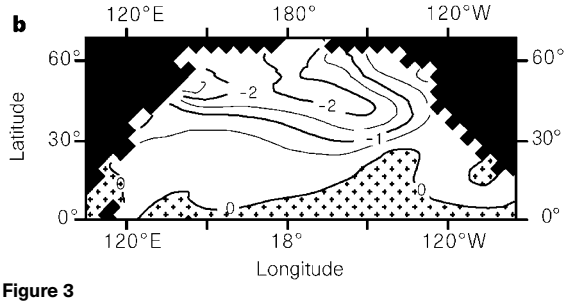

Figure 3

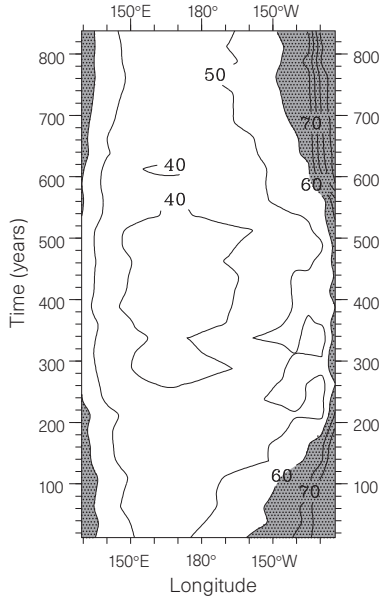

Figure 4