EFFECTS OF SULPHYDRYL REAGENTS ON THE STRUCTURE OF DEHISTONIZED METAPHASE CHROMOSOMES

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SUMMARY

Dehistonized metaphase chromosomes lose their apparent axial organization (the 'scaffold') and sediment more slowly following exposure to β-mercaptoethanol (BME). We have subsequently treated BME chromosomes with reagents that oxidize protein sulphydryls to disulphides, and found that if calcium is also present during the oxidation an apparently similar axial structure is restored following dehistonization, as seen by microscopic examination. In general, however, we do not find that oxidation restores the higher sedimentation rate of dehistonized control chromosomes. Analysis of residual core protein in dehistonized chromosomes by sodium dodecyl sulphate/polyacrylamide gel electrophoresis fails to detect any differences in polypeptide composition related to the state of oxidation or to the presence or absence of visible axial organization. Combining our results with those of other workers, we conclude that the axial structure evident in dehistonized metaphase chromosomes is maintained, at least partially, by inter-protein cross-linking, although in vivo this may not be via simple disulphide bridges. Additional factors, which we have not yet characterized, but which possibly include heavy metal ions, appear to be involved in the axial organization existing in vivo.

INTRODUCTION

The organization of chromatin in eukaryotic chromosomes is not static. A particularly striking change in the degree of chromatin condensation occurs during cell division, at both mitosis and meiosis, and it has been suggested that a possible mechanism for achieving this might involve the formation of interprotein disulphide bridges (e.g. see Dounce, Chanda & Townes, 1973). There is a body of biochemical and cytochemical evidence to show that the -SS-/SH ratio is higher in condensed chromatin, whether in interphase or at mitosis, although there are a number of conflicting reports in the literature also (for a review, see Sumner, 1983). The difficulty is in determining the in vivo oxidation state – sulphhydryl groups may become oxidized, or disulphide bridges reduced, in the process of sample preparation. During the course of our work on the structure of metaphase chromosomes, we have examined the effects of adding disulphide reducing agents, such as β-mercaptoethanol (BME) and dithiothreitol (DTT) to chromosomes isolated by a variety of procedures (e.g. see Jeppesen, Bankier & Sanders, 1978; Gooderham & Jeppesen, 1983), and have been unable to detect any alteration in

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morphology or other properties of native chromosomes, following exposure to these
agents (unpublished observations). It has been reported that reduction of disulphide
bonds leads to swelling in methanol:acetic-fixed chromosomes (Dounce et al. 1973;
Sumner, 1973), but acid fixation can be expected to have considerably altered the
state of the chromosomes under these conditions.

When metaphase chromosomes that have not been exposed to BME or DTT are
treated with 2m-NaCl to remove histones, as described before (Gooderham &
Jeppesen, 1983; Paulson & Laemmli, 1977), many of the resulting structures
appear, by both light and electron microscopy, as a 'halo' of DNA surrounding an
axial filament or 'scaffold', although some structures have a much more expanded
appearance (Gooderham & Jeppesen, 1983; Hadlaczky, Sumner & Ross, 1981). We
have shown that these axial regions not only have an apparently high concentration
of DNA, as indicated by their bright fluorescence with DNA fluorochromes such as
Hoechst '33258', but that they also contain the bulk of the non-histone protein
(NHP) that is resistant to extraction by 2m-NaCl (Gooderham & Jeppesen, 1983).
The NHP may be freed of over 99% of its associated DNA by nuclease digestion,
resulting in the structures we have called chromosome 'cores' (Jeppesen & Bankier,
1979). There is evidence from a number of sources of axial organization of
chromatin in metaphase chromosomes (Satya-Prakash, Hsu & Pathak, 1980;
Adolph, 1980), and we think it likely that the axial filaments in dehistonized
chromosomes, and hence chromosome cores, might retain the elements of this
higher-order structure, which we envisage as an interaction between NHP and
specific DNA sequences (Jeppesen et al. 1978). As we shall show later, dehis-
tonization of metaphase chromosomes that have been exposed to either BME or
DTT leads to structures that are all of a highly expanded form, with little
resemblance to chromosomes, and having no visibly identifiable axial organization.
It has also been reported that there is a reduction in sedimentation coefficient with a
loss of associated proteins in chromosome scaffolds prepared from BME-treated
chromosomes (Lewis & Laemmli, 1982).

These observations of the effects of apparently reducing disulphide bonds in
dehistonized mitotic chromosomes are remarkably similar to what would be
predicted by earlier models of chromosome structure, involving a central protein
core maintained by disulphide bridging (Dounce et al. 1973; Sobell, 1973). However,
Lewis & Laemmli (1982), based on observations with the chelating agents
α-phenanthroline (OP) and necuproine, have proposed that BME and DTT act not
by reducing disulphide bridges, but by chelating a metal ion contained as a
metalloprotein component of the chromosome scaffold, although their results do
implicate the involvement of protein sulphydryl groups. They have further
suggested that the metal chelated is copper, since they show that cupric ions are
capable of restoring a high sedimentation rate to dehistonized BME-treated
chromosomes. We report here the results of treating Chinese hamster metaphase
chromosomes isolated in the presence of BME with agents specifically selected to
oxidize free protein sulphhydryls to disulphide bonds, and compare these with the
effects of copper. We shall demonstrate that in the presence of calcium, both cupric
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ions and oxidizing agents are indistinguishable in reproducing axial filaments clearly visible in the light microscope following dehistonization, and morphologically closely resembling those observed in non-BME-treated dehistonized chromosomes. Since we detected no significant differences in polypeptide composition, we conclude that axial filaments are a consequence of protein cross-linking. Of the procedures we have investigated, however, only cupric ions lead to an apparent partial restoration of high sedimentation rate in dehistonized structures, although this is also produced in the absence of calcium, when axial filaments are not evident. The implications of these observations with reference to chromosome structure in vivo will be discussed.

MATERIALS AND METHODS

Metaphase chromosome isolation

CHO-K1 Chinese hamster ovary cells (ATCC no. CCL 61) were adapted for spinner culture, and grown in modified Eagle's Minimum Essential Medium for Suspension Cultures (Flow Laboratories), supplemented with 10% foetal calf serum, 30 μg/ml l-proline, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Mitotic enrichment was achieved by partial synchronization of cultures as follows: cells were grown to stationary phase, then diluted twofold with fresh medium; after 8 h incubation, colcemid (Fluka) was added to 0.1 μg/ml, and growth continued for a further 16 h. In this way, up to 50% of cells in metaphase were obtained. For tritium labelling of chromosomal DNA, [methyl-3H]thymidine (5 Ci/mmol, Amersham International) was added to the culture medium to a final activity of 0.5 μCi/ml at the time of twofold dilution.

Metaphase chromosomes were isolated by the 'physiological' procedure described previously (Gooderham & Jeppesen, 1983), with the following modifications. Approx. 2 x 10⁸ total cells were harvested by centrifugation at 1000 rev./min and resuspended in 2 x 10 ml samples of fresh culture medium. After 30 min on ice the cells were pelleted by centrifugation again, and trypsinized for 5 min at 37°C (Gooderham & Jeppesen, 1983). After washing each sample with a further 10 ml of fresh culture medium to inactivate trypsin activity, the cells were pelleted once more, and each sample was resuspended in 10 ml of 50 mM KCl, which for one sample contained 7 mM β-mercaptoethanol (BME). After 10 min of hypotonic treatment at 37°C, the swollen cells were cooled, collected by centrifugation at 1000 rev./min and resuspended in cold 120 mM-NaCl, 10 mM-Tris-HCl (pH 8), 2 mM-CaCl₂, again with the inclusion of 7 mM-BME to the previously similarly treated sample. Cell lysis was carried out after the addition of 0.1% (v/v) Triton X-100 by passing the cells through a hypodermic needle several times (Gooderham & Jeppesen, 1983). Excess calcium was removed with EDTA, and the chromosomes purified by the one-step 10% to 50% glycerol gradient centrifugation described before (Gooderham & Jeppesen, 1983). For this and all subsequent procedures, both samples ('BME chromosomes', and non-BME or 'standard' chromosomes) were treated identically in parallel, with no further exposure to BME. The chromosome peaks were collected from the glyceral gradients and dialysed against 120 mM-KCl, 20 mM-NaCl, 10 mM-Tris-HCl (pH 8), 0.1% (v/v) Triton X-100, (KCM). To minimize chance oxidation, all solutions were degassed under reduced pressure before use.

Although the proportion of interphase nuclei in the crude lysate from suspension cultures was much higher than obtained previously from monolayer 'wash off' mitotic cells (Gooderham & Jeppesen, 1983), nuclei pelleted at the bottom of the glyceral gradient, and the chromosome preparations described here appeared by both microscopic examination and polyacrylamide gel polypeptide analysis to be equally clean.

Treatment with oxidizing and other agents

Where required, after removing glyceral by dialysis, samples of standard and/or BME chromosomes were variously treated before dehistonization, as follows. The chromosomes...
suspended in KCM were incubated for 2 h on ice with the appropriate agent, with occasional gentle agitation to prevent chromosomes settling and aggregating. The final concentrations of agents used for these treatments were: 7 mM β-mercaptoethanol; 3 mM dithiothreitol; 10 mM EDTA; 3 mM o-phenanthroline; 0.1% (w/v) ammonium persulphate; 3 mM oxidized glutathione (GSSG); 0.1 mM CuSO₄; 0.01 mM–0.1 mM copper–OP complex (Cu(OP)₂). In addition, where indicated in the text, 1 mM-MgCl₂ or 1 mM-CaCl₂ were also included.

Dehistonization and preparation of core proteins

Following treatment, if appropriate, as described above, the chromosome suspension containing approximately 10 μg/ml DNA was extracted with 2 M NaCl to remove histones essentially as described previously (Gooderham & Jeppesen, 1983). After adding 2/3 vol. of 5 M NaCl, gently mixing, and standing for 30 min on ice, the dehistonized chromosomes were separated from dissociated protein by centrifugation through a solution containing 2 M NaCl, 2.5% (w/v) sucrose, 10 mM-Tris HCl (pH 8), 0.1% (v/v) Triton X-100 (NaCl/sucrose). To prepare core proteins for sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis, 20 ml of 2 M NaCl chromosome suspension was layered onto 10 ml of NaCl/sucrose supported on 0.3 ml of 0.6 M-Metrizamide (Nyegaard & Co., Oslo), 2 M NaCl, 10 mM-Tris HCl (pH 8), 0.1% (v/v) Triton X-100, centrifuged for 4 h at 3000 rev./min in a Sorvall AH 627 swingout rotor, and the dehistonized chromosomes collected from the Metrizamide/sucrose boundary with a Pasteur pipette (Gooderham & Jeppesen, 1983). After dialysis at 4°C for 16 h against 10 mM-Tris HCl (pH 8), 1 M-MgCl₂, 0.1% (v/v) Triton X-100 to remove salt, DNA was liberated from chromosome core protein by digesting on ice for 30 min with 10 μg/ml DNase I (Sigma). Enzyme activity was terminated by addition of EDTA to 10 mM, and core protein collected by centrifugation at 25 000 rev./min (approx. 50 000 × g), in a Sorvall AH 650 swingout rotor. SDS/polyacrylamide gel electrophoresis and sample preparation were as previously described (Gooderham & Jeppesen, 1983). Low molecular weight marker proteins were obtained from Pharmacia.

To follow the sedimentation properties of dehistonized ³H-labelled chromosomes, a smaller-scale version of the centrifugation procedure described above was used; 1 ml of 2 M NaCl chromosome suspension (activity 1 × 10⁶ to 2 × 10⁶ disintegrations/min) was layered onto 3.5 ml NaCl/sucrose, supported on 0.1 ml of 0.6 M-Metrizamide solution, contained in a 5.5 ml pollyallomer centrifuge tube. The tube was then centrifuged at 3000 rev./min for 3 h at 4°C in a Sorvall AH 650 rotor, after which 1 ml fractions were carefully removed from the top using a wide-bore Pasteur pipette. (Other methods of collecting fractions containing the viscous dehistonized material proved unsatisfactory.) Each total fraction was added to 2.5 ml Aquasol (New England Nuclear), shaken briefly to obtain a clear gel, and the radioactivity was measured by scintillation counting.

For preparation of microscopic specimens, 1 ml of NaCl/sucrose containing 4% (w/v) formaldehyde was added to a 15 mm × 50 mm flat-bottom glass vial holding a 13 mm circular glass coverslip; 0.1 ml of 2 M NaCl chromosome suspension was gently layered on top, and the dehistonized chromosomes were centrifuged onto the coverslip at 3000 rev./min for 5 min in a swing-out bench centrifuge. After allowing to fix by standing 30 min at room temperature, the coverslip was removed, rinsed in distilled water and stained with Hoechst 33258 as described before (Jeppesen et al. 1978).

RESULTS

The axial organization in non-BME-treated Chinese hamster metaphase chromosomes ('standard' chromosomes), following dissociation of histones with 2 M NaCl, is most clearly shown in the light microscope by using a DNA fluorochrome such as Hoechst 33258, as illustrated in Fig. 1A. The majority of the resulting structures have a typical appearance of a diffuse 'halo' of DNA surrounding a more brightly fluorescent axial filament. The protein-specific fluorochrome fluorescein isothiocyanate (FITC) also stains the NHP-rich axial filaments
Fig. 1. Abolition of visible chromosome 'cores' by β-mercaptoethanol. Dehistonized Chinese hamster metaphase chromosomes were stained with Hoechst 33258 and observed by blue-light fluorescence. A. Standard chromosome isolation procedure; B, chromosomes isolated in the presence of 7 mM-BME. approx. ×3000.
(Gooderham & Jeppesen, 1983), but their identification is more difficult without DNA halos to locate the dehistonized structures. Fig. 1B shows Hoechst 33258 fluorescence of dehistonized chromosomes isolated and treated exactly as those in Fig. 1A, except for the addition of 7 mM-BME during hypotonic swelling, and at the cell lysis stage ("BME chromosomes"). The subsequent glycerol gradient centrifugation, overnight dialysis versus KCM, and 2 M-NaCl histone dissociation did not contain BME. Although the DNA appears to be present as discrete packages, presumably corresponding to individual chromosomes, the structures are highly expanded, and axial filaments are no longer evident. If the same preparation is stained instead for protein with FITC, no fluorescence can be detected above background (unpublished observation).

As reported by others (Lewis & Laemmli, 1982), this change in morphology is accompanied by a reduction in sedimentation rate for the dehistonized structures. Fig. 2 shows the distribution of counts when Chinese hamster chromosomes labelled in vivo with [methyl-3H]thymidine are extracted with 2 M-NaCl and centrifuged at low speed through 2-5% sucrose supported on a 0-6 M-Metrizamide cushion, both solutions also including 2 mM-NaCl. A clear alteration in the sedimentation profiles of

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**Fig. 2.** Decrease in sedimentation rate of dehistonized metaphase chromosomes following exposure to BME. 3H-labelled Chinese hamster metaphase chromosomes were layered onto 2 M-NaCl/2.5% sucrose solution as described in the text, centrifuged for 3 h at 3000 rev./min and fractions (from the top) were assayed for radioactivity. A, Standard chromosome isolation procedure; B, chromosomes isolated in the presence of 7 mM-BME.

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**Fig. 3.** Restoration of morphological chromosome cores in dehistonized BME chromosomes by pre-treatment with cupric ions or oxidation, demonstrating co-requirement for calcium. Chinese hamster metaphase chromosomes isolated in the presence of BME, were treated with: A, 0.1 mM-CuSO4 alone; B, 0.1 mM-CuSO4 + 1 mM-CaCl2; C, 1 mM-CaCl2 alone; D, 3 mM-GSSG + 1 mM-CaCl2 (all treatments for 2 h on ice), before extracting with 2 M-NaCl, and staining with Hoechst 33258 as described for Fig. 1. approx. X3000.
Fig. 3
dehistonized chromosomes is evident following exposure to BME: standard chromosomes (Fig. 2A) sediment to the Metrizamide cushion, whereas BME chromosomes (Fig. 2B) fail to penetrate far into the 2.5% sucrose solution. Similar effects on the morphology and sedimentation rate of dehistonized chromosomes are produced if BME is added to standard chromosomes at any stage up to and including the 2M-NaCl extraction step; 3 mM-DTT and 3 mM-OP were also found to produce identical results to BME (not shown), but 10 mM-EDTA has no effect.

BME chromosomes were treated in a number of ways before extracting histones with 2M-NaCl, and the effects on the resulting dehistonized structures observed. Although we were primarily interested in alterations to the NHP axial filaments, we chose the DNA fluorochrome Hoechst 33258 for the morphological assay for the reasons noted above. Fig. 3 compares four pre-treatments. Incubation with 0.1 mM-CuSO4 alone for 2 h (Lewis & Laemmli, 1982) has no apparent effect on our preparations of BME chromosomes (Fig. 3A), but if 1 mM-CaCl2 is also included (Fig. 3B), a high proportion of dehistonized chromosomes exhibit brightly fluorescent axial filaments, similar to those seen after dehistonization of standard chromosomes (Fig. 1A); 1 mM-CaCl2 by itself has no effect (Fig. 3C). Magnesium ions may be substituted for calcium, but a lower proportion of dehistonized structures with axial filaments is subsequently obtained (not shown here).

If BME chromosomes are treated for 2 h with oxidized glutathione (GSSG) to oxidize free protein sulphydryls to disulphide bonds, axial filaments are also seen following extraction of histones with 2M-NaCl (Fig. 3D). As in the case of CuSO4 treatment described above, the presence of calcium (or less efficiently, magnesium) is also required. In addition to using GSSG, we have also oxidized BME chromosomes with the less-specific agent ammonium persulphate, and the complex formed between cupric ions and OP (Cu(OP)$_2$), which, in the presence of free oxygen, is specific for oxidizing sulphhydrlys to disulphide bonds (Kobashi, 1968). Both of these procedures, in the presence of calcium, also lead to dehistonized structures with axial filaments, similar to those of Fig. 3B and D (not illustrated). Of the procedures we have used to reproduce axial filaments following dehistonization of BME chromosomes, the most efficient are CuSO4/Ca, and Cu(OP)$_2$/O$_2$/Ca, the latter effective at a concentration of Cu(OP)$_2$ as low as 0.01 mM. Interestingly, if excess OP is used in the mixture with CuSO4, then axial filaments are not observed, which is consistent with the observation of Lewis & Laemmli (1982), and our own results, that OP treatment of chromosomes itself destroys evidence of axial structure following dehistonization. It should be stressed that the treatments described here to reverse the effects of BME are only effective if applied to BME chromosomes before dehistonization takes place. The loss of axial structure induced by BME appears non-reversible following dehistonization.

In order to see if the apparent reversal of the effects of BME on the morphology of dehistonized chromosomes is reflected in their sedimentation behaviour, 3H-labelled BME chromosomes variously treated as in Fig. 3A–D were extracted with 2M-NaCl and centrifuged in sucrose/Metrizamide as described above, and the distributions of 3H radioactivity were determined. The results are shown in Fig. 4A–D, respectively.
Fig. 4. Non-correlation of sedimentation rates with restoration of morphological cores in BME-treated dehistonized chromosomes. A-D. \(^{3}H\)-labelled Chinese hamster metaphase chromosomes prepared in the presence of BME were treated identically to those illustrated in Fig. 3A-D, and the sedimentation rates after dehistonization were compared, as described in the legend to Fig. 2.

Pretreatment with 0.1 mM-CuSO\(_{4}\), in both the absence and presence of calcium, leads to significantly changed sedimentation profiles (Fig. 4A and B, respectively) compared to untreated BME chromosomes (Fig. 2B), with large fractions of the \(^{3}H\) radioactivity being recovered from the Metrizamide cushion. Although the proportion sedimenting is somewhat variable from experiment to experiment, it is in
general greater when calcium is present during the pretreatment. However, the difference in sedimentation profile produced by the inclusion of calcium is very much less marked than the very clear difference in morphology that was noted above: namely, fluorescent axial filaments, when calcium is included. Treatment of BME chromosomes with calcium alone leads to a shoulder of slowly sedimenting radioactivity that just enters the sucrose solution (Fig. 4c), which is not seen with untreated BME chromosomes. A virtually identical profile is obtained if GSSG is included with the calcium (Fig. 4d), although as shown above, this latter treatment leads to retention of morphologically distinct axial fluorescence (Fig. 3d). Incubation with GSSG alone before dehistonization produces no observable change in sedimentation profile compared to untreated BME chromosomes (not shown). Extending the times of reaction with GSSG, with or without calcium, has no apparent further effect. We conclude, therefore, that there is very little correlation between the presence of visible axial filaments, and the sedimentation rate of dehistonized chromosomes. Although CuSO₄ appears to restore the sedimentation better than the other agents we have used, the effect is obtained even in the absence of visible axial structure, and may be due to a more compact conformation of DNA in the presence of copper ions. A similar, but lesser, effect appears also to be produced by calcium ions, leading to the shoulders seen in Fig. 4c and d.

These results suggest that the very significant difference in sedimentation profiles after dehistonization of standard chromosomes compared to BME-treated chromosomes (Fig. 2) is related to some property other than the presence or absence of a visible fluorescent core. In order to see if this property could be protein composition, polypeptide analyses of residual proteins from dehistonized standard and BME chromosomes were carried out by SDS/polyacrylamide gel electrophoresis. Previously (Gooderham & Jeppesen, 1983), this was achieved by first digesting dehistonized chromosomes with DNase I, collecting the resulting chromosome 'cores' by centrifugation at 10000 gav, and then preparing the pellet for electrophoresis. Although this procedure is satisfactory for dehistonized standard chromosomes, it leads to a very poor recovery of protein in the case of dehistonized BME chromosomes. CuSO₄/Ca and GSSG/Ca pretreatments lead to increased recoveries, roughly proportional to the fraction of dehistonized chromosomes exhibiting axial fluorescence. The yields of cores estimated visually by fluorescence microscopy of FITC-stained DNase I-digested dehistonized chromosomes (Gooderham & Jeppesen, 1983) also parallels the recovery of protein at 10000 gav, there being an almost total absence of cores having a characteristic metaphase morphology from untreated BME chromosomes. Carrying out the sedimentation of DNase I-digested material at 50000 gav instead, however, leads to efficient recovery of protein from all samples. These observations argue strongly that chromosome cores, which contain the bulk of the chromosomal proteins remaining after dehistonization of standard chromosomes, are held together by cross-linking, and that reaction with BME results in the dissociation of cores into smaller particles. Treatment of BME chromosomes with CuSO₄/Ca and GSSG/Ca appears to reintroduce a degree of cross-linking.
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Fig. 5. SDS/15% polyacrylamide gel electrophoresis of chromosome core polypeptides, stained with Coomassie Brilliant Blue. Lanes 1 and 8: molecular weight (×10^3) markers (phosphorylase b, 94000; albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 21000; a-lactalbumin, 14400). Lane 2: standard chromosome isolation (untreated). Lane 3: chromosomes isolated in the presence of BME. Lane 4: standard chromosomes, pre-treated with 0-1 mM-CuSO_4 + 1 mM-CaCl_2 before 2 M-NaCl extraction. Lane 5: BME chromosomes, pre-treated with 0-1 mM-CuSO_4 + 1 mM-CaCl_2. Lane 6: standard chromosomes, pre-treated with 3 mM-GSSG + 1 mM-CaCl_2. Lane 7: BME chromosomes, pre-treated with 3 mM-GSSG + 1 mM-CaCl_2. (All pre-treatments for 2 h on ice.) Annotations are referred to in the text.

SDS/polyacrylamide gel electrophoresis of the residual proteins of variously pretreated dehistonized chromosomes is shown in Fig. 5. The polypeptide compositions of Chinese hamster metaphase chromosome cores prepared as described here are more complex than those of HeLa scaffolds prepared from polyamine chromosomes, as described by Lewis & Laemmli (1982). Two major groups of core polypeptides, which we have reported previously (Gooderham & Jeppesen, 1983), migrating at approximately 70 and 50×10^3 M_\text{r} (lane 2, II and III, respectively), are found in dehistonized chromosomes derived in each of the ways described in the legend to Fig. 5. (The differences in overall yields evident in the electrophoreograms illustrated are not significant, and relate to losses incurred at various stages in handling the material.) The relative intensities of individual polypeptide bands vary somewhat within these groups, in an apparently non-systematic way: for example,
the band arrowed in lane 3 is relatively strong in lanes 3, 4 and 7, but weaker in lanes 2, 5 and 6. In some samples, a relatively strong actin band is found (A, lane 2) (Gooderham & Jeppesen, 1983), which seems to correlate with the presence of a band close to the origin (A', lane 2). It is possible that this latter band is myosin, which migrates in a similar position on one-dimension gels (unpublished observation), but further characterization is necessary to establish its identity. Actin, and the putative myosin, are strong contaminants of unpurified chromosomes, and collect mainly in fractions 1–3 during the glycerol gradient chromosome purification step. Presumably therefore, the presence of these bands in dehistonized chromosome preparations is mostly, if not wholly, accounted for by contamination. For reasons we cannot explain, in all cases where chromosomes were treated with CuSO₄ or GSSG in the presence of calcium before dehistonization (lanes 4–7) the yields of actin and the putative myosin are lower than from untreated chromosomes (lanes 2 and 3). This observation does indicate, however, that such treatments do not merely cause a random cross-linking of proteins present in the chromosome preparation to produce an artefactual insoluble protein core. Just below the position of the putative myosin is a group of polypeptides (I, lane 2), which is common to all samples of dehistonized chromosomes – again, the relative intensities of the individual components are somewhat variable. This region of the 15% gel is not well resolved, and molecular weights are not easy to estimate, but we would expect the major chromosome scaffold protein components Sc1 (170×10⁶ Mr) and Sc2 (135×10⁶ Mr) described by Lewis & Laemmli (1982) to migrate in this area, although we have not identified whether any of the group I polypeptides do indeed correspond to Sc1 or Sc2. Lewis & Laemmli (1982) reported a loss of these polypeptides in dehistonized chromosomes exposed to BME, which however are retained if the chromosomes are treated with copper before dehistonization. Fig. 5 fails to show exactly similar behaviour by any of the group I polypeptides, although we have noted one reproducible difference, comparing the protein profiles of differently prepared dehistonized chromosomes over several experiments, which may be related. An extra band is apparent just below the position of the putative myosin in BME chromosomes treated with CuSO₄/Ca before dehistonization (arrowed in lane 5). No similar bands are apparent in dehistonized standard chromosomes (lane 2), standard chromosomes treated with CuSO₄/Ca before dehistonization (lane 4), or in standard or BME chromosomes treated with GSSG/Ca (lanes 6 and 7).

DISCUSSION

Axial structure in dehistonized chromosomes

The most direct and striking evidence for the ‘scaffold’ model of chromosome structure is the visualization by both optical and electron microscopy of a residual NHP axial filament or core in dehistonized metaphase chromosomes (Paulson & Laemmli, 1977). As reported by others (Lewis & Laemmli, 1982), and clearly shown here, exposure to BME abolishes the morphological evidence for axial structure. We also show that nuclease digestion of dehistonized BME chromosomes
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does not liberate protein cores, recognizably derived from metaphase chromosomes, as we have described previously (Gooderham & Jeppesen, 1983). Instead, much smaller NHP particles requiring higher centrifugation speeds for sedimentation are obtained. Our interpretation of these results is that the NHP core in standard dehistonized chromosomes is held together by BME-labile cross-linking. Support for this interpretation is provided by our demonstration that axial structure, evident after dehistonization, and morphologically closely resembling that apparent in standard dehistonized chromosomes, can be simulated by oxidation of BME chromosomes in the presence of calcium ions, using conditions in which one would expect to convert free sulphydryl groups to disulphide bridges. These observations suggest that the chromosome core in standard dehistonized chromosomes might also be maintained by cross-linking between protein sulphydryls. We do not know why calcium or, less effectively, magnesium ions are required during oxidation to reproduce axial NHP filaments in dehistonized chromosomes, but to create the possibility of a disulphide bond forming two sulphydryl groups need to be in close proximity, and it may be that divalent cations play a role in aligning adjacent protein molecules so that intermolecular disulphide bonds can form.

Our results clearly fail to demonstrate a specific requirement for copper, or for any other heavy metal, in regaining morphological chromosome cores by oxidation of BME chromosomes. We have not found it practicable to eliminate atmospheric oxygen completely when treating BME chromosomes with copper (Lewis & Laemmli, 1982), and have therefore not attempted to do so. The morphological effects of copper appear very similar to the effects of the sulphydryl oxidizing agents we have used. In particular, free CuSO₄ is indistinguishable in its effects from the copper–OP complex, which specifically catalyses the oxidation of sulphydryls to disulphide bonds by O₂ (Kobashi, 1968), and it therefore seems likely that in the experiments described here copper is also exerting its effect on the morphology of dehistonized BME chromosomes by catalysing the oxidation of sulphydryls. Lewis & Laemmli (1982) have reported oxidation by copper in the presence of O₂, and further support for this interpretation is provided by the finding that, in common with the other oxidative procedures we have used, the presence of calcium is necessary for the recovery of NHP axial filaments.

Sedimentation properties

Although oxidation in the presence of calcium is apparently sufficient to reverse the effects of BME on the morphology of dehistonized chromosomes, in general we are unable to demonstrate a corresponding reversion in the sedimentation properties to those of standard dehistonized chromosomes. We have found that sedimentation rate is no indication of whether a particular pretreatment leads to a recovery of visible axial structure in dehistonized BME chromosomes. Thus although treatment with CuSO₄ appears to give a sizeable fraction of dehistonized material reverting to high sedimentation rate, there is no significant difference whether axial filaments are evident (CuSO₄ + calcium) or not (CuSO₄ alone). On the other hand, whereas axial structure is clearly evident after oxidation by GSSG in the presence of calcium followed by dehistonization, only a marginal increase in sedimentation rate is achieved, which also occurs with calcium ions alone when no axial structure can be
detected. These changes in sedimentation rate seem to be influenced by the nature of any divalent cations that may be present, rather than by the recovery of visible axial structure. The protein compositions following dehistonization of oxidized and non-oxidized BME chromosomes are not significantly different from standard dehistonized chromosomes, and our inability to reverse the change in sedimentation rate cannot be accounted for by an irreversible loss of protein. We conclude that there is some additional factor present in standard dehistonized chromosomes that we cannot detect by fluorescence microscopy.

The NHP chromosome core

It has been argued that chromosome cores or scaffolds observed in dehistonized chromosomes are artefacts produced by the random deposition of insoluble or poorly soluble NHP along the chromatid axes, which are regions of high DNA concentration (Okada & Comings, 1980). Against this view is the evidence for a specific subset of chromosomal proteins retained after 2M-NaCl extraction (Goodeham & Jeppesen, 1983; Lewis & Laemmli, 1982). In addition, the conclusion drawn here that interprotein cross-linking is required to maintain the integrity of NHP axial cores makes the explanation that such structure has an entirely artefactual origin less likely. Moreover, we can find no evidence that, following dehistonization, either the total amount of residual NHP or the pattern of polypeptide bands differ significantly whether chromosomes are in an oxidized or reduced state, exhibiting identifiable axial cores or not. If apparent axial structure were a result of non-specific deposition and cross-linking of chromosomal proteins, we would expect an increase in the total amount of protein left unsolubilized after 2M-NaCl extraction of oxidized chromosomes compared with BME-treated chromosomes. Loss of visible core structure is not due to solubilization of protein, but rather to a breakage of cross-linking between protein molecules, which nevertheless remain associated with the DNA. The demonstrated requirement for calcium during oxidation of BME chromosomes also suggests that a non-random cross-linking of sulphydryls is necessary in order to regain axial cores following dehistonization. As the solubilization of chromosome cores for electrophoresis requires the presence of sulphhydryl reducing agents such as BME or DTT, we are unable to investigate the degree of cross-linking directly on gels.

Cross-linking in vivo

It seems clear that the NHP axial structure observed following dehistonization of oxidized BME chromosomes is held together by disulphide cross-linking. The simplest extrapolation is that NHP cores evident in non-BME-treated dehistonized chromosomes are also maintained by disulphide bridging, and that this reflects in vivo metaphase chromosome structure. This interpretation is attractive in that it agrees with previous models of chromatin condensation and chromosome structure, as discussed in the Introduction. However, it does not easily explain why the heavy-metal chelating agents OP and the closely related neocuproine (2,9-dimethyl-o-phenanthroline) abolish axial structure (Lewis & Laemmli, 1982). Similar exposure to the much stronger chelator EDTA (as an example, the log
stability constants for EDTA and OP complexes with cupric ion are 18.8 and 6.3, respectively) has no effect, and it could be argued that OP (and neocuproine) might function as a reducing agent. We are unaware of this activity having been reported previously, although OP appears to act as a redox intermediate in a number of catalytic oxidations (Kobashi, 1968). Further doubt is cast on disulphide bridging in vivo, since NaBH₄, which would be expected to reduce disulphides to sulphydryls, does not abolish scaffolds (Lewis & Laemmli, 1982). An alternative model, which is entirely speculative but is consistent with most of the available data, is that in vivo cross-linking between NHP is via a dimercaptide of the form:

protein–S–M–S–protein,

where M is a metal ion. This link would be labile to chelators (including sulphydryl reagents) through removal of M, but not susceptible to pure reducing agents such as NaBH₄. Cross-linking could be re-introduced either by replacing M, or a metal with similar properties, or by oxidizing the now free sulphydryl pair, suitably aligned under the influence of calcium ions, as we have demonstrated. If this model is correct, replacing M should lead to complete reversal of the effects of BME both on the morphology and on the sedimentation rate of dehistonized chromosomes. Although, in the presence of calcium, copper (Lewis & Laemmli, 1982) appears to fulfil the requirements for M, the evidence is complicated by two observations. First, cupric ions can catalyse the oxidation of sulphydryls, and it is difficult to eliminate this possibility completely, even when precautions are taken. Second, we show that cupric ions have an effect on the sedimentation rate of dehistonized chromosomes in the absence of evident axial structure. Further clarification of these points is necessary.

To summarize our conclusions, we believe that the axial cores visible by fluorescence and electron microscopy in dehistonized chromosomes, are maintained by cross-linking between NHP. The cross-linking is labile to sulphydryl reagents and certain chelators, such as OP, leading to a loss of visible structure. Morphologically similar axial cores may be reproduced in BME-treated chromosomes if the free sulphydryls are oxidized to disulphides in the presence of calcium before dehistonization, although in general this does not lead to a return to the high sedimentation rate of non-BME-treated dehistonized chromosomes. Neither exposure of chromosomes to BME, nor subsequent oxidation, leads to significantly different polypeptide profiles following dehistonization. Taking into account the results of Lewis & Laemmli (1982), we suggest that cross-linking in vivo may occur via heavy-metal dimercaptide bridges linking the same sulphydryl groups. The metal involved could be copper (Lewis & Laemmli, 1982), although we have no direct evidence to support this. We hope in the future to determine which proteins present in the core are involved in cross-linking.

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