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Higher Order Metaphase Chromosome Structure: Evidence for Metalloprotein Interactions

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Summary

One level of DNA organization in metaphase chromosomes is brought about by a scaffolding structure that is stabilized by metalloprotein interactions. Fast-sedimenting, histone-depleted structures (4000–7000 S), derived from metaphase chromosomes by extraction of the histones, are dissociated by metal chelators or by thiol reagents. The chromosomal (scaffolding) proteins responsible for constraining the DNA in this fast-sedimenting form are solubilized under the same conditions. Chromosomes isolated in a metal-depleted form, which generate slow-sedimenting, histone-depleted structures, can be specifically and reversibly stabilized by Cu^{2+}, but not by Mn^{2+}, Co^{2+}, Zn^{2+} or Hg^{2+}. Metal-depleted chromosomes can also be stabilized by Ca^{2+} (at 37°C), but this effect is less specific than that of Cu^{2+}. The scaffolding protein pattern that is reproducibly generated following treatment with Cu^{2+} is composed primarily of two high molecular weight proteins—Sc1 and Sc2 (170,000 and 135,000 daltons). The identification of this simple protein pattern has depended upon the development of new chromosome isolation methods that are highly effective in eliminating cytoskeletal contamination.

Introduction

There is accumulating evidence that the higher order folding of the basic 250 Å chromatin fiber takes the form of loops of 50–100 kb that are constrained by a protein framework during metaphase (Adolph et al., 1977a, 1977b; Paulson and Laemmli, 1977) and interphase (Benyajati and Worcel, 1976; Cook and Brazell, 1976; Igo-Kemenes and Zachau, 1978; Adolph, 1980a; Vogelstein et al., 1980; Lebkowski and Laemmli, 1982a, 1982b). The organization of the chromatin fiber in metaphase chromosomes can be described by a radial loop model (Laemmli et al., 1978) in which chromatin loops emanate from a central axis formed by a network of nonhistone (scaffolding) proteins that link the bases of the loops. Sedimentation studies show that the metaphase DNA is compacted by nonhistone proteins (Adolph et al., 1977a), and electron microscopy of histone-depleted chromosomes reveals a halo of DNA loops converging on a central skeletal structure, referred to as the scaffold, which retains the overall metaphase morphology (Paulson and Laemmli, 1977). More recent electron micrographs of transverse, thin sections through swollen, but unextracted chromosomes show a star-like arrangement of the chromatin fiber, consistent with the proposed radial loop model (Marsden and Laemmli, 1979; Adolph, 1980b). A central longitudinal "core" or scaffold has also been observed in mitotic chromosomes by using silver staining and light microscopy (Howell and Hsu, 1979; Satya-Prakash et al., 1980). This paper identifies the structural components of the scaffold and demonstrates that important metalloprotein interactions are involved in stabilizing the structure of histone-depleted chromosomes.

Results

Purification of Metaphase Chromosomes

We have developed three alternative chromosome isolation procedures to extend our previous studies. Each method employs a different buffer system, but the common strategy of all three methods is to homogenize hypotonically swollen cells and then to fractionate the lysate by a combination of sucrose and density gradients. The density gradient is formed by a colloidal suspension of silica (Percoll) in which chromosomes migrate to a position well separated from cytoskeletal material. Figure 1 shows an example of the electrophoretic pattern of the proteins at each step of a purification sequence in which chromosomes are isolated in the presence of polyamines and EDTA (polyamine chromosomes). The most prominent proteins of the total lysate (lane a) are the histones and those of the HeLa cell cytoskeleton. These proteins have been identified and characterized (Franke et al., 1979) and are composed primarily of actin (45,000 daltons [45 kd]) and the intermediate filaments vimentin (57 kd) and prekeratin (52 kd). Sedimentation of the cell lysate through a sucrose gradient results in the elimination of nuclei and aggregates of chromosomes that pellet to the bottom of the gradient. Soluble, nonchromosomal proteins from the total cell lysate remain in the supernatant of the sucrose gradient (lane b). Chromosomes that are purified through sucrose (lane c) still contain the contaminating cytoskeletal proteins. The actin band (A) is particularly prominent. Following sedimentation through the sucrose gradient, the chromosome band is removed, rehomogenized and layered onto a Percoll gradient. Cytoskeletal debris float at the top of the Percoll gradient, while chromosomes form a sharp band at their bouyant density close to the bottom of the gradient (lane d). This last purification step removes most if not all of the actin and the intermediate filaments.

In addition to the polyamine method described above, we have developed two other chromosome isolation procedures. One is an aqueous method using a modified reticulocyte standard buffer. The other is a modification of a method (Wray and Stubblefield,

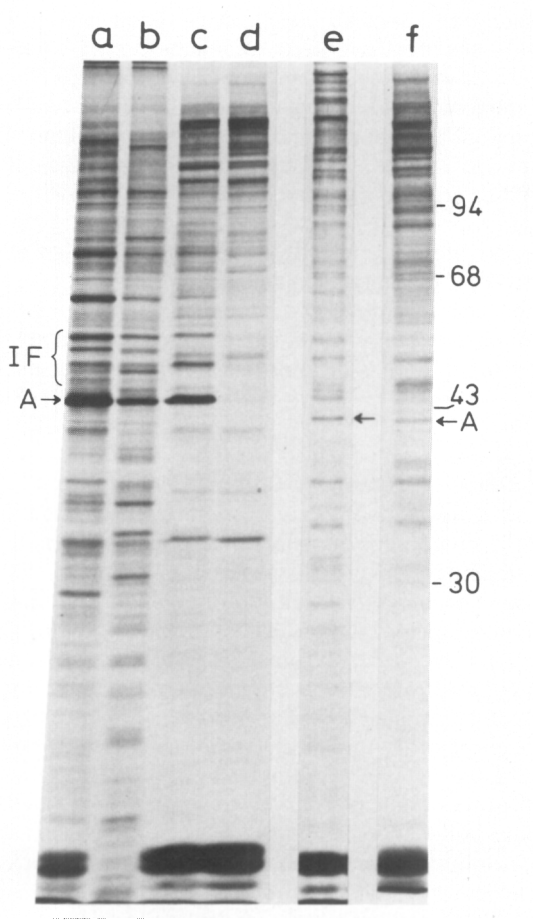

Figure 1. Polyacrylamide Gel Electrophoresis of the Proteins Associated with Chromosomes through a Purification Sequence

Chromosomes labeled with ^{35}S-methionine were purified by the polyamine method. Samples were collected at each step of the purification and prepared for electrophoresis in 12.5% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970). The gels were fluorographed by the procedure of Bonner and Laskey (1974). (Lane a) Metaphase cells homogenized in the presence of 0.1% digitonin; (lane b) supernatant of the sucrose gradient; (lane c) chromosome band of the sucrose gradient; (lane d) chromosome band of the Percoll gradient; (lane e) purified reticulocyte standard buffer chromosomes; (lane f) purified Mg-Hex chromosomes. A: actin. IF: intermediate filaments.

1970) in which chromosomes are stabilized by hexylene glycol and $MgCl_2$ (Mg-Hex) rather than $CaCl_2$. The protein patterns of purified chromosomes isolated by these two methods are shown in Figure 1, (lanes e and f). It can be seen that the cytoskeletal proteins adhere more tenaciously to these chromosomes despite purification through sucrose and Percoll gradients.

Dissociation of Histone-Depleted Chromosomes

We have demonstrated previously that histone-depleted chromosomes, obtained by extraction with a dextran sulfate–heparin lysis mixture, sediment as a peak between 4000 and 7000 S. We now show that addition of β-mercaptoethanol or dithiothreitol to the

Table 1. Sedimentation of Histone-Depleted Chromosomes as Isolated without further Treatment

Type of Chromosome	Fast Form	Slow Form
Reticulocyte standard buffer	+	–
Hexylene glycol and $MgCl_2$	+	–
Hexylene glycol and $CaCl_2$	+	–
Polyamine	–	+

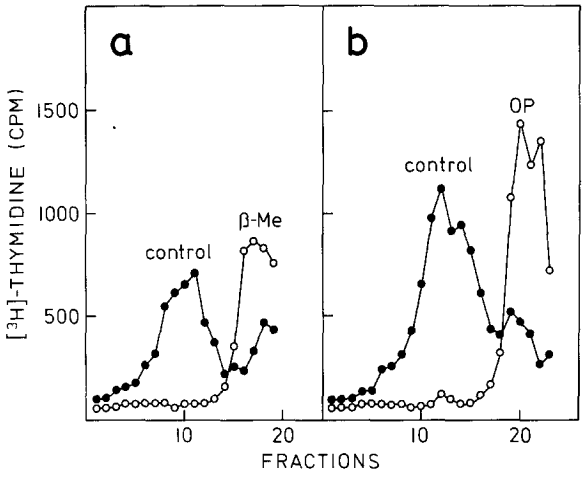

Figure 2. Dissociation of Histone-Depleted Chromosomes by β-mercaptoethanol and 1,10 phenanthroline (OP)

Aqueous (reticulocyte standard buffer) chromosomes, labeled with ^{3}H-thymidine, were treated with a dextran sulfate–heparin lysis buffer to remove histones. The extracted material was then sedimented through 5%–30% linear sucrose gradients containing 0.1 NaCl. Fraction number 1 is the bottom of the gradient. (a: ●——●) Control of histone-depleted chromosomes; (○——○) same as control but with 1% β-mercaptoethanol (β-Me) added to the dextran lysis buffer. (b: ●——●) Control of histone-depleted chromosomes; (○——○) same as control but with 3 mM OP added to the dextran lysis buffer.

lysis mixture leads to a dramatic reduction of the S value in the case of reticulocyte standard buffer and Mg-Hex chromosomes (Table 1). The gradient profile in Figure 2a shows that the DNA of the histone-depleted chromosomes not treated with β-mercaptoethanol sediments in the lower part of the sucrose gradient (4000 to 7000 S: the fast form). In contrast, histone-depleted chromosomes remain near the top of the sucrose gradient following addition of β-mercaptoethanol to the lysis mixture (Figure 2a). As little as 1 mM β-mercaptoethanol or dithiothreitol is sufficient to induce the shift, while as much as 140 mM does not lead to an additional shift in the gradient profile. The shift from the fast to the slow form suggests that the thiols are reducing structurally important disulfide bonds or, alternatively, acting as chelators to dissociate essential metalloprotein interactions. Strong support for the latter possibility comes from the finding that $NaBH_4$, a powerful reducing agent, has no effect (Table 2), while the two hydrophobic chelators, OP and neocuproine, can bring about the unfolding of the fast form (Table 2). The gradient

Table 2. Sedimentation of Histone-Depleted Reticulocyte Standard Buffer or Mg-Hex Chromosomes after Various Treatments

Treatment	Fast Form	Slow Form
No treatment	+	−
β-Mercaptoethanol (1 mM–140 mM)	−	+
OP (3 mM)	−	+
Neocuproine (3 mM)	−	+
$NaBH_4$ (25 mM)	+	−

Table 3. Identification of the Metal Required to Stabilize the Fast Sedimentation Form of Histone-Depleted Chromosomes

Treatment of Metal-Depleted Polyamine Chromosomes	Sedimentation Form of Histone-Depleted Chromosomes	
	Fast Form	Slow Form
No treatment	−	+
Plus $CuSO_4$ (10^{-3} M–10^{-8} M)	+	−
Plus $MnSO_4$ (10^{-3} M)	−	+
Plus $ZnSO_4$ (10^{-3} M)	−	+
Plus $Hg(CH_3COO^-)_2$ (10^{-3} M)	−	+
Plus $CoCl_2$ (10^{-3} M)	−	+
Plus $CaCl_2$ at 37°C (5×10^{-4} M)	+	−

profiles of the fast form and of the slow form obtained following addition of 3 mM OP to the lysis mixture are shown in Figure 2b. These results imply that metal ions are required to stabilize the scaffold.

We have previously shown by fluorescence and electron microscopy that histone-depleted chromosomes retain the morphological features of expanded metaphase chromosomes and consist of a central scaffolding to which the DNA is attached in loops (Paulson and Laemmli, 1977). We repeated these structural studies and found that the histone-depleted chromosomes (fast form) derived from reticulocyte standard buffer or Mg-Hex chromosomes are morphologically similar to those studied previously. Examination of the slow form by fluorescence microscopy reveals that these structures are very expanded and retain little of the metaphase chromosome shape. Although further work is required to study the structure of the slow form in detail, this observation indicates that chelation leads to destruction of the metaphase morphology of the histone-depleted chromosomes.

Stabilization of Histone-Depleted Chromosomes by Copper and Calcium

Metal-depleted chromosomes can be obtained by isolation of Mg-Hex or reticulocyte standard buffer chromosomes with β-mercaptoethanol (see Experimental Procedures). Such chromosomes generate histone-depleted structures of the slow form and thus provide a means to test whether addition of a given metal restores the fast sedimentation form. Polyamine chromosomes are also suitable for these experiments, since these chromosomes, isolated in the presence of EDTA, appear metal-depleted. That is, they generate histone-depleted structures of the slow form without addition of β-mercaptoethanol during the isolation (Table 1).

Various metallic salts have been added to such metal-depleted chromosomes to test whether such treatment would restore the fast sedimentation form. Among the metals tested, which include Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Hg^{2+}, only Cu^{2+} is effective in regenerating the fast histone-depleted structure (Table 3). An example of such an experiment is illustrated in Figure 3. It is evident that histone-depleted structures derived from chromosomes treated with Cu^{2+} sediment at the fast position. In contrast, addition of Zn^{2+} has no effect, since histone-depleted chromosomes treated with this metal remain at the slow position. Determination of the concentration dependence of the Cu^{2+} treatment has shown that as little as 1×10^{-8} M $CuSO_4$ is sufficient to "rescue" metal-depleted chromosomes, while as much as 1 mM $MnSO_4$ or $ZnSO_4$ has no effect. All metal additions were performed under an N_2 atmosphere to reduce the oxidizing effect of Cu^{2+}. If this precaution is taken, addition of the

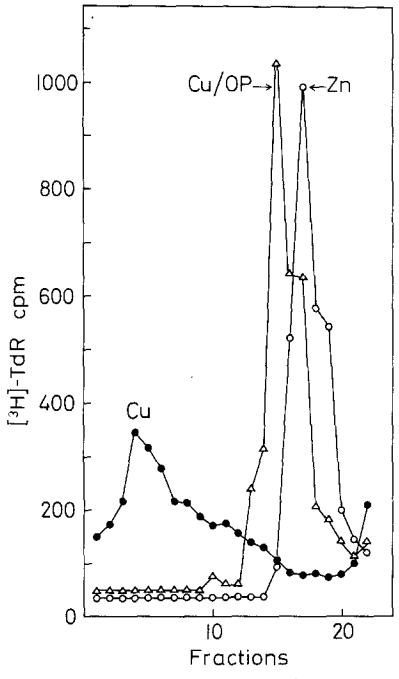

Figure 3. Rescue of Polyamine Histone-Depleted Chromosomes by Cu^{2+}

Polyamine chromosomes, labeled with ^{3}H-thymidine, were treated with $CuSO_4$ or $ZnSO_4$ prior to extraction of the histones with dextran sulfate. Sedimentation was carried out on 5%–30% linear sucrose gradients containing 0.1 M NaCl. (●—●) Histone-depleted chromosomes after addition of 0.1 mM $CuSO_4$; (○—○) after addition of $ZnSO_4$; (△—△) after addition of 0.1 mM $CuSO_4$ and extraction in lysis buffer containing 3 mM OP.

chelators OP or neocuproine to Cu^{2+}-treated chromosomes leads to a reversible dissociation and unfolding of the induced fast form (Figure 3). Omission of this precaution leads to oxidation, since the Cu^{2+}-induced fast form is no longer reversible by metal chelation, but requires a reducing agent such as β-mercaptoethanol for reversion.

We also tested the effect of Ca^{2+} on the metal-depleted polyamine chromosomes and found that exposure of these chromosomes to 0.5 mM $CaCl_2$ generates the fast sedimentation form (Table 3). In contrast to Cu^{2+}, however, incubation at 37°C is required in the presence of Ca^{2+} to induce the fast form. Interestingly, we find that this Ca^{2+} effect can be reversed if polyamine chromosomes are used, but not if the chromosomes are isolated in the presence of hexylene glycol or reticulocyte standard buffer.

To determine whether sulfhydryl groups are involved in the reversible binding of Cu^{2+} and Ca^{2+} to histone-depleted chromosomes, we treated metal-depleted chromosomes with either 0.5 mM $Hg(CH_3COO^-)_2$ or 5 mM iodoacetamide to see whether such treatment would block "rescue" by Cu^{2+}. The gradient profiles presented in Figure 4 demonstrate that pretreatment of chromosomes with Hg^{2+} prior to exposure to Cu^{2+} blocks the rescue; that is, the histone-depleted chromosomes sediment at the slow position. If the order is reversed, then the fast form is obtained. Similarly, pretreatment of metal-depleted chromosomes with iodoacetamide also prevents rescue by Cu^{2+} and Ca^{2+}. These findings suggest that the binding of both Cu^{2+} and Ca^{2+} to histone-depleted chromosomes depends upon the availability of free sulfhydryl groups.

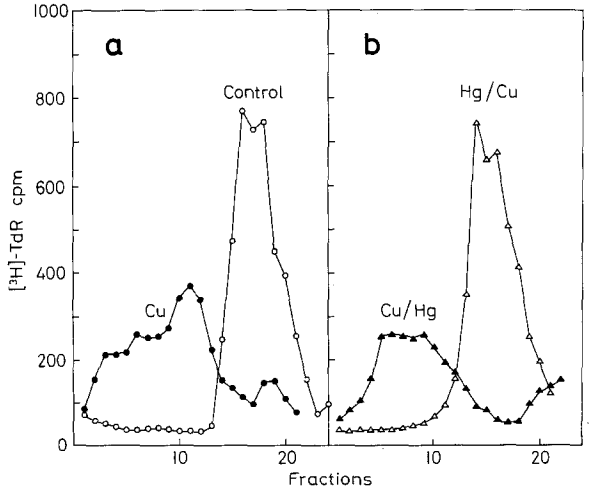

Figure 4. Mercury Blocks Rescue of Histone-Depleted Chromosomes by Cu^{2+}

Polyamine chromosomes labeled with ^3H-thymidine were treated with dextran sulfate lysis buffer after the addition of metals. Sedimentation was as described in Figure 2. (a: O——O) Control of histone-depleted chromosomes; (●——●) fast-sedimenting histone-depleted chromosomes after the addition of 0.1 mM $CuSO_4$. (b: △——△) 0.1 mM $CuSO_4$ was added to chromosomes that had been resuspended in reticulocyte standard buffer containing 0.5 mM $Hg(CH_3COO^-)_2$; (▲——▲) chromosomes were treated with 0.1 mM $CuSO_4$ followed by 0.5 mM $Hg(CH_3COO^-)_2$.

Dissociation of the Metaphase Scaffolding by Metal Chelation

Available evidence suggests that the structural integrity of the histone-depleted chromosome (fast form) is brought about by a network or scaffolding of nonhistone proteins to which the DNA is bound. This scaffolding can be isolated as a fast-sedimenting structure following nuclease digestion and extraction of histones with dextran sulfate–heparin or high salt lysis buffers. We demonstrated above that metal chelation unfolds the fast sedimentation form of the histone-depleted chromosomes. We will show now that such treatment also leads to the dissociation of the scaffolding of nonhistone proteins.

The addition of either β-mercaptoethanol or OP to the lysis mixture dissociates the scaffolding isolated from either reticulocyte standard buffer or Mg-Hex chromosomes. Figure 5 shows the protein patterns of intact reticulocyte standard buffer chromosomes (lane a) and of the scaffolding (lane b). The effect of the extraction buffer is to remove all the histones and many of the nonhistone proteins. Remaining in the scaffold is a subset of proteins constituting about 8%–12% of the total chromosomal proteins. Prominent among those that remain are two high molecular weight proteins labeled Sc1 and Sc2. Addition of either β-mercaptoethanol or OP, but not $NaBH_4$, dissociates the scaffolding, as evidenced by the absence of the high molecular weight proteins following such treatments (Figure 5, lanes c and d, respectively). Contaminating cytoplasmic impurities composed primarily of actin and the heterogeneous group of intermediate filaments remain in the sedimentable fraction. Identification of the cytoplasmic origin of these proteins is based on their strong enrichment in a fraction that floats on top of the Percoll gradient. The protein pattern of this cytoskeletal fraction, which is shown in Figure 5 (lane e), is devoid of chromosomes, as seen by the absence of histones. The percentage of cytoplasmic contamination contained in the reticulocyte standard buffer or Mg-Hex scaffold varies depending on the degree of purification achieved for the chromosomes, but it is usually about 40% of the proteins isolated as the scaffold.

Stabilization of the Scaffolding Proteins by Metals

We have shown that polyamine chromosomes generate histone-depleted structures of the slow form without addition of β-mercaptoethanol or the metal chelators. Such structures can be converted to the fast sedimentation form following "rescue" by Cu^{2+}. These findings imply that polyamine chromosomes are metal-depleted; consequently, we would expect such chromosomes to contain no scaffolding unless

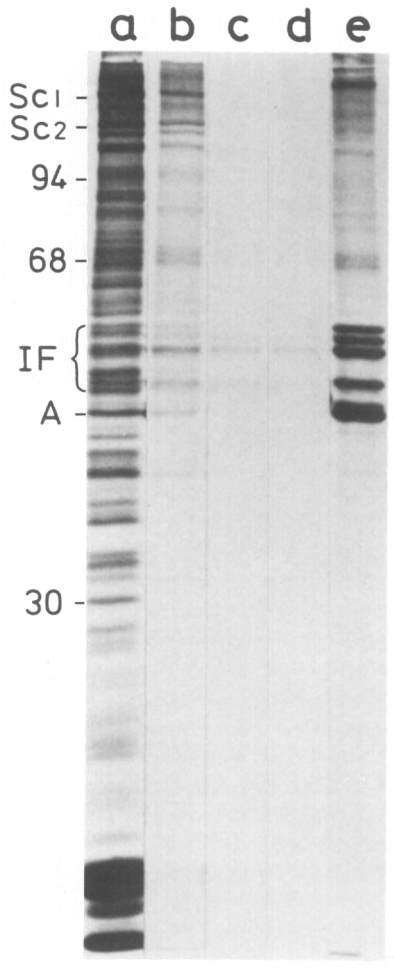

Figure 5. Dissociation of Reticulocyte Standard Buffer Scaffolds by β-mercaptoethanol and OP

Reticulocyte standard buffer chromosomes labeled with ^{35}S-methionine were digested with micrococcal nuclease and then treated with a 2 M NaCl lysis buffer to extract the histones. The resulting scaffolds were pelleted out of the lysis mixture and prepared for electrophoresis as in Figure 1. (Lane a) Reticulocyte standard buffer chromosomes; (lane b) reticulocyte standard buffer scaffolds; (lane c) scaffolds treated with 1% β-mercaptoethanol in the lysis buffer; (lane d) scaffolds treated with 3 mM OP in the lysis buffer; (lane e) cytoskeletal proteins removed from chromosomes by a Percoll gradient. A: actin. IF: intermediate filaments. The major scaffolding proteins are labeled Sc1 (170 kd) and Sc2 (135 kd).

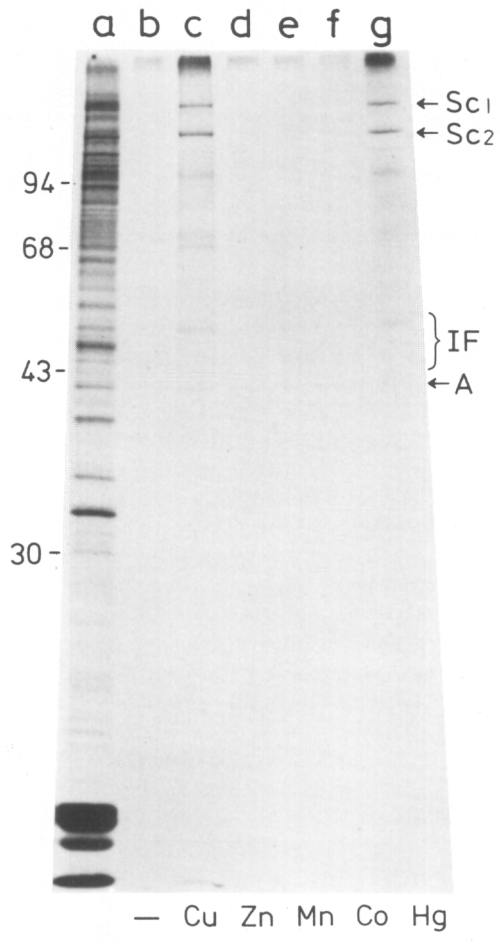

Figure 6. Stabilizing Effects of Cu^{2+} and Hg^{2+} on the Scaffolding Proteins of Polyamine Chromosomes

Polyamine chromosomes labeled with ^{35}S-methionine were treated with 0.1 mM $CuSO_4$, $ZnSO_4$, $MnSO_4$, $CoCl_2$ and $Hg(CH_3OO^-)_2$ under an N_2 atmosphere for 10 min at 4°C. Following the addition of 1 mM EDTA to stop the reaction, the chromosomes were digested with micrococcal nuclease and histone-extracted in the 2 M NaCl lysis buffer. The resulting scaffolds were pelleted out of the lysis mixture and prepared for electrophoresis as in Figure 1. (Lane a) Polyamine chromosomes; (lane b) scaffolds prepared from native polyamine chromosomes; (lane c) scaffolds prepared from polyamine chromosomes after treatment with $CuSO_4$; (lane d) after treatment with $ZnSO_4$; (lane e) after treatment with $MnSO_4$; (lane F) after treatment with $CoCl_2$; (lane g) after treatment with $Hg(CH_3COO^-)_2$. A, IF, Sc1 and Sc2 indicate bands as labeled in Figure 5.

exposed to Cu^{2+}. To test this hypothesis, and the metal specificity, chromosomes were treated with Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} or Hg^{2+}, and scaffolds were prepared. Figure 6 shows that no sedimentable scaffold can be isolated from polyamine chromosomes unless they are first treated with Cu^{2+} (lane c). Treatment with Mn^{2+}, Co^{2+} or Zn^{2+} has no effect on stabilizing the association of the scaffolding proteins (lanes d, e and f). The result obtained following exposure to Hg^{2+} is interesting in that such treatment leads to the formation of scaffolding identical in composition to that obtained by Cu^{2+} (compare lanes c and g).

It is evident from the gel pattern in Figure 6 that the protein composition of the scaffolding obtained from polyamine chromosomes is quite simple, constituting 3%–4% of the total chromosomal proteins. The pattern is reproducible and is represented by two prominent bands, Sc1 and Sc2 (170 kd and 135 kd), a series of minor bands and a small amount of cytoskeletal contamination.

Metal-depleted chromosomes can also be prepared by the isolation of reticulocyte standard buffer or Mg-Hex chromosomes in the presence of 1 mM β-mercaptoethanol. These chromosomes are similar to the polyamine chromosomes in that they generate his-

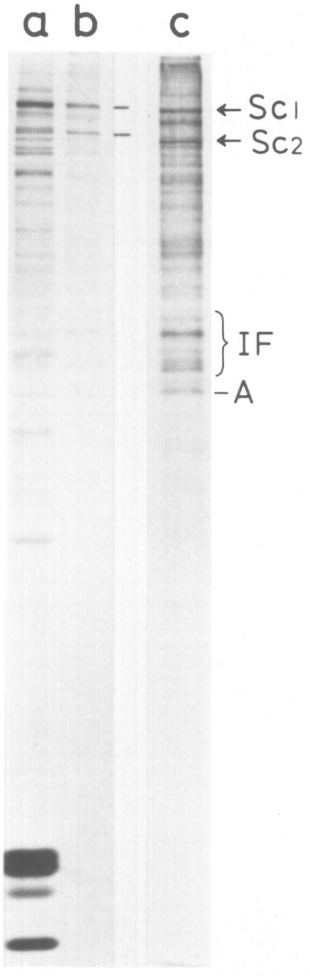

Figure 7. Scaffolding Proteins from Cu^{2+}-Treated Polyamine and Reticulocyte Standard Buffer Chromosomes

Scaffolds were prepared from polyamine chromosomes labeled with ^{35}S-methionine, as described in Figure 6. Scaffolds were made from reticulocyte standard buffer chromosomes that had been isolated in the presence of β-mercaptoethanol by treating the final chromosomes with 1 mM $CuSO_4$ prior to digestion with micrococcal nuclease and extraction of the histones. Both types of scaffolds were pelleted from the lysis mixture and prepared for electrophoresis as described in Figure 1. (Lane a) Purified polyamine chromosomes; (lane b) Cu^{2+}-treated polyamine scaffolds; (lane c) Cu^{2+}-treated reticulocyte standard buffer scaffolds. A, IF, Sc1 and Sc2 indicate bands as labeled in Figure 5.

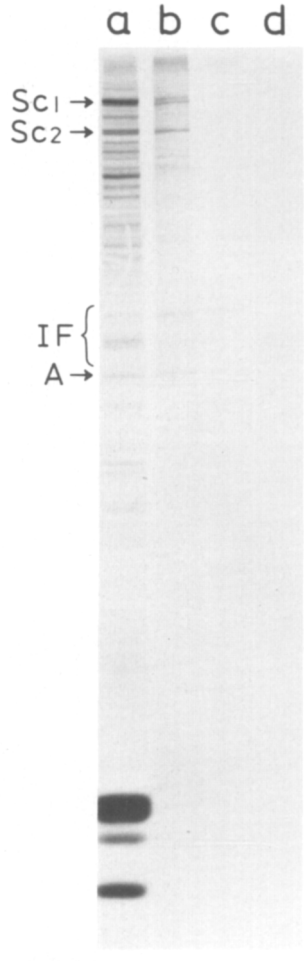

Figure 8. Dissociation of Cu^{2+}-Stabilized Polyamine Scaffolds by β-mercaptoethanol and OP

Scaffolds were prepared from polyamine chromosomes labeled with ^{35}S-methionine and treated with Cu^{2+}, as described in Figure 6. (Lane a) Polyamine chromosomes; (lane b) control of Cu^{2+}-treated polyamine scaffolds; (lane c) same as control but with 1% β-mercaptoethanol added to the lysis buffer; (lane d) same as control but with 3 mM OP added to the lysis buffer. A, IF, Sc1 and Sc2 indicate bands as labeled in Figure 5.

tone-depleted structures of the slow form and contain no scaffolding unless Cu^{2+} is added back. The protein pattern of the scaffold that results from Cu^{2+}-rescue of reticulocyte standard buffer chromosomes is shown in Figure 7 (lane c). This pattern is the same as that of the scaffold from reticulocyte standard buffer chromosomes not isolated in the presence of β-mercaptoethanol and thus not rescued with Cu^{2+} (compare Figure 5, lane b to Figure 7, lane c). In contrast to the simple pattern obtained from the polyamine chromosomes (Figure 7, lane b), scaffolds isolated from reticulocyte standard buffer chromosomes display a more complex pattern. The dramatic difference between the two scaffold patterns is partly due to the heavier cytoskeletal contamination in the reticulocyte standard buffer scaffold.

Sedimentation established that the rescue of histone-depleted chromosomes is reversible by the metal chelators OP and neocuproine if the additions are made under N_2 atmosphere (see above). Figure 8 extends this evidence to the protein level. It is clear from this experiment that the scaffolding generated by exposure to Cu^{2+} (lane b) is reversibly dissociated either by β-mercaptoethanol (lane c) or by OP (lane d). This finding shows that the rescue by Cu^{2+} is reversible by chelation and is thus not due to random oxidation and crosslinking of proteins.

Stabilization of the Scaffolding by Ca^{2+}

We have shown above that the fast sedimentation form of the histone-depleted structure can be induced by the incubation of the polyamine or other metal-depleted chromosomes at 37°C with Ca^{2+}. We now show that incubation of polyamine chromosomes at 37°C with $CaCl_2$ concentrations ranging from 10^{-8} to 10^{-3} M stabilizes the scaffolding proteins. Incubation of chromosomes at 37°C without Ca^{2+} or with Ca^{2+} at 4°C has no effect on stabilizing the scaffolding proteins. The effect of Ca^{2+}, however, is quite different from that of Cu^{2+}, as seen in Figure 9. At a concentration of 10^{-4} M $CaCl_2$, the protein pattern is quite complex (lane b), consisting of many nonhistone proteins as well as a small amount of histones. At concentrations of 10^{-6} and 10^{-8} M (lanes c and d), the pattern becomes progressively less complex, as shown, for example, by the disappearance of the protein band labeled X. At the lowest Ca^{2+} concentration (10^{-8} M), the scaffold pattern is identical to that obtained with Cu^{2+} at the higher concentration of 10^{-4} M (lane f). Thus the effect of Cu^{2+} appears to be more specific. The Ca^{2+}-induced stabilization of the scaffold derived from polyamine chromosomes is reversible by the addition of β-mercaptoethanol to the lysis mixture (lane e). This observed reversibility is in contrast to our observations with either reticulocyte standard buffer or Mg-Hex chromosomes, since exposure of these chromosomes to Ca^{2+} generates histone-depleted structures that are not dissociable by β-mercaptoethanol.

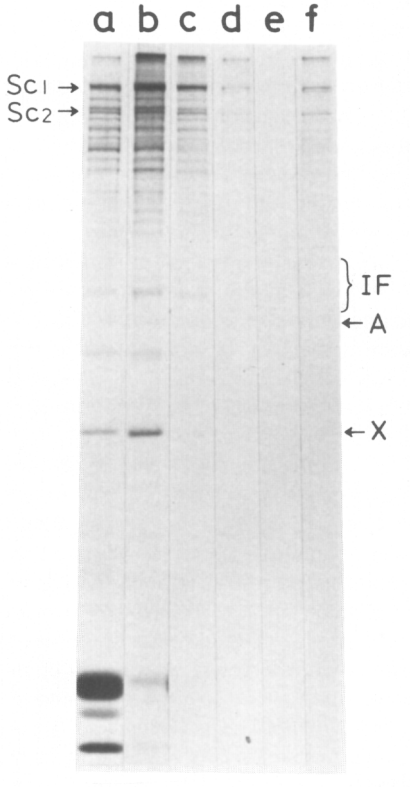

Figure 9. Effects of Incubation with Ca^{2+} at 37°C on Polyamine Scaffolding Proteins

Polyamine chromosomes labeled with ^{35}S-methionine were digested with micrococcal nuclease and treated with various concentrations of $CaCl_2$ for 10 min at 37°C. Following extraction in the 2 M NaCl lysis buffer, the resulting scaffolds were pelleted and prepared for electrophoresis. (Lane a) Polyamine chromosomes; (lane b) scaffolds following treatment with 10^{-4} M $CaCl_2$; (lane c) scaffolds following treatment with 10^{-6} M $CaCl_2$; (lane d) scaffolds following treatment with 10^{-8} M $CaCl_2$; (lane e) scaffolds following treatment with 10^{-6} M $CaCl_2$, but with 1% β-mercaptoethanol added to the lysis buffer; (lane f) scaffolds prepared from Cu^{2+}-treated polyamine chromosomes. A, IF, Sc1 and Sc2 indicate bands as labeled in Figure 5. The disappearance of the protein labeled X emphasizes the effect of decreasing the Ca^{2+} concentration.

Discussion

Chromosomes Isolated by Three New Methods Yield Nonhistone Residual Scaffolds

We have shown by sedimentation studies that the DNA of metaphase chromosomes isolated by newly developed methods retains a compact morphology following extraction of the histones. These histone-depleted chromosomes can be isolated as fast-sedimenting structures (4000–7000 S) on sucrose gradients. The (scaffolding) proteins responsible for constraining the DNA in this fast-sedimenting structure can be isolated independently following digestion of the DNA and extraction of the histones. These findings are in agreement with our previously published results, which used chromosomes isolated by an entirely different method (Adolph et al., 1977a, 1977b; Paulson and Laemmli, 1977). The reproducibility of these results eliminates the possibility raised by the earlier studies that artifactual protein interactions induced by hexylene glycol maintain the structure of the histone-depleted chromosomes.

The availability of highly purified chromosomes is essential for a thorough characterization of the scaffolding structure and, in particular, of the proteins associated with the structure. It has been necessary to develop improved purification procedures, since chromosomes prepared by the traditional methods of differential centrifugation generate scaffolds containing contaminating cytoskeletal proteins as the major components. The combination of sucrose and Percoll gradients, which is used in all three chromosome isolation procedures presented here, is effective in eliminating most cytoplasmic contamination. Since the sedimentation behavior of chromosomes following histone extraction is not altered by the elimination of the cytoskeletal proteins, we conclude that the bulk of these proteins have no structural role in histone-depleted chromosomes.

The purest chromosomes we obtain are isolated in the presence of polyamines and EDTA (polyamine chromosomes). The absence of divalent cations in this

method facilitates the dissociation of cellular components and leads to a dramatic reduction in the material adhering to the periphery of the chromosomes. Thus the protein patterns both of the intact chromosomes and of the scaffolds prepared from polyamine chromosomes are less complex and are relatively free of cytoskeletal contamination.

The Scaffolding Requires Specific Metal Ions for Stability

For chromosomes isolated by either the reticulocyte standard buffer or Mg-Hex procedures, the addition of β-mercaptoethanol, dithiothreitol or the metal chelators OP and neocuproine to the histone extraction medium causes an unfolding of the fast-sedimenting structures. This is witnessed as a large reduction in the S value and by a loss of the metaphase morphology as seen in the fluorescence microscope. It is unlikely that this observed unfolding is due to DNA degradation known to be caused by OP under certain conditions (Que et al., 1980; Marshall et al., 1981). Neocuproine, which does not have the same capacity to degrade DNA, unfolds these structures as efficiently. Furthermore, the EDTA present during exposure to OP strongly inhibits the hydrolyzing capacity of this metal chelator. The unfolding of histone-depleted chromosomes by thiols must be due to chelation rather than reduction of disulfide bonds, since the metal chelators can mimic the effect of the thiols. Consistent with this view is the finding that the strong reducing agent $NaBH_4$ has no effect in dissociating the histone-depleted chromosomes. Our protein studies show that the unfolding of the fast sedimentation form is due to dissociation of the protein scaffolding, since the metal chelation that leads to the unfolding of the histone-depleted chromosomes also solubilizes the protein scaffolding.

Strong support for the involvement of a metal in stabilizing the scaffolding structure comes from experiments with metal-depleted chromosomes. These chromosomes are obtained by isolating reticulocyte standard buffer or Mg-Hex chromosomes in the presence of β-mercaptoethanol. Alternatively, metal-depleted chromosomes can be isolated by the polyamine method. Addition of as little as 10^{-8} M Cu^{2+} restores the compact, fast-sedimenting, histone-depleted structures, while addition of as much as 10^{-3} M Zn^{2+}, Mn^{2+}, Co^{2+} or Hg^{2+} has no effect. Similarly, a protein scaffolding can be obtained following exposure of these chromosomes to Cu^{2+} or Hg^{2+}, but not to the other metals. Since metal-depleted chromosomes cannot be rescued with Hg^{2+} to generate the fast sedimentation form, the effect of Hg^{2+} on the scaffolding proteins is believed to be due to a nonspecific stabilization of the proteins through -S-Hg-S- bonds. We have shown that the rescue of chromosomes by Cu^{2+} is not due to a random oxidation and crosslinking of proteins, since the effect of Cu^{2+} is reversible by metal chelation.

We do not know if the effect of Cu^{2+} is of biological significance. The specificity of this ion, as well as the effect of the metal chelators on dissociating the scaffolding structure, argue in favor of a biological role. Cu^{2+} has been detected by analytical means both in metaphase chromosomes (Cantor and Hearst, 1966) and in nuclei (Bryan et al., 1981). One could propose that the association of Cu^{2+} with chromosomes occurs during the isolation. We have attempted to rule out this possibility by isolating chromosomes in buffers treated with an ion exchange resin with a high specificity for Cu^{2+} (Chelex 100). Chromosomes isolated in this way retain their sensitivity to metal chelation. This finding supports the argument that metals occur naturally in chromosomes.

Effect of Ca^{2+} on the Scaffolding Structure

Incubation of metal-depleted chromosomes at 37°C with Ca^{2+} stabilizes the scaffolding structure. This effect has been shown by the fast sedimentation of histone-depleted chromosomes and by the independent isolation of the scaffolding proteins following exposure of metal-depleted chromosomes to Ca^{2+} at 37°C. At high concentrations (10^{-3}–10^{-4} M), Ca^{2+} appears to be less specific than Cu^{2+}, since massive scaffolds, composed of many protein components, are formed. At lower, near physiological concentrations (10^{-6}–10^{-7} M), the scaffold pattern becomes less complex and approaches that obtained with Cu^{2+}. It is also noteworthy that Ca^{2+} does not have the oxidizing capacity of Cu^{2+}. This adds further support to our view that the stabilization by Cu^{2+} is not brought about by oxidation.

The effect of Ca^{2+} on the complexity of the protein pattern of the scaffold has demonstrated that exposure of chromosomes to Ca^{2+} alters the solubility properties of many nonhistone proteins. This finding explains why the protein pattern of the scaffold published previously (Adolph et al., 1977a, 1977b) was more massive and complex. The chromosomes used in those experiments were isolated by a method requiring an incubation step at 37°C in the presence of hexylene glycol and 0.5 mM $CaCl_2$ (Wray and Stubblefield, 1970). Similar observations on the effects of Ca^{2+} have been made in a study of the nuclear scaffold (Lebkowski and Laemmli, 1982a, 1982b). The complexity of the nuclear scaffold protein pattern can also be controlled by the concentration of Ca^{2+} to which the nuclei are exposed prior to extraction. We believe that this observed effect of Ca^{2+} has been a major source of confusion in the study of the nuclear matrix.

The Scaffold Protein Composition

Our protein studies have shown that the scaffold isolated from highly purified polyamine chromosomes is composed primarily of two prominent bands, Sc1 (170 kd) and Sc2 (135 kd); a set of minor proteins; and a small amount of cytoplasmic contamination. These

two proteins are also found in scaffolds derived from reticulocyte standard buffer and Mg-Hex chromosomes, although the protein pattern is more complex due to cytoskeletal contamination. The conditions that generate a reproducible scaffold from polyamine chromosomes (namely, the addition of 10^{-4} M Cu^{2+}) suffice to stabilize the histone-depleted chromosomes in the fast sedimentation form. We conclude that the minimal set of proteins could be sufficient to maintain the structure. That is, additional proteins that cosediment with the more massive scaffold do not contribute structurally to the histone-depleted chromosomes. The discrete protein pattern observed in the minimal scaffold eliminates the possibility that the scaffold is brought about by a random association of proteins.

It has been suggested that the scaffold is an isolation artifact brought about by the aggregation of nonhistone proteins in high salt (Comings and Okada, 1979; Hadlaczky et al., 1981). This possibility is not valid, since identical results are obtained for the sedimentation of the histone-depleted chromosomes and for the scaffolding protein pattern by using either high or low salt extraction buffers (Adolph et al., 1977a, 1977b; Paulson and Laemmli, 1977; and this report). In the lysis mixture containing dextran sulfate and heparin, extraction occurs at low ionic strength by competition of the negatively charged polymers for proteins associated with DNA, while in the 2 M NaCl lysis mixture, extraction occurs primarily by dissociation of electrostatic interactions.

Although there is no complete proof that the scaffolding interactions observed at the level of the histone-depleted complex are of structural importance in organizing chromatin in whole chromosomes, the observations as a whole strongly favor this view. Possibly the most direct support for the scaffold concept comes from structural studies of intact chromosomes. Recent thin sections of intact chromosomes clearly show that the chromatin is radially arranged and converges in the center to form a lampbrush type of configuration (Marsden and Laemmli, 1979; Adolph, 1980b). This was predicted by our previously proposed loop model (Laemmli et al., 1978), in which the chromatin loops are radially arranged around a central axis. The axis is composed of the scaffolding proteins, which crosslink the bases of the loops.

Relationship to Nuclear Structure

We recently reported evidence that the DNA of histone-depleted structures derived from nuclei is organized on two levels. One level is thought to be brought about by the repetitive attachment of the DNA to the peripheral lamina. On the other level, the DNA is stabilized by a metalloprotein structure involving either Cu^{2+} or Ca^{2+} (Lebkowski and Laemmli, 1982a, 1982b). These observations suggest that histone-depleted nuclei and chromosomes share common structural components. We would like to think that the interactions that are sensitive to chelation in nuclei represent the interphase equivalent of the metaphase scaffolding. As a possible model of chromosome condensation, one could propose that the chelation-sensitive interphase scaffold reorganizes to drive chromosome condensation. The nuclear lamina is known to dissociate late in the G2 phase of the cell cycle as chromosome condensation proceeds (Gerace et al., 1978; Gerace and Blobel, 1980; Jost and Johnson, 1981). Thus the level of chromatin organization that is imposed by the nuclear lamina is lost in metaphase while the chelation-sensitive scaffold structure rearranges to bring about chromosome condensation.

Experimental Procedures

Isolation of Metaphase Chromosomes with Hexylene Glycol

HeLa S3 cells were maintained in suspension culture at 37°C with RPMI-1640 medium supplemented with 10% newborn calf serum, 100 units/ml penicillin and 100 mcg/ml streptomycin. The cells were labeled with ^{3}H-thymidine at 0.5 μCi/ml for 24 hr before isolating the chromosomes and with ^{35}S-methionine at 1–2 μCi/ml for 48 hr. The cells were blocked in metaphase at a concentration of 2–3 × 10^5 cells/ml with colcemid at a final concentration of 0.06 μg/ml for 12–16 hr.

Metaphase chromosomes were prepared by purification through a series of density and velocity gradients by using a modification of the buffer system developed by Wray and Stubblefield (1970). The cells (500 ml) were collected in two tubes by centrifugation (1400 × g, 5 min), washed once in 40 ml/tube with an aqueous buffer (reticulocyte standard buffer) containing 10 mM Tris–HCl (pH 7.4), 10 mM NaCl and 5 mM $MgCl_2$ at room temperature, pelleted (1400 × g, 5 min) and incubated for 15 min in 40 ml/tube of reticulocyte standard buffer at room temperature. The cells were collected (1400 × g, 5 min), and each pellet was washed in 40 ml per pellet of Mg-Hex buffer containing 1 M hexylene glycol, 0.1 mM PIPES (pH 6.5) and 1 mM $MgCl_2$ at 4°C. Subsequent steps were carried out at 4°C. Following centrifugation (1400 × g, 5 min), the swollen cells were resuspended in 40 ml per pellet of Mg-Hex buffer containing 0.1% amonyx LO, 0.5% sodium deoxycholate (Mg-Hex lysis buffer) and 2.5 μg/ml boiled RNAase A. The cells were lysed by 15 strokes with a Dounce tissue homogenizer (50 ml capacity) by using a type A pestle. Each lysate was given a low speed spin (400 × g, 5 min) to remove nuclei. Chromosomes in the supernatant were collected by centrifugation (900 × g, 15 min), combined into 5 ml of Mg-Hex lysis buffer, rehomogenized (20 strokes, 15 ml Dounce homogenizer) and given an additional low spin (180 × g, 5 min). The supernatant was added to 5 ml of 60% Percoll (v/v) in 17% sucrose, 1 M hexylene glycol, 1 mM PIPES (pH 6.5), 5 mM $MgCl_2$, 0.1% amonyx LO and 0.05% sodium deoxycholate and layered onto 30 ml of the same 60% Percoll solution. The Percoll mixture was centrifuged in 50 ml polycarbonate tubes in a fixed angle JA-20 rotor (Beckman J-21 centrifuge) at 20,000 rpm for 30 min to establish a density gradient. The chromosomes formed a diffuse band near the bottom of the gradient, while cytoskeletal debris remained at the top and in variable aggregates in the middle. The chromosome layer was removed in about 10 ml and diluted 1:2 with Mg-Hex lysis buffer containing 2.5 mM $MgCl_2$. The chromosomes were concentrated (1400 × g, 30 min), resuspended in 1 ml of Mg-Hex lysis buffer and layered onto 12 ml of a 5%–60% sucrose gradient in Mg-Hex lysis buffer containing 1 mM PIPES (pH 6.5). The sucrose gradient was constructed in a 15 ml polystyrene conical tube and spun for 30 min (800 × g). The diffuse chromosome band occupying the middle third of the gradient was removed, diluted 1:2 with Mg-Hex lysis buffer and concentrated by centrifugation (1400 × g, 30 min). The resulting chromosome pellet was resuspended in 1 ml of Mg-Hex lysis buffer and used within 1–2 hr of preparation. All solutions contained 0.1 mM phenylmethylsulfonyl fluoride, 1% thiodiglycol and 10 Kallikrein inhibitor units (KIU)/ml Trasylol. Metal-depleted hexylene glycol chromosomes were prepared by including 1 mM β-mercaptoethanol in all isolation buffers.

These chromosomes were washed twice and resuspended finally in Mg-Hex lysis buffer containing 0.5 mM β-mercaptoethanol.

Isolation of Chromosomes in Aqueous Solutions

Cells were blocked, harvested, washed once and soaked for 15 min in reticulocyte standard buffer at room temperature, as described above. Each of the two pellets derived from 500 ml of cells was lysed at 4°C by 15 strokes with a Dounce tissue homogenizer in 40 ml of reticulocyte standard buffer containing 17% sucrose, 0.5 mM Ca^{2+}, 0.1% digitonin and 2.5 μg/ml boiled RNAase A. Following a low speed spin (400 × g, 5 min) to remove nuclei, and a high speed spin (900 × g, 15 min) to concentrate the chromosomes, the two pellets were combined in 10 ml of 20% Percoll (v/v) in isolation buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 0.1% digitonin) containing 17% sucrose. The chromosomes were rehomogenized (15 strokes, 15 ml capacity Dounce homogenizer) and given an additional low speed spin. The supernatant was layered onto 30 ml of 60% Percoll (v/v) in isolation buffer containing 17% sucrose and spun in the Beckman J-21 centrifuge, as described above for the hexylene glycol procedure. The diffuse chromosome band (see above) was collected, diluted 1:2 with isolation buffer and concentrated (400 × g, 30 min). The resulting pellet was resuspended in reticulocyte standard buffer containing 0.1% digitonin and processed as described above through a sucrose gradient buffered by reticulocyte standard buffer and 0.1% digitonin. The chromosomes were resuspended finally in reticulocyte standard buffer and 0.1% digitonin. All solutions contained 0.1 mM phenylmethylsulfonyl fluoride and 1% thiodiglycol and 10 KIU/ml Trasylol. Metal-depleted reticulocyte standard buffer chromosomes were prepared by including 1 mM β-mercaptoethanol in all isolation buffers. The chromosomes were washed twice and resuspended finally in reticulocyte standard buffer containing 0.1% digitonin and 0.5 mM β-mercaptoethanol.

Isolation of Polyamine Chromosomes

Metaphase chromosomes were purified through Percoll and sucrose gradients, as described above, by using a polyamine buffer system outlined by Blumenthal et al. (1979). Cells were blocked and harvested as described above. Each of the two pellets derived from 500 ml of cells was washed three times at 25°C in 15 ml of solution I containing 7.5 mM Tris–HCl (pH 7.4), 0.1 mM spermine, 0.25 mM spermidine, 1 mM EDTA (pH 7.4) and 40 mM KCl. The cells were collected after each wash by spinning for 5 min at 1400 × g. Each pellet of washed, swollen cells was resuspended and vortexed for 15 sec in 10 ml of twice-concentrated solution I containing 0.1% digitonin and 10 μg/ml boiled RNAase A at 4°C. All subsequent steps were carried out at 4°C. The vortexed cells were lysed by five gentle strokes followed by five vigorous strokes with pestle B of the 15 ml capacity Dounce homogenizer. Too vigorous homogenization at first while the chromosomes are still partially expanded often results in obvious damage to the chromosomes. Following a low speed spin to remove nuclei (180 × g, 5 min), the two supernatants containing the chromosomes were layered gently onto two 37 ml, 15%–60% sucrose gradients in solution III, which contained 5 mM Tris–HCl (pH 7.4), 0.25 mM spermidine, 2 mM K-EDTA (pH 7.4), 2 mM KCl and 0.1% digitonin. The gradients were spun in a Clay Adams Dynac centrifuge for 5 min at 400 × g, followed by 30 min at 1100 × g. In the gradient, cytoskeletal material remained at the top, nuclei and large aggregates sedimented to the bottom and the partially purified chromosomes migrated to the middle where they formed a diffuse, often quite faint, band. This band was removed in 10 ml, made 0.8 mM in spermine, 2 mM in spermidine and rehomogenized (10 strokes, 50 ml capacity Dounce homogenizer) with 10 ml of 89% (v/v) Percoll in 5 mM Tris–HCl (pH 7.4), 2 mM K-EDTA (pH 7.4), 0.8 mM spermine, 2 mM spermidine and 0.1% digitonin. After thorough mixing with an additional 15 ml of the Percoll solution, each 35 ml Percoll mixture was spun in 50 ml polycarbonate tubes in the fixed angle JA-20 rotor (Beckman J-21 centrifuge) at 20,000 rpm for 30 min. The density gradient that was formed separated cytoskeletal debris and chromosomes into two very sharp bands, with the cytoskeletal debris at the top and the chromosomes at the bottom of the gradient. Chromosomes were removed from each gradient in approximately 10 ml, diluted 1:2 with solution III and concentrated by centrifugation (1400 × g, 45 min). The two pellets were washed twice and resuspended in 2 ml of solution IV, containing 5 mM Tris–HCl (pH 7.4), 0.25 mM spermidine, 2 mM KCl and 0.1% amonyx LO. Alternatively, the chromosomes were resuspended in reticulocyte standard buffer and 0.1% digitonin. All solutions contained 1% thiodiglycol and 10 KIU/ml Trasylol. Phenylmethylsulfonyl fluoride (0.1 mM) was included in all buffers up to and including the Percoll gradient. Chromosomes prepared with polyamines were not as well dispersed as those made with hexylene glycol or aqueous buffers, but the yield was 3–4 times higher. Typically, 2 ml of a solution of 10 OD_{260} units/ml were obtained from 500 ml of cells by using the polyamine method.

Sedimentation of Histone-Depleted Chromosomes

Chromosomes labeled with ^{3}H-thymidine (5–10 OD_{260} units) were diluted 1:100 at 4°C into 0.2 ml of lysis buffer containing either 2 M NaCl or 2 mg/ml dextran sulfate–0.2 mg/ml heparin, both in 10 mM Tris–HCl (pH 9), 10 mM EDTA, 0.1% amonyx LO, 1% thiodiglycol, 0.1 mM phenylmethylsulfonyl fluoride and 10 KIU/ml Trasylol. It was important not to exceed more than 1 μg of DNA per gradient in order to avoid the viscosity generated by unfolding of the DNA. After combining the chromosomes and the lysis buffer by gently rolling them in a siliconized (5% dichlorodimethylsilane in CCl_4) glass tube, the mixture was layered onto a linear 5 ml gradient of 5%–30% sucrose in 0.1 M NaCl, 10 mM Tris–HCl (pH 9), 10 mM EDTA, 0.1% amonyx LO, 1% thiodiglycol, 0.1 mM phenylmethylsulfonyl fluoride and 10 KIU/ml Trasylol. A layer (0.2 ml) of 2.5% sucrose in the gradient buffer without NaCl was used to separate the lysis mixture containing the chromosomes from the gradient. After 30 min of incubation, the gradients were spun at 4°C in siliconized, 5 ml nitrocellulose tubes in the Beckman SW50.1 rotor at 5000 rpm for 2 hr. The gradients were collected from the bottom, and the radioactivity in each fraction was counted.

Isolation of the Scaffolding Proteins from Metaphase Chromosomes

Chromosomes (5–10 A_{260} units) isolated by any one of the three methods described above were treated with staphylococcal nuclease or DNAase I, 40 μg/ml, for 30 min at 4°C. Histones were extracted by lysis in either 2 M NaCl or 2 mg/ml dextran sulfate–0.2 mg/ml heparin, both in 10 mM Tris–HCl (pH 9), 10 mM EDTA, 0.1% amonyx, 10 KIU/ml Trasylol, 0.1 mM phenylmethylsulfonyl fluoride and 1% thiodiglycol. Typically, 100 to 500 μl of nuclease-treated chromosomes were mixed with 4.5 ml of the extraction buffer, incubated for 20 min at 4°C and spun in 5 ml siliconized nitrocellulose tubes for 30 min at 10,000 rpm in the Beckman SW50.1 rotor at 4°C. The pellets were washed with 80% ethanol and 50 mM NaCl and centrifuged again at 10,000 rpm for 30 min. Isolation of metaphase scaffolds was also achieved by mixing the chromosomes 1:1 with twice-concentrated lysis buffer, followed by layering of the mixture onto 5 ml of a 5%–60% sucrose gradient prepared in lysis buffer. The gradient was spun in 5 ml, siliconized nitrocellulose tubes in the Beckman SW50.1 rotor for 1 hr at 20,000 rpm. The gradients were collected from the bottom, the fractions were pooled and the proteins were precipitated with trichloroacetic acid.

Treatment of Chromosomes with Metals and Chelating Agents

All metal additions were performed either on intact chromosomes or on nuclease-treated chromosomes prior to the extraction of the histones in lysis buffer. Scaffolds and histone-depleted chromosomes from polyamine chromosomes were routinely prepared by the addition of 0.1 mM $CuSO_4$ under an N_2 atmosphere. The reaction was stopped after 10 min by the addition of tenfold excess of EDTA. Scaffolds and histone-depleted chromosomes from reticulocyte standard buffer or Mg-Hex chromosomes isolated in the presence of β-mercaptoethanol were prepared by addition of 1 mM $CuSO_4$ to overcome the 0.5 mM β-mercaptoethanol present in the final resuspension buffer. The procedure for testing the effects of other metals was identical to that used for Cu^{2+}. The dissociation by chelation of scaffolds and histone-depleted chromosomes was carried out by the addition of 1–140 mM

β-mercaptoethanol, 10 mM dithiothreitol and 3 mM OP or 3 mM neocuproine to the lysis buffers used to extract histones. Stabilization by Ca^{2+} was achieved by incubation of nuclease-treated or intact chromosomes with $CaCl_2$ (10^{-3}–10^{-8} M) for 10 min at 37°C prior to extraction of histones.

Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970), with the use of a modified procedure. The stacking gel contained 1 mM EDTA, 0.1% thiodiglycol and 8 M urea. The separating gel contained 1 mM EDTA and 0.1% thiodiglycol, and the well buffer contained 14 mM β-mercaptoethanol. These precautions help prevent oxidation during the course of electrophoresis and result in higher resolution of the gel. The gels were fluorographed by the method of Bonner and Laskey (1974).

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