Large-scale chromatin structural domains within mitotic and interphase chromosomes in vivo and in vitro

Andrew S. Belmont *, Michael B. Braunfeld, John W. Sedat, and David A. Agard
Department of Biochemistry and Biophysics and the Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94143-0448, USA

Abstract. Higher-order chromatin structural domains approximately 130 nm in width are observed as prominent components of both Drosophila melanogaster and human mitotic chromosomes using buffer conditions which preserve chromosome morphology as determined by light microscopic comparison with chromosomes within living cells. Spatially discrete chromatin structural domains of similar size also exist as prominent components within interphase nuclei prepared under equivalent conditions. Examination of chromosomes during the anaphase-telophase transition suggests that chromosomes decondense largely through the progressive straightening or uncoiling of these large-scale chromatin domains. A quantitative analysis of the size distribution of these higher-order domains in telophase nuclei indicated a mean width of 126 ± 36 nm. Three-dimensional views using stereopairs of chromosomes and interphase nuclei from 0.5 μm thick sections suggest that these large-scale chromatin domains consist of 30 nm fibers packed by tight folding into larger, linear, fiber-like elements. Reduction in vitro of either polyamine or divalent cation concentrations within two different buffer systems results in a loss of these large-scale domains, with no higher-order chromatin organization evident above the 20–30 nm fiber. Under these conditions the DNA distribution within mitotic chromosomes and interphase nuclei appears significantly diffuse relative to the appearance by light microscopy within living cells, or, by electron microscopy, within cells fixed directly without permeabilization in buffer. These results suggest that these large-scale chromatin structural domains are fundamental elements of chromosome architecture in vivo.

Introduction

Within the higher eukaryotic chromosome, DNA is folded through DNA-protein interactions into multiple levels of organization. Together these yield greater than a 10000 : 1 linear condensation of DNA into the fully compacted metaphase chromatin. The lowest and best characterized level of packaging is the nucleosome, accounting for a 7 : 1 packaging ratio and consisting of 145 bp of DNA wrapped around the exterior of a protein octamer core (Richmond et al. 1984). A higher-order folding of nucleosomes yields the next level of organization, a fiber of approximately 30 nm diameter, for which several models involving one- or two-start helical foldings have been proposed (Woodcock et al. 1984; Widom and Klug 1985; Williams et al. 1986); experimentally, an additional compaction of about 6 : 1 has been measured for this fiber.

This leaves the majority of folding, greater than 250 : 1, accounted for by the highest levels of folding, referred to in this paper as the large-scale chromatin organization. A number of models have been proposed for this level of folding, including an irregular transverse folding of the 30 nm fiber (Dupraw 1965), a radial loop organization of the 30 nm fiber (Marsden and Laemmli 1979; Adolph 1980), and a helical folding of an underlying 200–250 nm diameter chromonema fiber, which itself is organized as a helical (Sedat and Manuelidis 1978) or radial loop folding (Rattner and Lin 1985) of the 30 nm fiber. Reflecting the number of contradictory models proposed, our current structural understanding of chromatin organization is the most incomplete for this highest level of folding.

A major difficulty in studying higher order chromatin folding is the selection of suitable buffer conditions. At each of the organizational levels described above, chromatin conformation is quite sensitive to variations in ionic conditions. This sensitivity increases with the higher levels of folding. Thus over a range of cation concentrations in which the nucleosome is stable, the higher order 30 nm chromatin fiber can be completely unfolded (Widom 1986). Similarly, over a narrower range of cation concentrations in which the 30 nm fiber remains condensed, a folding/unfolding transition involving the packing of these 30 nm fibers within intact mitotic chromosomes or interphase nuclei is observed (Adolph 1980; Zatsepina et al. 1983; Langmore and Paulson 1983). These last transitions can occur under physiological, or near physiological, buffer conditions.

In practice, this means that different investigators using different, but commonly accepted, nuclear isolation buffers, have observed quite different chromosome structures, with the maximum ambiguity existing for the highest level of chromatin folding, the arrangement of 30 nm fibers. Unfortunately, with just a few exceptions (Langmore and Paulson 1983; Paulson and Langmore 1983), there has been relatively little effort devoted to relating the structures observed in vitro to the actual large-scale chromatin organization existing in vivo. The reasons are clear. Traditional light microscopy methods are limited not only by resolution, but more significantly by out-of-focus blurring from thick specimens which obscures even global changes in chromosome

Offprint requests to: D.A. Agard

* Current address: Department of Cell and Structural Biology University of Illinois, Urbana, Urbana, IL 61801, USA
architecture; on the other hand, electron microscopy methods are limited not only by the inherent potential for artifacts resulting from fixation and embedding, but also by the lack of a chromatin specific, electron-dense stain.

In this paper, we have taken advantage of new technologies to redress this issue. Using a combination of three-dimensional, optical sectioning microscopy (Agard 1984; Hiraoka et al. 1987; Agard et al. 1989), conventional and intermediate voltage electron microscopy, and a systematic titration of large-scale chromatid condensation/decondensation, we provide evidence that a large fraction of 30 nm diameter chromatid fibers within mitotic and interphase chromosomes in vivo are folded into spatially discrete, large-scale domains measuring on the order of 130 nm in diameter.

Materials and methods

Buffers. The four buffers used in isolation of nuclei/chromosomes were: (1) Buffer A, 80 mM KCl, 20 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 15 mM Pipes buffer, 15 mM β-mercaptoethanol, 0.5 mM spermine, 0.2 mM spermidine, 10 µg/ml turkey egg white protease inhibitor (Sigma), adjusted to pH 7.0. For experiments in which the spermidine, spermine concentrations were varied with this buffer system, the spermine : spermidine ratio was held constant and the concentrations of polyamines varied from 0–3 × the values listed above. (2) PBS, Dulbecco’s phosphate-buffered saline solution, 2.68 mM KCl, 1.47 mM KH₂PO₄, 137 mM NaCl, 8.06 mM Na₂HPO₄, 7H₂O, 0.9 mM CaCl₂, 0.49 mM MgCl₂·6H₂O plus 15 mM β-mercaptoethanol, and 10 µg/ml turkey egg white protease inhibitor. The above buffer minus Ca, Mg was referred to as CMF-PBS. For experiments in which the Ca, Mg concentrations were varied, the ratio of Ca : Mg was held constant, and the divalent cation concentrations varied between 0–2 × the values listed above. (3) For still higher concentrations of Ca, Mg, the phosphate in PBS buffer was replaced with Pipes, yielding Buffer B, 145 mM NaCl, 4.15 mM KCl, 15 mM Pipes buffer, 0.9 mM CaCl₂, 0.49 mM MgCl₂·6H₂O, 15 mM β-mercaptoethanol, and 10 µg/ml turkey egg white protease inhibitor, adjusted to pH 7.4. Again for the higher divalent cation concentrations the ratio of Ca : Mg was held fixed and the concentrations of divalent cations varied between 1–6 × the values listed above. (4) Buffer C, a sucrose buffer with low univalent cation concentrations, 0.25 M sucrose, 10 mM Pipes, 1.5 mM MgCl₂, 1.0 mM CaCl₂, plus 10 µg/ml turkey egg white protease inhibitor, adjusted to pH 6.8. Variations in Ca, Mg concentrations were carried out at a fixed Ca : Mg ratio as described above.

Tissue culture. Drosophila Kc cells were grown at 24°C in Falcon 75 cm² flasks using Eschschler medium. For isolated chromosome preparations, cells in log phase growth from ten 75 cm² flasks were treated with 2.5 µg/ml Colcemid (Sigma) for 1.5 h or with 0.5 µg/ml Colcemid for 4 h, then harvested by shaking free the loosely attached cells. These dosages of Colcemid represented suboptimal concentrations for blocking mitosis, with maximum mitotic block requiring 5.0 µg/ml. Lower dosages were selected to minimize the hypercondensation observed with Colcemid block. Control experiments using no Colcemid were also done.

Human Hela cells were grown at 37°C in a 5% CO₂ atmosphere using Eagle’s minimum essential medium with Earle’s salts supplemented with 10% newborn calf serum and 1 × nonessential amino acids. For chromosome preparations no drugs were used; cells were either harvested in log phase growth using a 0.05% trypsin, 0.02% EDTA saline solution, or cell cycle synchronized populations were obtained by selective detachment (Terasima and Tolmach 1963). “S-phase” cells were prepared using a double thymidine block, to arrest the majority of cells in early S-phase (Tobey et al. 1967). Specifically, cells were plated at approximately 1/10 confluent density and 32 h later thymidine was added to 2 mM. Twelve hours later the medium was removed, culture flasks washed with Hank’s balanced salt solution, and new medium added. After another 12 h, 2 mM thymidine was added again, and cells harvested 12 h later.

Preparation of Drosophila chromosomes. Drosophila Kc chromosomes were isolated in Buffer A by sucrose gradient and fixed overnight at 4°C, as described previously (Bellmont et al. 1987).

For isolation of Colcemid-arrested, precellular blastoderm Drosophila embryonic chromosomes, Oregon R stock embryos were obtained from population cages, harvested, washed, and their chorions removed according to standard procedures (Elgin and Miller 1978). A collection period of 1.5 h was used, with an additional aging of 1 h after the end of collection to ensure embryos at the syncytial blastoderm or earlier embryonic stage (Foe and Alberts 1983).

Embryos were permeabilized by immersion in heptane for 2.5 min, washed, and resuspended in 4 : 1 Eschschler medium: H₂O supplemented with 10 µg/ml colchicine (Sigma) (Hanson 1978). After 30 min complete mitotic block was achieved, and embryos were washed and resuspended in cold Buffer A with 1 mM PMSF (phenylmethylsulfonyl fluoride), and lightly homogenized to break open the embryos. Then 0.1% digitonin and 0.16% Brij 58 were added and the suspension briefly vortexed. The suspension was pelleted, washed in Buffer A, and fixed overnight in a 3 : 1 Buffer A: 8% glutaraldehyde (Polysciences) solution at 4°C.

Preparation of Hela mitotic and interphase cells. Cells in complete medium were pelleted at room temperature, then washed twice in cold isolation buffer. For detergent-treated cells, the pellet was resuspended at a density of 6–12 × 10⁵ cells/ml in isolation buffer supplemented with 0.1% digitonin, 0.16% Brij 58, and 0.25 mM PMSF and placed on ice for 5 min. In some instances this suspension was vortexed briefly in order to obtain isolated chromosomes and nuclei. Similar results were obtained with either detergent-treated whole cells or isolated chromosomes and nuclei. The suspension was pelleted at low speed and gently resuspended in isolation buffer.

For the majority of experiments described in this paper, fixation involved the addition of 8% glutaraldehyde to the isolation buffer to a final concentration of 2%. This addition was made either directly to the cell suspension or immediately prior to mixing the fixation solution with the cell suspension, when using isolation buffers containing polyamines. Addition just several minutes prior to mixing the fixation solution with the cells resulted in chromosome and nuclear morphology typical of lower polyamine concentrations; this presumably reflects the high reactivity of polyamines with the glutaraldehyde. After 5 min fixation on ice
the suspension was pelleted, placed in fresh fixative solution, and incubated at 4°C overnight. Alternatively, fixation was carried out using 0.04% glutaraldehyde for 4 h at 4°C in isolation buffer. (After the initial 5–10 min fixation in glutaraldehyde cells were pelleted as described above.) The pellet was then washed, without resuspension, in several changes of fresh isolation buffer without β-mercaptoethanol and fixed overnight in isolation buffer plus 0.05% OsO₄ at 4°C.

In the case of non-detergent-treated cell preparations, glutaraldehyde to a final concentration of 2% was added instead of detergent and PMSF, and after 5 min on ice the cells were pelleted and fixed in 2% glutaraldehyde overnight. All operations, after the first spin at room temperature, were carried out at 4°C or on ice.

**Electron microscopy.** Dehydration, infiltration, embedding, and sectioning were carried out as described previously (Belmont et al. 1987). Grids were stained with uranyl acetate and lead citrate. Sections 80–200 nm thick were examined with a Philips 400 microscope, operated at 100–120 kV and using a 30 μm objective aperture. Thicker sections were examined at 300 kV with a Philips 430 microscope. Kodak EM 4489 film was used with a 2 : 1 dilution of Kodak D-19 developer. For consistency of display, electron micrographs were printed in reverse contrast; inner negatives were made using Kodak Tech Pan 2415 film developed either with Technidol or HC-110 developer.

**Light microscopy.** For examination of living cells, Hela cells were grown in a Bionique tissue culture chamber (Bionique Laboratories, Saranac Lake, NY). Thirty minutes before examination, Hoechst 33342 was added directly to the culture medium to a final dye concentration of 2 μg/ml; the medium was also supplemented with 25 mM Hepes, pH 7.4. By the criteria of trypan blue exclusion, cell viability was greater than 99% 4 h after shifting to room temperature and CO₂ concentration; actual times for data collection were shorter.

For examination of intact, nonpermeabilized cells, Hela cells were grown directly on a no. 1 1/2 glass coverslip, washed twice in buffer, fixed in 1% glutaraldehyde for 1 h at room temperature, washed twice in buffer, and stained with 0.2 μg/ml of DAPI (4',6'-diamidino-2-phenylindole). Cells examined after detergent permeabilization, were prepared similarly; after washing twice in buffer, coverslips were incubated 5 min in buffer plus 0.1% digitonin, 0.1% Brij 58, 0.1 mM PMSF, washed twice in buffer, and then fixed and stained. (Similar results were also obtained from cells harvested with trypsin/EDTA, processed as described in preparation of Hela mitotic and interphase cells, fixed on ice 1 h with 1% glutaraldehyde, washed in buffer, and then embedded in 1% agarose.)

Epon sections were stained with 0.1 μg/ml DAPI. Grids were mounted section side up using no. 1 1/2 coverslips, and examined within 1–2 h after mounting.

A Zeiss Axiomat light microscope, modified for computer-controlled stage, focus, and data collection, was used (Hiraoka et al. 1987). Digital pictures were obtained with a liquid nitrogen-cooled, charge-coupled device (CCD) camera (Photometrics, Tucson, Ariz.), providing low light capability and an extremely linear response. A detailed description of this special camera, including its configuration with the Axiomat and our Vax 8650 computer, its performance and operation, and flat fielding methods, is described elsewhere (Hiraoka et al. 1987). A Leitz Planapo 63×/NA = 1.4 oil immersion lens was used, together with a 365 nm band pass excitation filter, a 395 nm dichroic mirror, and a combination of a 425 nm long pass and a 560 nm short pass emission filter.

**Histogram measurements.** To obtain measurements of the widths of large-scale chromatin domains, samples were prepared as described above, with the exception that nonpermeabilized cells were fixed in 2% glutaraldehyde for 4 h at 4°C, washed in PBS, and then postfixed in 1% OsO₄ for 1 h on ice, to provide greater contrast.

Electron microscope negatives were digitized at 12 bit grey scale resolution using a Perkin-Elmer Micro-1010 G densitometer with a 30 × 30 μm source aperture and a 25 × 25 μm sensor aperture. Images were visualized on a Deanza graphics monitor, and width measurements made by calculating the distance in pixel units between 2 points on either side of the fiber. Microscope magnification was calibrated for each session using a diffraction grating. For display of the histograms, distances were rounded to the nearest pixel and then binned in groups of 2 (pixels).

**Results**

In a previous study of mitotic chromosome structure (Belmont et al. 1987), we described higher levels of chromatin organization than had been appreciated in most earlier electron microscopy investigations. Specifically, local folding of 24 nm diameter chromatin fibers into condensed structures as large as 120 nm in diameter was visualized within stereopairs and axial tomographic reconstructions. A major feature in our experimental procedures was the choice of sample buffer.

Our choice of buffer conditions had been based on previous work (Sedat and Manuelidis 1978), using as a criterion for buffer selection the preservation of the high degree of structural order within the bands of polytene chromosomes, as assayed by polarization light microscopy. Buffer A, a high K⁺, polyamine buffer originally described by Burgoine et al. (1971), was selected from a number of commonly used nuclear isolation buffers. In light of previous results, we decided to reinvestigate this issue of buffer conditions, focusing directly on the effects on diploid chromosome structure.

**Light microscopy observations of chromosome morphology within living cells**

To begin our analysis we examined living cells, stained with a membrane permeable, DNA specific fluorochrome, Hoechst 33342, using light microscopy optical sectioning. Six to eight consecutive pictures of an individual cell were taken, each separated by 0.25 μm in focus. Similar optical sectioning data sets were collected for fixed cells in various buffers; fixation was either immediate or after permeabilization by brief detergent treatment. In this case, a related, DNA specific fluorochrome, DAPI, was used which was not permeable to cell membranes, but which faded less than Hoechst 33342.

Mitotic chromosomes in both living cells and nonpermeabilized cells fixed in either CMF-PBS or buffer A showed similar morphology—sharply defined borders and
significant banding substructure. In cells permeabilized in buffer A before fixation, this appearance was unchanged. By contrast, in cells permeabilized in CMF-PBS mitotic chromosomes lost all visible substructure and their boundaries became very diffuse.

These results are illustrated in Figure 1. To accentuate structural details, a computational algorithm (Agard 1984) was applied to reduce optical blurring caused by chromosomal regions outside the focal plane. This algorithm uses a difference method which approximates the out-of-focus contribution to the image at a given focal plane, by quantities estimated from the two adjacent optical sections above and below this focal plane; these estimated, low spatial frequency, out-of-focus contributions are then subtracted from the original image, yielding a “deblurred” optical section.

Similar observations were made with regard to the appearance of chromatin staining within interphase nuclei. In living cells, nonpermeabilized cells fixed in either buffer A or CMF-PBS, or cells permeabilized with detergent in buffer A, a “thready” fluorescent pattern was evident. In nuclei from cells fixed after permeabilization by detergent in CMF-PBS buffer, only a diffuse fluorescence staining was seen. These results are summarized in Figure 2.

These results suggest that the large-scale chromatin organization, at the level of resolution provided by light mi-
microscopy, is preserved in any reasonable isotonic buffer if cells are fixed intact. Once chromosomes are exposed to a given buffer condition by cell permeabilization, however, quite dramatic changes in large-scale chromatin organization can be induced, even by commonly accepted buffers whose ionic strengths are within physiological ranges. At the level of discrimination provided by this technique, large-scale chromatin organization in mitotic and interphase chromosomes exposed to buffer A cannot be distinguished from the in vivo state. Chromatin in permeabilized cells exposed to CMF-PBS buffer, however, is obviously greatly perturbed from its in vivo conformation.

Electron microscopy visualization of the effects of cell permeabilization on large-scale chromatin structure

Mitotic chromosomes. How do these buffer-induced perturbations in the large-scale chromatin organization appear at the higher spatial resolution of the electron microscope? In Figure 3 we show a comparison of mitotic chromosome morphology within cells fixed before, during, or after permeabilization in CMF-PBS. Cells washed several times in CMF-PBS and fixed directly with glutaraldehyde (Fig. 3A) again show smooth, sharply defined chromosome borders even at the much higher resolution of the electron microscope. Although the nonselectivity of the uranyl and lead staining results in significant nonchromosomal staining obscuring the finer details of chromosome structure, large-scale chromatin structural domains can be seen oriented roughly transverse to the chromosome axis. Mitotic chromosomes in cells washed and then fixed directly in buffer A show a morphology which is identical, at this level of analysis (data not shown).

When cells in CMF-PBS were processed with the simultaneous addition of fixative and detergent, similar results were obtained (Fig. 3B). Adding fixative just 30 s after addition of the detergent, results in chromosomes with a very different, decondensed conformation (Fig. 3C). Specifically, chromosome borders are now obviously diffuse; a dense central core is seen, within which chromatin fibers cannot be traced, surrounded by a less condensed peripheral region, where the chromatin fibers appear radially oriented over the short regions in which they can be traced. No obvious substructure larger than the 30 nm fiber is recognized. This “radial loop” appearance is quite similar to that previously described for chromosomes slightly swollen in low Na, 1-1.5 mM MgCl₂ buffers (Marsden and Laemmli 1979; Adolph 1980). In sharp contrast, mitotic chromosomes within cells permeabilized in buffer A (Fig. 5) appear quite similar to chromosomes within nonpermeabilized cells.

Interphase nuclei. Unfortunately, a comparison similar to that described above for mitotic chromosomes is not easily obtained for interphase nuclei. This is due to the nonspecificity of uranyl and lead electron microscopy stains (Hayat 1981), which highlight chromatin, and ribonucleoprotein structures, as well as protein and lipid structures containing no nucleic acids. As a result, a significant nucleoplasmic background staining exists (see Fig. 3), that includes fibril-

Fig. 2A–D. Optical sections of nuclei, deblurred by a nearest-neighbor algorithm. A Living cell stained with Hoechst 33342. B Detergent-permeabilized cell in buffer A, stained with DAPI. C Cell fixed in CMF-PBS, stained with DAPI. D Cell permeabilized before fixation in CMF-PBS, stained with DAPI. Note the preservation of “thread-like” staining in A–C, including cells permeabilized in buffer A before fixation, versus significant loss of discrete structures in CMF-PBS in D. Scale, crosses separated by 300 nm.
lar structures with diameters 10–30 nm (varying as a function of fixation conditions; Skaer and Whytock 1976, 1977) which can easily be confused with decondensed regions of the genome. A significant fraction of this background has been attributed to soluble nuclear proteins artifically precipitating during fixation (Skaer and Whytock 1976).

Therefore to compare the actual large-scale chromatin organization in sections of interphase nuclei within cells fixed either directly or after detergent permeabilization, we used a combination of both light and electron microscopy. Epon sections, 200 nm thick and already mounted on electron microscope grids, were stained first with a DNA specific fluorochrome, DAPI, and images of nuclei obtained with the Zeiss Axiosmat light microscope and CCD system. The grids were then washed, stained with uranyl and lead, and examined in the electron microscope, where the very same nuclei seen in the light microscope were identified and photographed.

Nuclei in nonpermeabilized cells fixed in either PBS (Fig. 4A) or buffer A (data not shown) show a similar thready DAPI fluorescence image, where the diameters of these threads correspond to the 200 nm diffraction-limited resolution of the microscope. As expected, the electron microscopic images show heavily stained “heterochromatin” and lightly stained “euchromatin” patterns. However, these correspond poorly to the DNA distribution revealed by the DAPI staining. In particular, many regions of DNA staining visualized in the light microscope are poorly contrasted, if at all, within the euchromatic regions of the electron micrograph. Also the apparent fraction of DNA distributed within the euchromatic region of the nucleus is seen to be significantly higher than the actual fraction revealed by the fluorescence staining, with most of the DNA concentrated in the thread-like staining patterns, even in the euchromatic regions. In most areas of heterochromatin, however, fiber-like structural domains measuring from 80–150 nm in diameter are seen which correspond closely to the diffraction-limited threads seen in the light microscopic image.

Examination in the light microscope of detergent-permeabilized nuclei using buffer A (Fig. 4C) shows a thready appearance identical to that seen in the nonpermeabilized cells. At the electron microscope level, most of the euchromatin staining pattern is now gone, and there is a nearly 1:1 correspondence between spatially distinct, chromatin large-scale domains and the DAPI fluorescent threads. In contrast, nuclei from cells permeabilized in CMF-PBS (Fig. 4B) show in the electron microscope an absence of any large-scale chromatin organization, with light microscopy displaying a near continuous fluorescence distribution. Both the electron and light microscopic appearance of these nuclei are easily distinguished from that seen within cells fixed without permeabilization.

These results, for both the mitotic chromosomes and the interphase nuclei, are consistent with the previously described light microscopic observations on living and fixed, but nonembedded cells. Cells fixed intact show similar large-scale chromatin conformations independent of the buffer used; once permeabilized, however, different buffers

---

Fig. 3A–C. Changes in chromatin organization following cell permeabilization by detergent prior to fixation. Cells suspended in CMF-PBS were fixed directly (A), treated with detergent simultaneously with the addition of fixative (B), or fixed 30 s after addition of detergent (C). Cells fixed directly in PBS, or any of the buffers tested (data not shown) show chromosomes with large-scale chromatin organization (arrows A, B) similar to that observed in chromosomes isolated in Buffer A. Chromosome borders were smooth and well defined, and large-scale domains approximately 130 nm in diameter were observed. Chromosomes exposed to detergent with fixative showed the same appearance, but chromosomes within cells permeabilized just seconds after detergent showed an unraveling of large-scale chromatin organization. Bar represents 260 nm
of similar ionic strengths can produce radically different chromosome morphologies. Again buffer A produces a morphology which is very close to that seen within intact cells fixed without any prior permeabilization.

**Appearance of human and Drosophila chromosomes isolated in buffer A**

In Figure 5 a closer examination of mitotic chromosomes isolated in buffer A is shown. Either isolated chromosomes or mitotic plates were fixed, embedded in Epon, and sectioned. Samples in the top panel were prepared using either Kc cells, an established embryonic *Drosophila melanogaster* tissue culture line, or *D. melanogaster* precellular blastoderm embryos arrested in metaphase by Colcemid treatment; in the bottom panel samples were prepared from Hela cells, a transformed human tissue culture line, synchronized in mitosis by selective detachment (Terasima and Tolmachev 1963), avoiding the use of any drugs.

Although the *Drosophila* genome is approximately 20 x smaller than the human genome (Lewin 1980), with approximately 1/3 the DNA content per chromosome, the similarities in structure are striking. The chromatid diameters of the *Drosophila* chromosomes appear somewhat smaller, but both show the sharply defined chromosome borders mentioned above, and both show the presence of structural domains, oriented largely transversely to the chromosome axis, and measuring 80–160 nm in width. Subjectively, the majority of these domains appear to be between 120–140 nm in diameter (see arrows in Fig. 5 and histograms in Fig. 7).

Variations in the isolation protocol, including disruption of the mitotic plate via detergent treatment and mechanical shear forces, followed by chromosome isolation in sucrose gradients, and direct embedding of detergent-extracted, but intact, mitotic plates, all yielded metaphase chromosomes of similar morphology. Similar results were also obtained from metaphase chromosomes isolated from precellular blastoderm *Drosophila* embryos arrested by Colcemid, as well as from exponentially growing Kc cells without Colcemid treatment. The appearance of these large-scale structural domains, therefore, cannot be attributed to a mechanically induced distortion of chromosome structure, or to a nonphysiological, Colcemid-induced state. As described below, the observed substructure was also maintained when a different fixation protocol was used (Marsden and Laemmli 1979).

**Transition from mitosis to interphase**

As shown in Figure 4, similar large-scale domains were visualized within interphase nuclei. In Figure 6 interphase nu-
nuclei prepared in buffer A are shown more clearly, as well as the transition from anaphase through telophase, using nuclei prepared from Hela cells. Although nuclei from cellular blastoderm or older Drosophila embryos and from Kc cells show quite similar large-scale domains, the interpretation of the images is less straightforward due to the possibility of tight homologous pairing during interphase (Halfer and Barigozzi 1973) and the uncertainty in determining the cell cycle stage. In Hela cells, the intimate homologous pairing that exists in Drosophila is not present (Cremer et al. 1982; Moroi et al. 1981), and cell cycle synchronization is more easily accomplished.

Beginning in anaphase, the packing between large-scale domains loosens, and the chromosomes appear to decondense largely through the progressive straightening or uncoiling of these higher-order chromatin structural domains. This unfolding coincides with nuclear envelope formation and nuclear enlargement.

Size distribution of large-scale chromatin structural domains

Measurements were made of large-scale chromatin structural domains in cells fixed directly in PBS without prior permeabilization, and in cells permeabilized in buffer A before fixation. Telophase nuclei were chosen because they are easily recognized and it would therefore be possible to make comparisons between nuclei all in nearly the same cell cycle stage. This is important due to the pronounced cell cycle
variations in large-scale chromatin organization discussed below (see Fig. 6).

To facilitate visualization of discrete large-scale chromatin structural domains, OsO₄ fixation was used for the nonpermeabilized cell sample because it provides somewhat greater contrast above the nonspecific staining background. "Fibers" chosen for measurement were linear, spatially discrete structures with nearly parallel edges. The results, in histogram form, are shown in Figure 7. The observed mean (mode) for fiber widths was 126 (114) nm with a standard deviation of 34.5 nm for the nonpermeabilized cells; for the nuclei in buffer A, the mean (mode) was 139 (134) nm with a standard deviation of 26.3 nm. Because these measurements are made from projections through 200 nm thick sections in which these large-scale chromatin domains are oriented randomly, it is likely that the actual width distribution is narrower than that estimated from these projected images.

The coefficient of variation (standard deviation/mean) for these measurements, 19% for nuclei in buffer A and 27% for nuclei from nonpermeabilized cells, compares well with values of 25% for 30 nm fibers from erythrocyte chromatin in 20 mM NaCl (Woodcock et al. 1984) and 10% for the more ordered 30 nm Necturus erythrocyte chromatin in 75 mM univalent cation (Williams et al. 1986). Together with the subjective impressions of fiber-like parallel edges over lengths greater than several hundred nanometers, these results indicate that the observed tight folding of chromatin 30 nm fibers, in a significant fraction of the total chromatin, is occurring within structured, rather than random, aggregates.

Due to the possibilities of slight differences in cell cycle stages between the two sample populations, a careful, quantitative comparison of these statistics was not done. It is clear, however, that these large-scale chromatin domains are of comparable levels of condensation in intact and permeabilized cells prepared in buffer A.

Examination of exponentially growing, nonsynchron-
figs. 7A, B. Histograms of large-scale domain size in telophase Hela nuclei, as measured from 200 nm thick Epon sections (see text). A Within cells fixed in PBS without any prior permeabilization. B Within cells permeabilized in buffer A, prior to fixation.

ized cell populations revealed that the majority of nuclei contain similar large-scale chromatin domains, now clearly visualized as spatially discrete, linear structures, although there was a large variation in the size distribution of these domains in different nuclei. This variation was largely cell cycle, rather than procedural, in origin: using a double thymidine block to arrest the majority of cells in S-phase, a more homogeneous nuclear population was observed. Most of the nuclei had a greatly reduced fraction of chromatin present as 100–160 nm diameter domains, with an increase in individual 24 nm fibers and 40–100 nm domains. This is consistent with other measures of chromatin condensation, including affinity for intercalating agents (Pederson 1972; Nicolini et al. 1975), circular dichroism (Nicolini et al. 1975), DNase I susceptibility (Pederson 1972), and overall nuclear size and shape (Kendall et al. 1977), all of which have shown chromatin condensation to reach a minimum at S-phase. Nuclei obtained from a cell population synchronized by selective detachment and fixed at 3–4 h after mitosis in G1 also demonstrated a more homogeneous nuclear morphology, with less heterogeneity in the size distribution of large-scale domains and a larger population of approximately 130 nm diameter domains (data not shown). Two representative nuclei demonstrating the two extremes of this observed variation are shown in Figure 6D, E. This large-scale interphase and mitotic chromatin organization was not dependent on detergent treatment. Eliminating either digitonin or Brij 58 from the protocol, or eliminating Brij and reducing the digitonin fivefold had no effect on the appearance of either mitotic chromosomes or inter-

phase nuclei. Similarly, experiments in which cells were permeabilized to buffer through mechanical disruption in a Dounce homogenizer, rather than by detergent treatment, produced no change in morphology.

Titration of large-scale organization by varying the polyamine or divalent cation concentrations

To understand the origin of the differences in large-scale chromatin organization in chromosomes prepared in buffer A or CMF-PBS, several variant buffers were also tested. Figures 8, 9 show examples of mitotic chromosomes and interphase nuclei prepared by identical protocols but varying the amounts of the polyamines, spermine and spermidine, in the normal buffer A formulation.

Mitotic chromosomes prepared in buffer A with no polyamines show a radial loop appearance similar to that found in CMF-PBS or that previously described for chromosomes slightly swollen in low Na, 1–1.5 mM MgCl₂ buffers (Marsden and Laemmli 1979; Adolph 1980). The chromosome borders are substantially more irregular than when polyamines are present.

As the polyamine concentrations are increased, localized, irregular regions of loosely packed 20–30 nm diameter chromatin fibers appear within interphase nuclei; at higher polyamine concentrations these regions become further compacted, with sharper boundaries and more uniform widths along a linear axis. In mitotic chromosomes, increased polyamine concentrations result in increased chromatin packing and smoother chromosome borders. At polyamine concentrations 0.5 × the normal buffer A concentrations, large-scale domains are discernible, becoming more easily distinguishable as concentrations increase further. Presumably this is a consequence of increased condensation, where the increased compaction of large-scale domains results in better separation of adjacent domains and greater boundary contrast. At 2 × concentrations these large-scale domains are significantly more easily observed within mitotic chromosomes than for normal buffer A. The average domain width appears to have shifted to approximately 80–100 nm from 120–140 nm in 1 × buffer A. This suggests a use of overcondensation to highlight the larger levels of chromosome architecture.

A similar titration of large-scale chromatin structural domains was observed using a low Na, sucrose buffer and varying the divalent cation concentrations. With 1.0 mM Ca²⁺, 1.5 mM Mg²⁺ (buffer C), spatially discrete, large-scale chromatin domains, mostly 80–100 nm in diameter, are seen in both mitotic chromosomes and interphase nuclei. At lower concentrations (0.75 mM Ca²⁺, 1.12 mM Mg²⁺) a radial loop conformation is obtained for mitotic chromosomes similar to that produced in buffer A with no polyamines (see Fig. 10).

PBS with varying Ca and Mg levels was also tested, using up to 6 × the normal divalent cation concentrations. As expected, at low divalent concentrations (<0.9 mM Ca²⁺, 0.5 mM Mg²⁺) mitotic chromosomes have a radial loop appearance and interphase nuclei show dispersed chromatin. At higher divalent cation concentrations, mitotic chromosomes form compact, dense structures, but without discrete substructure. Under these conditions, interphase nuclei show local chromatin bodies 100 nm diameter, but more irregular in width than what is observed in buffer A or buffer C (data not shown).
Variation of fixation conditions

The above results document a pronounced dependence of large-scale chromatin organization on the exact concentrations of divalent or polyamines. The observed variations in large-scale chromatin organization mimic to a large extent the differences reported in chromosome structure in the literature. One additional difference between our experimental conditions and those used by others (Marsden and Laemmli 1979; Adolph 1980) was the fixation protocol.

Fig. 8A–D. Titration of large-scale chromatin organization within mitotic chromosomes by variation of polyamine concentrations. Hela metaphase chromosomes prepared using 0 × (A), 0.5 × (B), 1 × (C), or 2 × (D) the usual spermine, spermidine concentrations in Buffer A. Sections are 200 nm thick. At low polyamine concentrations, 20–30 nm diameter fibers are the highest level of chromatin organization apparent. At higher polyamine concentrations, large-scale domains are observed which decrease in diameter with increasing polyamine concentrations. Bar represents 250 nm.

Fig. 9A–D. Titration of large-scale chromatin organization within Hela interphase nuclei (see Fig. 6). Nuclei were prepared 0 × (A), 0.25 × (B), 1 × (C), or 2 × (D) the usual spermine, spermidine concentrations for Buffer A. At given polyamine concentrations, nuclei show the same size large-scale domains recognized in mitotic chromosomes. Bar represents 1 μm.
As a last control, therefore, the previously described experiments involving titration of spermine and spermidine with buffer A, or Ca and Mg with buffer C, were repeated using a fixation protocol described by Marsden and Laemmli (1979). At standard spermine and spermidine levels with buffer A, or Ca and Mg levels with buffer C, the fixation protocol made no obvious difference. At low polyamine concentrations in buffer A there was a significant improvement in the preservation of the 20–30 nm diameter chromatin fibers with the Marsden-Laemmli protocol, but no change in the overall distribution of chromatin fibers within mitotic chromosomes or interphase nuclei. At intermediate polyamine levels, results were similar, although there was a slight decrease in the concentrations of polyamines necessary to achieve a given level of condensation. This is not surprising given the 50× difference in glutaraldehyde concentrations and the reactivity of polyamines to glutaraldehyde (see Materials and methods). At low Ca, Mg levels with buffer C differences using the Marsden-Laemmli fixation protocol, if any, were small.

Visualization of the large-scale chromatin structural domains as spatially discrete, linear elements

The preceding figures demonstrated results obtained from 200 nm thick Epon sections. Because the width of these sections is only slightly larger than the diameter of these domains, it is difficult to appreciate the spatial extent and three-dimensional shape of these features.

In Figure 11, stereopairs from 500 nm thick sections are presented. Due to the greater contrast between adjacent large-scale domains, a stereo pair of a metaphase chromosome prepared in buffer A with 2× spermine and spermidine concentrations is shown in Figure 11A. Shown in B is a section from an interphase nucleus prepared in normal buffer A. The large-scale chromatin structural domains can be visualized as fiber-like structures, with roughly cylindrical cross sections, extending along the fiber axis over distances greater than several hundred nanometers, before they are obscured by overlapping structures or exit the physical section. In favorable regions the internal packing of 24 nm diameter chromatin fibers can be observed as well.

Discussion

We have provided evidence that a large fraction of chromatin is packaged in vivo within spatially discrete, large-scale domains, with a mean width of approximately 130 nm in telophase nuclei. In mitotic chromosomes and other interphase cell cycle stages, similar large-scale domains of qualitatively similar size are present. This conclusion is based on the following observations:
1. By fluorescence light microscopy, the appearance of both mitotic and interphase nuclei within living cells is indistinguishable from that observed within cells fixed in either CMF-PBS or a high K, polyamine buffer, buffer A. Similarly, in Epon-embedded specimens prepared for the electron microscope, chromosome morphology is similar within cells fixed in either buffer. By contrast, when cells are permeabilized before fixation, significant differences are observed. In CMF-PBS, chromosome borders become noticeably diffuse and large-scale chromatin domains, appearing as threads within interphase nuclei in living cells, disappear; in the electron microscope no obvious substructure above the level of the 30 nm fiber is seen. Alternatively, in buffer A the light microscope appearance of both mitotic chromosomes and interphase nuclei is unchanged from that observed in living cells, while the electron microscope appearance closely resembles that seen with either buffer in non-permeabilized cells. Structural domains, approximately 120–140 nm in width, are seen oriented largely transverse to the chromosome axis in mitotic chromosomes. Within interphase nuclei, similarly sized domains, present as spatially discrete entities, are observed which correspond to DAPI-staining threads seen in the light microscope. Measurements from telophase nuclei yield a mean width for these domains of 126 nm from nonpermeabilized cells versus 139 nm from cells permeabilized in buffer A before fixation.

2. In stereopairs from 500 nm thick Epon sections, examined with an intermediate voltage microscope, these large-scale structural domains appear to be fiber-like structures, and are seen extending linearly for greater than several hundred nanometers, before crossing or overlapping with

Fig. 11A, B. Stereopairs from Semithick Epon sections 500 nm thick of a Hela mitotic chromosome prepared in Buffer A with 2× the normal spermine, spermidine concentrations (A) and a Hela interphase nucleus prepared in normal Buffer A (B). Arrows point to large-scale domains. The nucleus in B was from a cell population synchronized in early G1 (2.5–3.5 h after mitosis). Pictures were taken at 300 kV. Negatives used in A were digitized, mass normalized, and digitally contrast enhanced as described previously (Nicolini et al. 1975). (The small, dot-like features scattered over (A) are gold beads placed on the section surfaces for use as fiducial marks in topographic reconstructions.) Bar represents 100 nm in A and 520 nm in B.
other structures. Even within interphase nuclei, the paths of these domains are tightly coiled and kinked, explaining the failure to perceive these spatially discrete, linear domains from thin sections. For the same reason, it is likely that these large-scale, fiber-like structural domains extend for even longer distances, which cannot be appreciated from stereopairs due to the problem of overlap.

Preliminary experiments using low temperature embedding methods (−60°C) with Lowicryl K-11 resin on fixed specimens rapidly frozen in liquid propane show similar-large-scale structure. In addition, a recent axial tomographic reconstruction from a 0.5 μm thick section of an early G1 Hela nucleus has shown large-scale domains which can be traced over long distances (A. Belmont, J.W. Sedat, D.A. Agard, unpublished data).

Relationship to previous studies

Titration of the spermine, spermidine concentrations produced pronounced changes in the large-scale chromatin organization. At low polyamine concentrations, all apparent large-scale organization above the 24–30 nm diameter chromatin fiber was lost within mitotic chromosomes. However, only a small change in overall chromosome width was observed. Interphase nuclei showed chromatin distributed throughout their interior; again no large-scale organization above the 24–30 nm diameter chromatin fiber was observed. With increasing spermine, spermidine concentrations, large-scale features became increasingly visible and progressively more uniform in size and shape in both mitotic and interphase chromosomes. Mitotic chromosomes required higher polyamine concentrations for this larger scale chromatin organization to become apparent, presumably due to the difficulty in visualizing closely packed structural units. At the level of overall morphology, these polyamine-dependent conformational changes were reversible. A similar trend was observed with titration of Ca, Mg levels using Buffer C, a low Na, sucrose isolation buffer, although structural preservation was better with Buffer A.

This demonstration of widely varying chromosome morphologies produced by simple buffer variations reconciles our results with conflicting reports in the literature. It is also consistent with previous comparisons, by low angle X-ray diffraction, of chromatin packing within living cells versus mitotic chromosomes and interphase nuclei isolated in various buffers (Langmore and Paulson 1983). In that study, the authors followed a reflection corresponding to a 30–40 nm spacing (attributed to close packing of adjacent 30 nm chromatin fibers) during titration of Mg levels. At low Mg levels, while the reflections attributed to the 30 nm fiber itself remained unchanged, the reflection related to close packing of the 30 nm fibers either disappeared or shifted to a 50–60 nm spacing; this corresponded to dispersal of chromocenters within erythrocyte nuclei. Buffer conditions similar to buffer A or C, which preserved the large-scale chromatin domains described in this paper, preserved the 30–40 nm spacing of fibers characteristic of the pattern present in living cells.

While our results clearly demonstrate that certain buffers, which result in mitotic chromosomes with a dispersed, radial loop appearance, greatly perturb chromosome structure from its in vivo conformation, they do not necessarily define the optimal buffers for preserving in vivo conformation. The light microscopy observations are not sufficient in resolution to distinguish between buffers A or C, for example. Our conclusions with regard to higher resolution conformation depend on observations within embedded samples of cells fixed directly without permeabilization. There still may be slight perturbations in the large-scale chromatin organization introduced by fixation, dehydration, and embedding artifacts; larger perturbations are ruled out by the light microscopy results. Also the problem of nonspecific staining by uranyl and lead stains, and cell cycle variation, makes difficult precise, quantitative comparison of sizes of large-scale chromatin domains in nonpermeabilized cells versus cells permeabilized in various buffers. The important point to be stressed, however, is that independently of whether the actual mean domain width is 100 or 140 nm, these large-scale chromatin domains are a fundamental feature of chromosome architecture in vivo.

Previous conclusions that distinct chromatin folding motifs above the 30 nm diameter fiber do not exist, therefore appear to be based on artifacts created by inappropriate buffer conditions.

Implications with regard to chromosome structure

Our observations described in this paper are inconsistent with a radial loop model (Marsden and Laemmli 1979; Adolph 1980) of mitotic chromosome structure. The 120–140 nm diameter chromatin domains cannot be explained as a subtle artifact from superposition of radial loops organized in some secondary lateral packaging, because despite their convoluted paths they can be visualized within interphase nuclei as discrete, cylindrical structures extending linearly over distances from 200–500 nm. Work now in progress, involving three-dimensional reconstructions from electron microscopic tomographic data, also suggests that these 130 nm domains are not themselves organized as simple radial loop structures (A. Belmont, J. Sedat, D. Agard, unpublished results).

The apparent radial loop organization of chromosomes in certain buffers can be explained simply as an unfolding and possible disassociation of these large-scale chromatin domains; given that the observed separation between adjacent approximately 120 nm diameter domains in metaphase chromosomes is no more than several tens of nanometers, any small structural perturbations could easily obscure this larger scale underlying organization.

We favor a model, therefore, where these large-scale domains reflect spatially distinct and fundamental chromatin-folding motifs. Mitotic chromosomes would progress out of and into interphase through the complex coiling and uncoiling or association and disassociation of these domains.

The detailed internal structure of these large domains still needs to be elucidated; within these domains, for instance, chromatin may still be organized as looped structures, although our preliminary results from three-dimensional reconstructions do not support a simple radial orientation for these loops. To trace directly the path of the basic 20–30 nm chromatin fiber within these structures, we are currently preparing high resolution three-dimensional reconstructions using over 100 tilted views (Belmont et al. 1987; A. Belmont, J. Sedat, D. Agard, unpublished results).

Acknowledgements. This work was supported by the Howard Hughes Medical Institute and grants GM-31627 (DAA) and GM-
25101 (JWS) from the National Institutes of Health, and a NSF Presidential Young Investigator grant (DAA). ASB was supported by a Damon Runyon-Walter Winchell Cancer Fund Fellowship, DRG-#769, and a National Institutes of Health postdoctoral grant, GM-11317-02. We wish to thank Dr. Elizabeth Blackburn for her critical reading of this manuscript.

References


Received December 1, 1988 / in revised form March 2, 1989
Accepted by S. Wolff