Interphase chromosome positions and structure during silencing, transcription and replication

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Introduction

Before 1975 there was little appreciation of the high degree of chromosomal folding within interphase nuclei. Classical cytologists were limited to descriptions of different chromatin staining patterns in cells of different lineage. Condensed chromatin, which was stained darkly by basic dyes, was known to include the inactivated X chromosome in female cells (Barr body). Such dense or heterochromatic regions of the nucleus were all assumed to be genetically silent. The other major compartment for DNA was called euchromatin. This 'good' chromatin was thought to be 'open' and possibly completely unravelled, a necessary structural prelude for RNA transcription and subsequent synthesis of proteins essential for life.

Biochemical fractionation of chromatin from disrupted nuclei and measurements of accessibility of chromatin to various molecules such as DNAases yielded insight into basic histone–DNA interactions. At the same time sophisticated Fourier transformation and reconstitution experiments elucidated the structural details of DNA–histone complexes as they form nucleosome fibres. However, nucleosome threads were beyond the limits of conventional light microscopy, having diameters or widths of about 10 nm (Manuelidis and Chen 1990; see Chapters 1–3). How these threads are folded into visible interphase chromosome fibres, or placed within large euchromatic and heterochromatic regions of the nucleus, remains a fundamental mystery.

Several paradoxes suggest that the term euchromatin may be too broad or insufficiently precise. For example, only a small portion of the mammalian genome (less than 5 per cent) is directly involved in transcription for protein production, yet in some cells, such as large neurones, the nucleus is almost entirely euchromatic. Therefore, at least some transcriptionally inactive regions of DNA appear to be in a structurally similar state as active genes or exons. Second, metaphase chromosomes contain many bands that are relatively heterochromatic or euchromatic, based on their staining properties as well as their molecular signatures (Manuelidis 1990). Each of these visible intrachromosomal

metaphase bands contains ≥ 300 000 bp of DNA (Manuelidis and Chen 1990), yet these bands cannot be correlated in any simple way with the large (1–3 μm) cohesive regions of euchromatin and heterochromatin in each nucleus. Because each chromosome lies within a defined nuclear space or territory (Manuelidis 1985b; Cremer *et al.* 1988), its internal heterochromatic bands cannot be spatially related to the few large heterochromatic compartments in the nucleus. Indeed, when nuclear structure is preserved, euchromatin does not diffusely extend from each chromosome, but remains in close proximity to its parent.

The purpose of this chapter is to suggest that only very small structural changes are needed for transcriptional activity. More specifically, the following points will be made. First, both gene activity and silencing can involve highly local and subtle structural modulations, i.e. transcription can be associated with very small and possibly rapid transitions in structure that are not readily visible. In this process, the higher order folding of the chromosome remains intact. Second, the dominance or proportion of specific types of non-coding ('junk') DNA can define either the silencing or the specific recruitment of large intrachromosomal bands in the functioning nucleus. Third, chromosome arms with an overwhelming preponderance of condensed heterochromatin can have a high degree of transcriptional activity. Surprisingly, many of these highly folded regions actively produce RNA transcripts. Fourth, in cultured cells there is a remarkable congression of these heterochromatic chromatids with each other and with the nuclear membrane. This suggests that recognition of like chromosome domains is achieved not only at a molecular level, but also at a global level of nuclear organization.

The above observations imply that our concepts of heterochromatin and euchromatin need major revision. They also beg for a more exact understanding of the types of chromosome unfolding that are required for transcription and replication. In this context I examine the predictions of a chromosome model for its fidelity to new observations. The structural changes that can be seen in chromosomes caught in the act of replication are compared with those seen during transcription. Transcription appears to be far more conservative of structure when judged within the confines of current techniques. In the following overview I cover some of the molecular and structural approaches currently used in our laboratory, and present a very limited sample of unpublished data concerning the above points.

Specific methods: advantages and limitations

The structure and nuclear location of different chromosome domains could not be addressed adequately before non-isotopic methods for labelling nucleic acids were introduced. There were only a few identified antibodies against nuclear proteins that could decorate specific chromosome regions (e.g. centromeres), as well as general fluorescent stains for DNA and RNA. The resolving power of

non-isotopic detection of specific sequences in the nucleus was already apparent by 1982 (Manuelidis *et al.* 1982). Additional methods for tagging DNA and RNA led to many applications, but the most pertinent for delineating interphase chromosome structure are three-dimensional (3D) preservation, and the use of various tags to delineate different functional states such as DNA replication or RNA transcription. Replication can be monitored by incorporation of BrdU during cellular DNA synthesis. At the same time, a specific chromosome region can be specifically labelled with another tag such as biotin or digoxigenin (Manuelidis and Borden 1988). Thus one can focus on events in a defined chromosome domain with known attributes. Similarly, RNA transcription can be simultaneously monitored with antisense probes for RNA, while the active source DNA is labelled with sense probes. However, because DNA is highly folded in the intact nucleus, one will not resolve molecularly adjacent motifs, or visualize very subtle structural changes at the most basic nucleosomal level. Indeed, only with swelling or disruption of native structure is it possible to resolve DNA loci separated by 50–200 kb (e.g. Yokota *et al.* 1995; Lawrence *et al.* 1990), a span greater than most exons. Furthermore, even when nuclear structure is preserved by isotonic aldehyde fixation, the necessary melting of DNA to provide access and reannealing of a labelled probe will distort structure. Fortunately, this change is minimal or not apparent by inspection, even at the ultrastructural level (Manuelidis 1984, 1991; Borden and Manuelidis 1988). Thus the first limitation for all sequence-specific DNA detection is its inability to describe very subtle structural alterations at the nucleosome or folded nucleosomal (solenoid) level. On the other hand, major unravelling of the larger compact interphase chromosome fibres can be evaluated, as shown later.

A second limitation is that fixation is required to capture the cell at an instant in time. A dynamic view is gained only by following a given domain over a relevant period of time. This can easily be done in a developmental setting to show large changes in nuclear organization, as was done in initial studies of neuronal maturation (Manuelidis 1985a). Nonetheless, there may be extremely rapid and local changes in substructure that cannot be evaluated by this approach. Alternative methods for the study of specific sequences in living cells are needed to address this issue.

Various enzyme-linked detectors can be used to examine hybridized domains at the ultrastructural level. We have found that peroxidase rather than larger gold ligands are most useful for this purpose, because they penetrate more consistently into highly folded and contracted heterochromatic regions (e.g. see Borden and Manuelidis 1988). Although electron microscopic (EM) examination provides a very good indication that the conditions we use for fixation and hybridization are non-disruptive, serial EM sections are usually required to delineate each labelled 3D chromosome domain. This approach is very labour intensive and is subject to alignment errors. High voltage EM of whole nuclei is also often inadequate, because more condensed chromosomes as well as other nuclear bodies can obscure the region of interest. Furthermore, the preparation of whole mount

nuclei removes the fundamental relationships and orientations to surrounding cells and to culture substrates. An example of these types of oriented relationships that would have been lost with nuclear isolation is illustrated in Plate 2 (e–g).

At the present time the best trade-off in terms of resolution and 3D visualization is confocal microscopy. More than one probe can be simultaneously evaluated and this is especially useful when the second fluorescent reporter is used to define a functional state. When the optical sectioning capacities of the confocal microscope are enhanced by deconvolution, and structures are studied in 3D rotations after reconstruction, a significant number of details and relationships can be brought forth. On the other hand, non-specific background fluorescence can be very high in tissues such as mammalian brain and this currently limits dual fluorescent detection to simpler samples such as cultured cells. Nevertheless, a number of experimental tissue culture models can be used for insertion of inducible genes, with controlled exposure to appropriate chemicals or treatments to affect cell physiology. We have used this approach to evaluate changes in an inducible and large transcriptional domain created by recombinant techniques, as briefly discussed below.

Confocal analysis

Figure 9.1 shows an overview of the process we use. For *in situ* hybridization we take great care to avoid swelling, protease treatment, and any drying of cells that can distort 3D structure. Only short detergent treatment after paraformaldehyde fixation is required to allow penetration of 100–400 nt long tagged sequences (Manuelidis and Ward 1984). We use a Leica confocal microscope because it has a very precise and reproducible piezoelectric stepper for collection of sequential optical sections. This machine can also rapidly display x–z as well as x–y sections for orientation of domains in the nucleus (see Plate 2). The correct z-axis proportions are periodically checked using 0.5 μm fluorescent beads. To ensure accurate overlays of each fluorescent signal, two fluorescent channels are simultaneously evaluated (green and red, typically FITC and either rhodamine or Texas red reporters). The whole nucleus is then resectioned with UV excitation for Dapi fluorescence of the whole nucleus (Fig. 9.1, step 3) using the same z-step origin and increments (0.15 or 0.25 μm steps). Our video sit camera (Cohu) for Dapi images has been mounted with a variable zoom lens and adjustment screws for reasonably precise video alignment (within two pixels) at any magnification. It can also be used to rapidly verify fluorochrome alignments of the red and green confocal channels by epifluorescence. A minimum of four frames of 512×512 pixels are collected for every z-axis step in each of the three fluorescent channels. The largest magnification to accommodate the structure of interest is used for each group of stacks, the laser is run at the lowest power setting possible to minimize photobleaching, and the pinhole is set to a relatively small size for optimal resolution. The video memory TCD board in our system

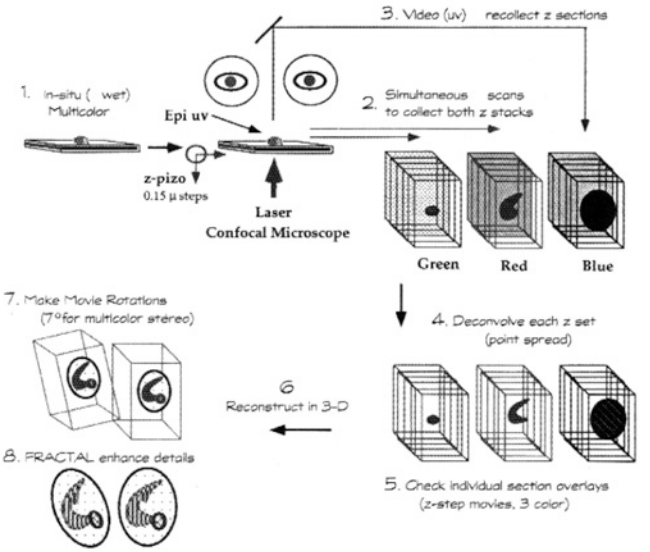

Fig. 9.1 Steps in the collection and analysis of confocal sections. For details see text.

allows us to collect as many as 40 slices for each of the three channels and is also later used to play three colour movies (Fig. 9.1, steps 5 and 7).

To obtain the most detailed information a point spread deconvolution is done on each serial image in the three channel stacks. This further removes out-of-focus fluorescence, but it is important that the brightness, contrast, and degree of deconvolution do not remove information (Fig. 9.1, step 4). Rapid deconvolutions are made possible using a 486 PC with an array processor (PL800, EighteenEight Labs) and deconvolution software (Vaytek Inc.). The resulting stacks are then displayed as individual three-colour sections to verify overlapping signals. The stacks, or relevant portions of the stacks, are also displayed as stereo pair rotations using maximum projection to reconstruct the 3D image for each stack. These primary data are far more informative and exact than interactive reconstructions used previously (Manuelidis and Borden 1988; Borden and Manuelidis 1988). Each rotation is also viewed as a movie or stereo movie with two or three colour channel overlays (Fig. 9.1, step 7). Fractal enlargement is sometimes used on regions of interest (software from Images Inc.). In principle,

fractal analysis will find statistically connected spatial patterns. Details such as loops made of solenoid-like fibres within, or extending only minimally from the basic interphase fibre, can be improved with this routine (see Fig. 9.3). For reference a solenoid fibre is about 30 nm thick and consists of a wound nucleosome fibre (see Fig. 9.2). Finally, no sharpening or other filters are used except for removal of random single pixel camera noise (Adobe Photoshop software).

Shared sequence signatures

The alphabet of coding DNA specifies gene products, but is insufficient for defining the orderly recruitment of genes on different chromosomes. Transcribed sequences are punctuated by non-coding sequences, and it is likely that paragraphs and chapters in the genome are recognized at least in part through repeated non-coding sequences. Different repeat motifs predominate in euchromatic and heterochromatic chromosome bands, and each repeat type is interspersed with particular classes of coding motifs. In human cells for example, tissue-specific genes that are developmentally regulated are generally found in the more heterochromatic and late replicating bands with abundant LINE sequences. In contrast, shorter interspersed Alu repeats and GC-rich sequences strongly favour the more euchromatic bands (Manuelidis and Ward 1984; Chen and Manuelidis 1989). Additional less numerous interspersed repeats can define a more limited subset of bands on only a few chromosomes. These types of observations led to the suggestion that different repeat motifs or 'sequence signatures' may be key elements in the regulation of genetic activity (Manuelidis 1990). In principle, as few as 10 unique repeats can combinatorially specify over 3×10^6 distinct or partially related domains on different chromosomes. Thus their strategic positioning could be part of a global indexing system. This system may provide the necessary recognition for multiple sets of genes recruited at a given time, as for example during development or stress.

Some of the following experiments show that it is possible to create different types of chromosome domains using recombinant approaches for inserting repeated DNA motifs. These domains are correctly recognized by the cell, and remarkably are organized in particular ways in the interphase nucleus. I will discuss the systematic examination of very heterochromatic to more euchromatic domains with reference to shared sequence signatures, replication time, and spatial positioning within the interphase nucleus. At the same time I will address the visible features of structural change that accompany a motif in G_1 and during transcription and replication. To understand or evaluate these transitions it is necessary to refer to the pertinent features of a folded chromosome model previously detailed (Manuelidis and Chen 1990; Manuelidis 1990).

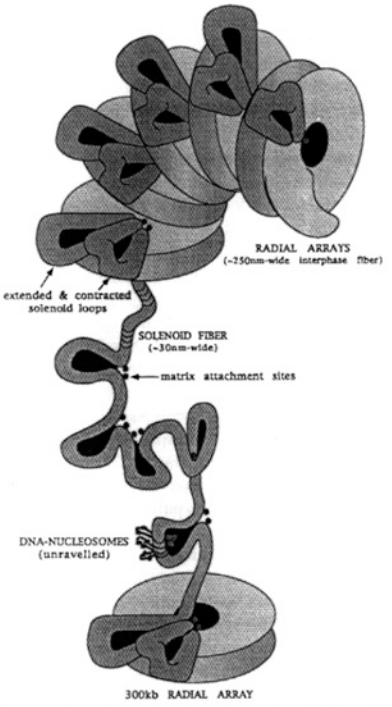

Fig. 9.2 Simplified model of part of an interphase and puffed haploid chromatid (top) giving rise to more unravelled solenoid fibres and completely unravelled nucleosomes (middle of diagram). Likely sites where matrix proteins may bind or tether DNA are also depicted at a few sites (filled circles). Alternatives addressed here include a puffing model where extended solenoid loops of about 120 kb permit access to proteins needed for transcription, but maintain the integrity of compact radial arrays. In contrast, greater unravelling disrupts the basic chromatid structure. Only a short portion of the curving intact chromatid is shown (about 250 nm in width when all its solenoid loops are contracted). In super heterochromatin the chromatid can coil upon itself (Manuelidis 1990). Such tight coils yield ~750 μm wide metaphase chromatids. For scale models and DNA compaction calculations see Manuelidis and Chen (1990).

How extended is an interphase chromosome?

In principle, the extent of chromosome unfolding needed for transcription or replication can be extreme. Figure 9.2 shows an idealized and simplified diagram

of the basic haploid interphase chromosome fibre or chromatid. We previously proposed that this fibre is formed by loops of smaller solenoid fibres that can extend out or 'puff' during transcription, as depicted in Fig. 9.2. Each extended solenoid loop contains about 120 kb of DNA and such puffed loops (top) would only slightly increase the diameter of the interphase fibre, to ~350 nm. For comparison, this fibre is about 250 nm wide in its most contracted and transcriptionally silent configuration during prophase. Each horizontal segment or turn is arbitrarily defined as a radial array and can accommodate about 300 Mb of DNA (Manuelidis and Chen 1990). Thus only a simple relaxation or puffing of the radial array would be needed to synthesize RNA from any surface of the ~30 nm solenoids (see interior black spaces that are in continuity with the surface uncoloured space). In contrast, a more conventional view is that solenoid fibres are very unravelled or completely open during transcription and replication, as depicted in the central portion of Fig. 9.2. In this case solenoid fibres would not be organized in coherent radial arrays, and thus DNA should be found at a distance from the interphase chromatid. Given the current methods, it is possible to find whether transcriptionally active or replicating DNA is very closely associated with the large and reasonably compact chromatids, or alternatively if it is unravelled. In the latter case the chromatid would be either interrupted by less structured solenoid and nucleosome threads, or these threads would extend well away from the chromosome periphery. The transcriptional studies below support a modulated small relaxation or puffing model for transcription, rather than major unravelling. Furthermore, even in the most extended fibres that are decorated with domain-specific probes, loops of small 30–40 nm wide fibres are visible (see Fig. 9.3, right arrow). Whether these solenoid loops are wound exactly as proposed (Manuelidis and Chen 1990) cannot be ascertained at the confocal level of resolution (*vide supra*).

At the other extreme are silent regions. These maintain a metaphase or contracted folding pattern. In this case the interphase chromatid has an additional helical turn (Manuelidis and Chen 1990; Manuelidis 1990). Some regions of the chromosome, such as those containing long silent tandem repeats of DNA, can be tightly coiled in interphase. Previous studies have shown that 7–11 Mb of silent DNA is organized in the predicted coils in interphase (Manuelidis 1991). Very tight and contracted coils have the final well-described width of each sister chromatid in metaphase (about 750 nm), a time at which genetic silencing is complete. Replication may represent the other extreme of permissible unfolding. Our model proposes that after replication each chromatid will be nested with its sister to yield a cohesive single thicker fibre in which each of the chromatids cannot be morphologically distinguished. The pulse labelling studies I have done with short exposures to BrdU are consistent with this view. However, during replication, which progresses very rapidly, some labelled regions are clearly unravelled. The reasons for greater, albeit transient, disruption of chromosome structure during replication compared to transcription are probably best understood from a biological perspective, as later discussed.

Long tandem repeats define super heterochromatin

Experiments with high copy number tandem repeats, such as major satellite DNAs on different chromosomes, have shown that these chromosome regions can adhere to each other in the interphase nucleus. The degree of association is extreme. Centromeric regions with little or possibly no coding DNA, as well as subcentromeric domains with ≥ 9 Mb of multiple short tandem DNA repeats, are compartmentalized together in the nucleus. Indeed, these repeats can be coalesced to such a degree that individual chromosome regions within these dense bodies are not recognizable as separate structural elements (Manuelidis 1984). Only by labelling repeats on a single chromosome is it possible to define the limits of each individual element in the large heterochromatic body. Interestingly, the 3D positioning of these highly heterochromatic bodies in the nucleus (hereafter called 'super heterochromatin') can be tissue and cell lineage specific. Different patterns of congregation are characteristic and unique for different neuronal and glial populations (Manuelidis 1984; Manuelidis and Borden 1988; Borden and Manuelidis 1988). Moreover, the pattern of super heterochromatin in each neuronal subtype is maintained in evolution regardless of the details of the alphabet or sequence of the tandem repeat (Manuelidis and Borden 1988).

To further define the necessary requirements for super heterochromatin we studied transgenic mice with an inserted repeat of about 11 Mb in length (Manuelidis 1991). This DNA was confined to a single chromosome locus that was distant from the centromere. A β-globin exon that was devoid of interspersed repeats was the sole element. Although each unit of this tandem repeat was relatively GC rich and longer than many major satellite DNAs, we hypothesized that this construct would make its chromosome locus behave as other centromeric loci with natural tandem repeats. Thus, this new domain should maintain a very heterochromatic or condensed state in G_1 cells. It should also be positioned together with other natural satellites in large bodies of super heterochromatin in the nucleus. These predictions were fulfilled. This enormous transgene was completely without effect on mouse development. Silencing appeared to be solidified by the physical segregation of this transgene into coalesced bodies with other centromeric satellite DNAs in the nucleus. Furthermore, the transgene was organized in a very cell type-specific manner as determined by assessing nuclear positions in different types of neurones (Manuelidis 1991). Electron microscopy also confirmed that these inserts formed more coiled fibres, as predicted by the model.

Although the experiments have not yet been done, I would suspect that interspersion of only a few copies of a promoter–exon sequence into these long tandem arrays would be insufficient to override the dominant parameters of silencing created by the long tandem repeats. In contrast, insertion of many tandem repeats of DNA containing promoter–exon–enhancer sequences can override the natural transcriptional characteristics of the local domain into which

they insert. Moreover, such genetically competent inserts can be specifically activated to produce RNA given the proper stimulus (*vide infra*). This is the contrary form of sequence dominance.

Interspersed repeats that define heterochromatic chromosome arms

It is not yet possible to insert multiple interspersed repeats in defined regions of the genome to test the importance of these repeats in chromosome recognition and function. However, nature has provided us with a remarkable example of almost completely 'heterochromatic' chromosome arms for study. In the Syrian hamster a defined set of chromosome arms show a 'constitutive' heterochromatic Giemsa staining pattern. I use the term β-heterochromatin here to distinguish these regions from large arrays of silent and truly constitutive super heterochromatin. All the β-heterochromatic arms of the Syrian hamster contain many interspersed copies of an endogenous retroviral intracisternal A particle (IAP) sequence, while other chromosome arms have few, if any, of these interspersed repeats (Taruscio and Manuelidis 1991). This feature makes it possible to completely delineate these arms without using dextran sulphate, a chemical necessary for adequate detection of single copy genes that disrupts and distorts interphase chromosome structure. IAP-labelled probes decorate the entire length of 10 different chromosome p-arms, as well as the entire q-arm of the X chromosome. In a comparative study of repeats it was pertinent to evaluate the structure and overall organization of these chromatids in interphase nuclei. For example, would they be organized together as like domains of super heterochromatin, or would they instead be widely dispersed in the nucleus? Double label studies of cells in G_1 and in S phase were also used to test the accuracy of the nested chromosome replication model. Additionally, because we had found that IAP sequences are actively transcribed both in the brain and in cultured cells, it was relevant to see if IAP RNA transcripts were produced only by a few unravelled domains, or if they arose from numerous sites along the compactly organized chromatids.

There was a remarkable congression of these β-heterochromatic IAP-rich chromosome arms in G_1 nuclei of cultured cells. These findings emphasize that 3D interphase collections of like chromosome domains are not restricted to super heterochromatic and transcriptionally inactive motifs. Thus, similar chromosome domains, defined by repeated coding DNA, can be organized together in the nucleus, possibly for functional purposes. The majority of the IAP-rich chromatids associated together at the nuclear edge, although some were in more interior portions of nucleus. Some of the arms so closely apposed the nuclear envelope that they could be used to define the peripheral border. In most cells the majority of these arms were at the top of the nucleus (pointing to the feeding medium) and only a few were at the bottom nearest coverslip attachment sites. Plate 2(g) for example shows this typical pattern where the IAP hybridization sites are depicted in red. In this reconstruction, representing about 2 μm of

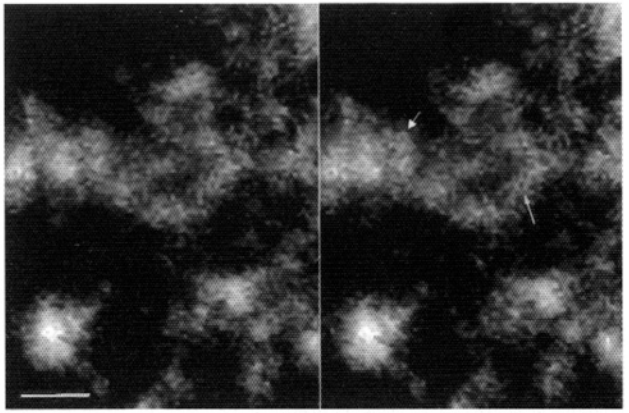

Fig. 9.3 Stereo pair of endogenous retroviral intracisternal A particle (IAP)-labelled chromatids in the nucleus after point spread deconvolution of serial sections and fractal magnification to visualize fine details. Cells were treated with Aza C for 4 h to increase IAP transcripts. The most extended labelled chromatids are shown, with a more compact chromatid at lower left for comparison. Note small loops (small circular features at right arrow), interpreted as solenoid loops of the chromatid. Left arrow points to a C-shaped interphase fibre, again containing small integral solenoid-like loops. Bar is 1 μm. Stereo for cross-eye viewing (turn upside down for viewing with stereo glasses).

through focus sections, the individual labelled chromatids are not well resolved from each other, especially because they have not been deconvolved as in Fig. 9.3. However, detailed structural studies showed more coiled 0.8–1 μm wide fibres (like super heterochromatin), as well as more relaxed interphase chromosome fibres that are about 0.4 μm wide in G_1 nuclei (Fig. 9.3). Note the numerous small loops or circles of smaller solenoid-width fibres within and at the surface of the relaxed interphase fibres (Fig. 9.3, right arrow). For reference, at the lower left of Fig. 9.3 a very compact and probably supercoiled IAP region is seen. None of the IAP solenoid loops extended far from their origin in G_1 cells. Interestingly, the degree of chromatid coiling was not readily related to the 3D position of the IAP chromatid in the nucleus.

Studies of cells pulsed with BrdU for only 15 min and then arrested by fixation were used to assess changes in the structure of these chromatids during replication. The general replication pattern of BrdU incorporation in early S-phase nuclei is characteristically diffuse, whereas when BrdU is incorporated later in replication, large and more compact bodies are typically labelled (e.g. see Bravo and Macdonald-Bravo 1987; Nakayasu and Berezney 1989). In Syrian

hamster cells all the β-heterochromatic arms, with the exception of the active X chromosome, replicate late in S phase (Taruscio and Manuelidis 1991). In late S-phase nuclei therefore most late replicating domains should be resolved as IAP chromatids, while in early S phase, unreplicated IAP-rich chromatids can be evaluated.

Plate 1(a) shows the structure of IAP-labelled chromatids in early S phase (in red, with no BrdU incorporation). The characteristic more diffuse pattern of early replication is seen in the rest of the nucleus (in green). When both BrdU (green) and IAP probe detectors co-localize the signal is yellow. Note many IAP chromatids are slender (0.3–0.4 μm wide) and some form semicircular motifs and more condensed thicker coils (arrows). Unlike super heterochromatin, many of the associating IAP chromatids maintain their individual structural identities. Although the surfaces of these chromatids are not completely smooth, there is not an extensive or diffuse halo of labelled surrounding fibres. In this nucleus the top portion with the nuclear membrane was omitted for greater clarity. Nevertheless, two clusters of chromatids are still seen abutting the nuclear membrane (at bottom and at arrow). Interestingly, a collection of early replicating domains also abuts the membrane (green bodies at right).

In late replicating cells the IAP chromatids are wider and sister chromatids cannot be distinguished from each other. Such intertwined thicker yellow double chromatids are seen in Plate 1(b), most of which are clustered in groups at the nuclear membrane and in the centre of the nucleus. Some replicating IAP chromatids however showed a more detailed fibre substructure suggestive of unravelling (e.g. Plate 1(b), arrow). Some of these small fibres had solenoid fibre widths (about 0.05 μm). These structural motifs may represent domains caught during active replication, a very rapid process. Interestingly, not all IAP chromatids replicated at exactly the same time. Plate 1(b) shows some red-only IAP chromatids that have not yet replicated. Differences in the time of IAP chromatid replication were not readily related to their 3D position in the nucleus. Replication laggards were seen in both central and peripheral positions.

The above findings are consistent with the nested replication model, indicate that more than one DNA sequence signature (besides the IAP motif) may determine precise timing of replication, and suggest that active DNA synthesis involves a transient partially unravelled state. The extent of unravelling with replication was solidified in studies of smaller and simpler chromosome domains (*vide infra*).

Rather unexpectedly, IAP RNAs were transcribed from highly compact chromosome domains. RNA transcripts coincided and closely adhered to compact DNA chromatids. Furthermore, transcription originated from multiple sites on different chromatids. It was also not restricted to chromatids with a particular position, such as those in more interior regions of the nucleus. For these studies we used both unstimulated cells, as well as cells exposed to 5-aza-2'-deoxycytidine (Aza C) for 16 h. In the latter case the IAP RNA transcripts are increased 5- to 10-fold as determined by Northern blotting (data not shown). The

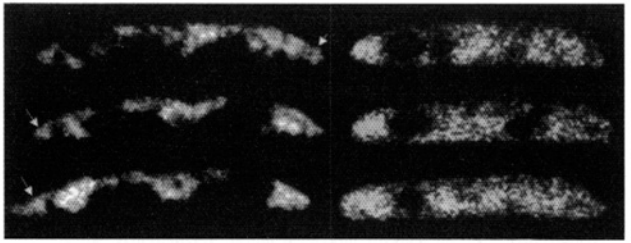

Fig. 9.4 Individual serial *x–z* sections (not deconvoluted) showing IAP RNA transcripts in non-denatured preparations at left and corresponding confocal sections stained with propidium iodide at right. Most of the RNA corresponds to preferred positions of the IAP chromatids at the top of the nucleus. Arrows point to regions of cytoplasmic RNA as determined by merging sections. Aza C treatment for 4 h.

IAP cDNA was inserted into an SP6/T7 plasmid to allow synthesis of labelled antisense RNA run-offs (for hybridization to RNA transcripts) as well as synthesis of labelled probes in a sense orientation (for labelling of chromatid DNA). The recombinant plasmid was cleaved with appropriate restriction enzymes to ensure each labelled probe represented either the 3' or 5' end of the IAP sequence, and labelling controls (e.g. switching biotin or digoxigenin for RNA and DNA detection) gave comparable results.

To evaluate possible artefacts with denaturation, RNA transcripts alone were first evaluated. With no denaturation of nuclear DNA, antisense probes labelled numerous RNA transcripts in the nucleus and many of these were focally clustered in chromatid-size domains. Plate 2(a) shows a nucleus with relatively few transcripts (yellow) in a preparation counterstained with propidium iodide (red) to reveal total nuclear DNA. Figure 9.4 shows serial *x–z* sections in a cell with more abundant IAP transcripts (left), where the nuclear DNA of the corresponding slice is shown on the right. Note the transcripts correspond to the typical IAP positions at the top of the nucleus. In these *x–z* sections RNA was also detected in the remaining cytoplasm (arrows). In contrast, control sense probes yielded only a few random background dots in non-denatured nuclei (data not shown). The close association of the IAP transcripts with IAP chromatids was further solidified by double label studies. Plate 2(b) and (c) show a single confocal slice with IAP DNA in green and RNA transcripts in red. Although the antisense probe can also label denatured DNA, some regions were only green or red (i.e. in non-contiguous structures). This confirmed that each label differentially decorated RNA and DNA. In Plate 2(c), a merged image of RNA and DNA from two sequential sections of this nucleus, note that RNA is being transcribed from a compact central IAP chromatid as well as from reasonably compact IAP chromatids at the nuclear periphery. Thus, these studies gave the first indication that reasonably compact chromatids with negligible unravelling

are capable of transcription. Furthermore, transcription was not dependent on nuclear position and involved many different IAP chromatids. Therefore, the attributes needed for transcription at these multiple sites might be universally designated by the IAP sequence, or by other shared sequence motifs.

Several principles can be drawn from the above studies on repeated DNAs. First, the cell can recognize chromosomal repeats provided they are sufficiently long or abundant. Second, this recognition is not dependent on the exact nucleotide sequence, but rather on the overall sequence organization. Insertion of long tandem repeats of exons lacking promoters, enhancers, or interspersed Alu family repeats creates a new super heterochromatic domain. Such domains are compartmentalized into highly coalesced bodies in the nucleus, a strong structural and probably rigid form of silencing. Third, high copy interspersed repeats (e.g. more than 1000 copies IAP) can define a different type of structure and association. They can designate β-heterochromatin over distances as long as an entire chromatid arm. These interspersed repeats are involved in the congression of similarly constituted chromatids in the nucleus. However, β-heterochromatic chromatids retain their individual spatial identities. The less coalesced association of β-heterochromatic chromatids probably provides for a flexible and rapid induction of transcripts in response to environmental or developmental signals. Fourth, although replication times are not precisely synchronous, closely apposed heterochromatic chromatids can replicate with extraordinary synchrony, as shown in Plate 1(b). Interestingly, early replicating chromatids can also cluster together as shown in Plate 1(a) (large peripheral green body). Finally, attachment to the nuclear envelope is not specific for different types of chromatin.

Telomeric repeats

Thus far I have covered repeats that can modulate the position and replication time of very large (Mb) chromosome domains. To contrast the organization of smaller domains, we studied a unique set of repeats with limited copy numbers. Such studies were helpful in understanding some of the features needed to determine nuclear position and replication time. The less numerous tandem repeats found at the ends of chromosomes (telomeres) were chosen for this purpose. A canonical TTAGGG sequence is found at the end of mammalian metaphase chromosomes (Moyzis *et al.* 1988). Several studies have suggested that truncation of these telomere repeats leads to cell senescence, whereas additional telomeric copies are typically correlated with cell immortality (see Bocke 1990). Therefore, the biological consequences of these repeats could be very significant. However, this senescence–immortality concept may be more complex than originally indicated in studies where total telomere copies per cell were calculated. Individual chromosomes can have quite variable telomere repeat copies, as shown by pulsed field gel electrophoresis of large chromosome

domains (Manuelidis 1994). In the same study, some immortal cell lines with a limited life span *in vitro* also had fewer telomere copies than normal cells. Additionally, it is often assumed that telomeres are universally positioned on the nuclear membrane, but this is clearly not true in mammalian, plant, and yeast cells (Manuelidis and Borden 1988; Rawlings *et al.* 1991; Vourch *et al.* 1993; Palladino *et al.* 1993; Manuclidis 1994).

Momentarily putting aside the different arrangements of interphase chromosomes in different organisms, as well as those specific for cells of different lineage, it is possible to ask if small telomeric repeats define a unique pattern of organization, i.e. one that is independent of the variant local sequences on each chromosome. Alternatively, is the behaviour of each telomere determined by attributes of its associated domain, as a submissive rather than dominant partner? This question has relevance for genetic silencing, best known through position effect variegation in *Drosophila* (see Chapter 13). It may also be relevant for spreading transcriptional activation or recruitment of Mb regions of DNA at the termini of chromosomes. In this case, for example, a more euchromatic determinant (such as many adjacent Alu repeats) should dominate the behaviour of its telomere locus.

If telomere repeats are submissive because of their relatively short length, they could be controlled by adjacent heterochromatic repeats. In Syrian hamster cells telomeres that inhabit terminal regions of β-heterochromatic chromatids should collect together with their respective chromatids at positions near to the nuclear membrane at the top of the cell. In contrast, telomeric repeats at the ends of more euchromatic chromatids might be more separated and/or at different nuclear positions. Furthermore, submissive telomeres should have replication times that are determined by their specific contextual domains, i.e. they should replicate in synchrony with their individual chromosome locales. Replication time is one of the best indicators of functional activity, with most housekeeping genes replicating early and tissue-specific genes replicating later in the cell cycle (Holmquist 1989). I am aware of only two studies on the replication time of telomeres, and both studies used hybridization to blots of synchronized cells for this determination. Each showed vastly different results, with exclusively late or very variable replication times of telomeres (McCarroll and Fangman 1988; ten Hagen *et al.* 1990). The structural studies below show that both replication time and spatial positioning of telomeres in the nucleus reflect their contextual DNA domains. Moreover, the degree of unravelling that can occur with replications was further clarified in the study of these small elements.

Canonical telomeric repeats, as well as polymerase chain reaction amplification products synthesized with 5'-IAP and consensus mammalian telomere primers, were generated as probes. In both instances characteristic small foci of hybridization were seen at metaphase chromosome termini (data not shown). Plate 2(c) shows a reconstruction from 10 serial 0.2 μm x–z sections at the centre of an early replicating nucleus (BrdU in green, telomeres in red). This nucleus has a characteristic dispersed early replication pattern. For comparison, a late

replicating cell is shown in Plate 2(f), where the sections were collected starting close to the lateral edge of the nuclear membrane. Characteristically, the later replication pattern shows label in larger and more discrete regions of the nucleus. In both images the size of the telomeres is variable, with larger red domains indicating close apposition of telomeres from several chromosomes. This conclusion was further solidified by counting telomeres in interphase. The number of telomeres was equal to or less than one-third of the number of telomeres in metaphase spreads. Additionally, the size of most telomere domains in interphase was typically more than three-fold greater than found in metaphase chromosomes. Thus telomeres often associate with each other in mammalian interphase nuclei.

Several large and small telomere domains were not attached to the nuclear membrane. Even with very large telomere aggregates only a small portion of the domain was attached to or near the nuclear membrane (Plate 2(e) and (f)). This indicates that self recognition is more important for these termini than envelope association. Interestingly, many telomeres were oriented near the bottom of the nucleus (facing the coverslip) in both early and late replicating cells (Plate 2(e) and (f)). A similar nuclear position was also often seen in G_1 cells (data not shown). Thus telomeric domains can be positioned in the nucleus with a particular 3D orientation. This orientation, however, is not strictly polar or Rabl-like, in accord with previous studies of mammalian cells (e.g. Manuelidis and Borden 1988; Manuelidis 1994). In contrast, the larger telomere aggregates collected at the top of the nucleus in regions known to harbour β-heterochromatic chromatids. For example, Plate 2(f) shows two large telomere collections at the top of the nucleus. Thus the orientation of particular groups of telomeres in the nucleus appears to be specified by their local chromosome residence, with greater aggregation of telomeres on β-heterochromatin. IAP or other interspersed repeats are probably key signals for these associations and variant positions. Similarly, the numerous hamster B1 family of Alu repeats could have a role in the more limited congression of different chromatid termini at the bottom of the nucleus.

The replication time of telomeric regions was variable in these cells. Some telomeric clusters, especially those near the bottom of the cell, replicated early in S phase, while fewer of these replicated later in S (Plate 2(e) and (f), yellow versus red structures in each). These differences indicate that replication time is also determined by the characteristics of the adjacent DNA domain. For example, telomere clusters from β-heterochromatic chromatids at the top of the cell in Plate 2(f) (red and yellow) are late replicating (compare the position of these arms shown in red in Plate 2(g)), as are a few smaller and more dispersed telomeres at the bottom of the cell. The variable replication time of telomeres shown here is entirely in accord with blotting studies of telomeres in human cells (ten Hagen *et al.* 1990). In human cells telomeres lie beside both heterochromatic and euchromatic bands. Although the late-only replication pattern in yeast cells may seem discordant, yeast telomeres all localize in heterochromatic domains

(McCarroll and Fangman 1988). Thus the uniform late replication time in yeast may be a consequence of their surrounding DNA motifs.

Finally, small details of structure were seen within replicating but compact telomeres. Some double-labelled solenoid-like fibres showed a helical or winding pattern (Plate 2(e), arrow) in accord with the proposed model (see Fig. 9.2). Double-labelled threads with similar widths could also be unravelled. Indeed, in some cases the small telomeric domain was no longer recognizable as a cohesive structure (Plate 2(f), arrow). This image probably represents capture of a rapid transient unravelling within an actively replicating domain. It also demonstrates that the methods used are capable of detecting unravelled fibres. We have not yet observed this degree of unravelling with RNA transcription, even in single large recombinant regions, where transcription was experimentally induced (*vide infra*). Therefore, replication appears to be more disruptive of structure than transcription.

Insertion of tandem coding domains

In the systematic evaluation of different chromosome domains, it becomes essential to compare events in a large and well-defined euchromatic domain that is transcriptionally active. Because there were no available probes that decorated only a single euchromatic domain of more than 200 kb, we chose to create a large inducible coding domain in the above hamster cells (D. Hanlon and L. Manuelidis, unpublished data). To create domains that would be as easy to detect as some of the above repeats, we chose to concatomerize a single transcriptionally competent element to produce tandem arrays totalling more than 400 kb in length. These concatomers were transfected into cells. For rapid monitoring of experimental induction of transcripts we used the β-galacosidase reporter under the control of a heat shock promoter. The details of heat induction, RNA transcription kinetics, and subcloning of cells to select for large inserts positioned in a few selected β-heterochromatic or euchromatic loci will be reported elsewhere. However, these studies confirmed the maintenance of a cohesive chromatid structure with active transcription. Each large locus was similarly inducible, regardless of its local environment. Typically these loci formed round bodies in G_1. With heat-induced transcription these sites remained compact, but had slightly greater diameters than their silent uninduced counterparts. One of the few more uncoiled fibres induced to transcribe is shown in Plate 2(g) (heat shock domain in green and the IAP chromatids labelled in red). The decorated green fibre is consistent with a more relaxed or uncoiled interphase chromatid as previously modelled. Double labelling of heat shock RNA transcripts and construct DNA confirmed transcription from these relaxed as well as more coiled heat shock loci (data not shown). Thus, this more refined experiment confirmed the ability of compact folded chromatids to support robust transcription.

Interestingly, these recombinant heat shock loci were positioned in the interior of the nucleus and did not attach to the nuclear membrane. Furthermore, although

some of the artificial inserts were over 1 Mb, they did not associate with each other. Each haploid chromatid bearing the insert was positioned in separated regions of the nucleus. Thus, these repeats were insufficient to override the segregated positioning of their parent chromatids. Studies on replication in these loci were consistent with the structural observations made on other loci, as described above. At least some of these heat-inducible loci were late replicating, as would be expected for an inducible exon. The complete lack of expression of the heat shock β-galactosidase reporter in uninduced cells, even when inserts were in euchromatic positions, shows that these large domains are correctly recognized by the cell. It remains to be seen if smaller constructs in euchromatic loci are similarly recognized, or are inappropriately recruited with other housekeeping genes, i.e. are subject to spreading activation.

Discussion

The above studies show that the characteristics of reiterated DNA are important determinants of chromosomal positioning and replication time. In the transgenic model system new super heterochromatin was created by insertion of long tandem repeats and was correctly assigned to congealed large bodies of heterochromatin in interphase nuclei (Manuelidis 1991). These bodies represent the classic form of heterochromatin that is associated with silencing. How the cell recognizes, silences, and compartmentalizes these domains is not clear. However, the structure of these large bodies suggests that specific proteins participate in this process. Such proteins must include more than centromere-specific proteins that function during mitosis, because newly created DNA inserts that were far from the centromere were similarly compartmentalized in the nucleus. Indeed, they coalesced with centromere-rich bodies in a cell type-specific pattern. Interestingly, the yeast SWI6 and SIR proteins are intimately associated with centromeres, silent mating-type loci, and telomeres (see Chapter 15), and it is most pertinent that telomeres form similarly cohesive heterochromatic bodies in yeast nuclei (Palladino *et al.* 1993; Ekwall *et al.* 1995). I assume that homologous proteins are involved in the organization of super heterochromatin in mammalian cells. The cell type-specific positioning of these bodies in the nucleus could also involve additional proteins or binding elements that are found only in mammalian cells.

This type of silencing may be viewed as the relegation of uninteresting junk sequences into a meaningless nuclear compartment. This view is predicated on the notion that function is restricted to exons that encode protein products. However, this may be an unnecessarily narrow concept of function. It is possible that transcriptionally silent sequences, such as very long tandem repeats, are actively used in interphase to organize specific groups of chromatids in mammalian nuclei (Manuelidis 1991). This could provide a functional advantage, especially in cementing particular patterns of expression in cells of different lineage. Highly refined cells, such as different types of neurones, may depend on these locking mechanisms to prevent their slippage into less exacting (or less cell-specific) patterns. I presume these alternate patterns would be detrimental for survival of the organism.

At the same time, the ability to organize a particular subset of chromosomes together in interphase may also provide a greater range or flexibility of expression. The variable positioning of groups of chromosomes in different mammalian cells contrasts with the simpler and more uniform Rabl organization (polar centromere orientation) of interphase chromosomes in *Drosophila* embryos (Ellison and Howard, 1981). However, transcriptionally silent regions are not the sole signals for chromosome segregation in mammals. The obvious congression of entire chromatids with β-heterochromatin-specific repeats exemplifies another form of chromosome-specific segregation in interphase. Congression of specific chromatid domains appears to be driven by their specific sequence identities rather than by some recognition element at the nuclear membrane. All classic forms of chromatin (euchromatin, β-heterochromatin and super heterochromatin) have been shown to focally adhere to the nuclear membrane, but most of the individual elements in these bodies have no direct attachment to the membrane. Thus the driving force of the nuclear envelope or its proteins is less substantial in this organization.

The effect of more numerous repeats that define super heterochromatin and β-heterochromatin was compared to less numerous repeats at defined loci. Telomere-repeat domains behaved as submissive elements. They appear to be incapable of overriding their local sequence environment and acquire both the position and replication time of their individual locales. Nevertheless, they can also form cohesive aggregates with each other and in this sense they mimic their longer tandem cousins. In yeast cells many telomeres also collect together, and the repression of gene expression that correlates with this spatial association can be destroyed in mutants with deficiencies in the SIR3 and SIR4 proteins (Palladino *et al.* 1993). Moreover, these protein deficiencies lead to a more scattered or diffuse organization of telomeres throughout the nucleus. This is further evidence that aggregation indicates silencing and is solidified by specific protein associations. It also is a strong indication that greater positional dispersion can be part of the process of transcriptional activation.

Because telomeres in normal yeast cells are uniformly late replicating and next to heterochromatin, it will be of interest to find if these telomeres acquire an early replication pattern after deletion of the SIR3 and SIR4 genes. Such a study would clarify the role of these proteins in the designation of replication time. It is also pertinent to find whether telomere repeats that are inserted within chromosome arms replicate in synchrony with their locale, and whether insertion of many telomere copies can override or alter the native replication pattern. Such determinations are most relevant for mobile or variable copy number intra-chromosomal repeats. Finally, the instability of telomeres may provide one mechanism for differential expression in cells of different lineage. Although shortening of telomeres has been associated with senescence, the inherent

instability of telomeric silencing (Chien *et al.* 1993) could be part of a differentiation mechanism. Similarly, small intrachromosomal repeats that are unstable could likewise have modulatory effects on transcription, not only in differentiation but also in disease. It remains to be seen if accumulating short tandem repeats within exons, as in Huntington's disease (Gusella and MacDonald 1995), can lead to a spreading change in genetic activity.

Although Giemsa (G)-dark bands on mammalian chromosomes are often assumed to be transcriptionally silent (e.g. Palladino *et al.* 1993), transcriptional studies of very heterochromatic G-dark chromatids show this assumption is unwarranted. RNA emanates from numerous regions on most of these chromatids. Furthermore, the actively transcribing DNA is remarkably compact and unravelled chromatids are not apparent. This indicates that structural disruption of the basic interphase chromatid is not required for transcription. Study of more discrete recombinant loci with more than 400 kb of coding repeats arrayed in tandem further verified the maintenance of the basic interphase fibre during transcription. Detailed studies with deconvolution showed structures consistent with a limited puffing or extension of radial arrays, rather than chromatid unravelling to the solenoid or nucleosomal level. Neither of these studies in 3D preserved nuclei support the notion of pulled out long solenoid loops of more than 2 Mb *in vivo*, an interpretation based on mapping studies of hypotonically swollen nuclei (Yokota *et al.* 1995).

The visualization of more unravelled structures with replication shows that small solenoid-like fibres are detectable, as shown for example in the telomere domains. The reason for obvious unravelling during replication, but not during transcription, is a matter of speculation. A biological explanation, however, seems most appropriate. In a committed cell the conservation of structure during transcription would be advantageous for preserving individual and unique cell functions. In contrast, during replication the cell must be able to reformulate or reorganize its chromatin for proper functioning in new environments, or in new developmental roles. Thus, the greater flexibility during replication may provide a window of opportunity for more extensive remodelling with consequent changes in function. Moreover, multiple sites that replicate at the same time can be modified together. From the above studies such changes are likely to be at the molecular level and affect submicroscopic nucleosome structure, leaving larger chromatid folding relatively intact. Remodelling with acetylated histones (see Chapter 3) or phosphorylated chromosomal proteins for example, along with DNA modifications such as methylation (see Chapter 11), may be key players in this act. These types of changes are amply discussed by others in this volume.

Future developments

Several principles of interphase chromosome organization have become apparent in the last 15 years, yet many fundamental questions remain. The precise relationship between function and structure, and the molecular underpinnings of chromosome position effects need to be addressed. Most pertinent for such studies is the choice of meaningful biological systems that can be experimentally manipulated. Clearly, the molecular insertion of defined genetic domains in cultured cells, or in transgenic animals, will continue to be informative. Such experimental approaches are likely to clarify several key features of developmental regulation. Nevertheless, the largest challenge at present is to develop chromosome-specific probes that can be used in living cells. These would allow one to investigate responses to more transient and controlled physiological stimuli and to give a more comprehensive view of rapid structural and protein interactive changes.

Summary

Classic concepts of heterochromatin and euchromatin are inadequate for distinguishing the many functionally diverse regions of a chromosome. Three-dimensional confocal microscopy hybridization studies have illuminated the structure of different chromosomal segments in interphase nuclei. More specifically, genetically silent, transcriptionally competent, and actively replicating domains of large size were used to evaluate degrees of chromatid unfolding. Several conclusions can be made. First, although many metaphase chromosome regions stain with the characteristics of 'constitutive' heterochromatin, only some of these are completely silent in interphase. Silent large regions, such as those at the centromere, lose their individual identity as they are congealed together in the nucleus in visible dense bodies. This form of 'super heterochromatin' is driven by very long tandem repeats. It can be created *de novo* by insertion of long tandem repeats of promoterless open reading frames, even at non-centromeric sites. In contrast, very heterochromatic chromosome arms with many long interspersed repeats maintain their individual structural identity in interphase. Unlike super heterochromatin, these chromatids cannot be assigned to G-dark or ultrastructurally dense bodies in the nucleus. Nonetheless, these chromatids congregate with each other, most often at the upper nuclear periphery of cultured cells. Furthermore, abundant transcripts are produced along these β-heterochromatic arms. Remarkably, there was only a minimal change in their overall structure during transcription. Because these chromatids contain specific and abundant interspersed repeats, these repeats may signal a different type of congression that is used for the orderly recognition and recruitment of their cell-type specific genes.

Second, for comparison, more euchromatic large loci were created by recombinant means. Each of these megabase DNA domains contained multiple tandem repeats of a competent reporter gene under a heat shock promoter. These large domains did not associate with each other in interphase, regardless of their chromosome position in G-dark or G-light bands. However, they again maintained a coherent 3D structure with transcriptional activation. Only a small

increase in width, consistent with puffing of the chromatid fibre, was observed and there was no evidence for long unravelled loops of solenoid/nucleosome fibres. Thus again, transcription was conservative of structure.

Third, less numerous, conserved tandem repeats at chromosome termini were also evaluated. These termini behaved in an analogous manner as their longer 'super heterochromatic' cousins in that they often adhered to each other in interphase. However, telomeres were submissive in their position and replication patterns. For example, those next to β-heterochromatin domains replicated late, while telomeres on more euchromatic arms replicated early in S phase.

Finally, several defined domains were caught in the act of replication. During this process the chromatid became more unravelled than during transcription. The above studies suggest that genetic activity is controlled through 3D positioning of chromosomes with specific sequence signatures. Transcriptional activation has only a minimal effect on the overall structure of the interphase chromatid. In contrast, the more dramatic unravelling of the chromatid during S phase can be part of the process of molecular remodelling that provides a way for the cell to flexibly adapt to new environmental stimuli. During replication the cell can modify molecular details of the chromosome to recruit alternate gene families for differentiation or in response to disease.

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Plate 2 (*Facing*) In (a) IAP RNA transcripts were detected with antisense probes in non-denatured preparations. The RNA signals are yellow because total nuclear DNA was counterstained with propidium iodide (red). Note obvious compact clusters of RNA transcripts. Single corresponding confocal sections (b and c) showing hybridization to simultaneously detect DNA and RNA (green and red, respectively) in cells stimulated with Aza C. In (d) a merged image from the two confocal sections (b and c) is shown. RNA transcripts overlie the IAP chromatids both in the interior and at the periphery of the nucleus. Note that most the RNA overlies the more compact regions of the IAP chromatids at both interior and peripheral locations. In x–z reconstructions of early and late replicating cells (e and f, respectively) BrdU is green, with telomeres shown in red. Note replicating telomeres are present in both cells (yellow regions). Many of the telomeres in these cells are oriented close to the coverslip attachment (at bottom). Late but note synchronously replicating telomere clusters in (f) are consistent with those on termini of β-heterochromatic chromatids. In (e) the arrow shows fine substructure of delicate fibres in early replicating telomeres, while in (f) these fibres are markedly unravelled in some replicating telomeres (yellow, at arrow). In (g) IAP chromatids near the starting edge of the nucleus are labelled in red and begin to show up at the top of the nucleus (nearest to feeding medium). These sections also resolve the entire concatomerized heat shock locus (in green). This was one of the most extended loci found in heat shocked cells producing abundant transcripts. Notably no small fibres emanate from this locus. All transcribing heat shock DNA domains were similarly cohesive or even more compact in RNA–DNA double label studies (data not shown). Above sections were not deconvoluted. (See pp. 148, 157, 159–61.)