UNITS OF CHROMOSOME REPLICATION AND PACKING

ANN M. MULLINGER AND ROBERT T. JOHNSON
Cancer Research Campaign Mammalian Cell DNA Repair Group, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EF, U.K.

SUMMARY
Fusion between mitotic and S-phase cells induces the formation of prematurely condensed chromosomes (PCC) in the interphase partner. Viewed in the light microscope, S-phase PCC derived from the Indian muntjac appear to be fragmented and heterogeneous. In scanning electron micrographs prepared by an osmium impregnation technique, which avoids the need to sputter-coat the specimen, the S-phase fragments derived from an individual cell are resolved into about 1000 fibre aggregates, together with more dispersed fibres. Aggregates are roughly spherical and vary in diameter between about 0.25 and 1.6 μm. The spatial distribution of the aggregates shows some order: chains of single aggregates and, less commonly, duplicated chains occur. Regions of the PCC where the fibres are more dispersed are considered to be likely candidates for sites of replication at the time of fusion. The relationship between the condensed aggregate structure of the S-phase PCC and replication clusters is discussed, and also the assembly of aggregates to form metaphase chromosomes.

INTRODUCTION
Despite new information and the presentation of several models of metaphase chromosome organization (Paulson & Laemmli, 1977; Marsden & Laemmli, 1979; Mullinger & Johnson, 1980), there is still considerable uncertainty about the way in which the chromatin fibre is folded and organized within the chromatid. Prematurely condensed chromosomes (PCC) offer one way of studying the higher levels of chromatin organization; since these chromosomes are not as tightly packed as their metaphase counterparts they are likely to be more amenable to analysis by microscopical techniques. Condensation of interphase chromatin can be imposed by factors present in the metaphase cell and this is generally achieved by means of cell fusion (Johnson & Rao, 1970). The resulting PCC produced from the interphase partner fall into three general categories: G1 PCC with single, extended chromatids; elongated G2 PCC, more closely resembling mitotic chromosomes; and S-phase PCC, which usually appear fragmented (Johnson & Rao, 1970).

Each category of PCC can be further subdivided and, at least for G1 and G2 cells, there appears to be a continuum of PCC structure associated with the position of the interphase partner in the cycle at the time of fusion. For example, PCC from early through to late G1 cells appear progressively more attenuated and the reverse situation occurs in G2 (Schor, Johnson & Waldren, 1975; Hittleman & Rao, 1978; Sperling, 1982). While the relationship, if any, between the state of attenuation of G1 and G2 PCC and the genetic activity of the cell is uncertain, in the case of S-phase PCC the
size, staining intensity and disposition of the fragments are related to the precise position within S phase at the time of condensation and to the amount of replication that has already taken place (Johnson & Rao, 1972; Sperling & Rao, 1974; Sperling, 1982). Early S-phase PCC are characterized by extremely attenuated filaments and late S-phase PCC by elongated, duplicated chromosomes with gaps. Mid S-phase PCC, on the other hand, are particularly heterogeneous, with large and small blocks of stained material associated with attenuated regions having little staining. Replication occurs only in the less condensed regions of the PCC (Sperling & Rao, 1974; Röhme, 1975). Because of the correlation between DNA replication and the structure of S-phase PCC we have examined this material in more detail by means of scanning electron microscopy (SEM). This has recently become feasible with the development by Harrison, Britch, Allen & Harris (1981) and Harrison, Allen, Britch & Harris (1982) of an elegant preparative method for the study of metaphase chromosomes. The procedure, which eliminates the need to cover the specimen with a conducting layer, enables more of the fine-structural details to be resolved than with conventional techniques for preparation of bulk specimens for SEM. We have chosen to look at the PCC of the Indian muntjac (Muntjac muntiacus) because this species has a small number of large, conspicuous chromosomes and also because earlier light-microscope studies of S-phase PCC from these cells by Röhme (1974, 1975) suggested that it might be possible to trace chromatid continuity through the prematurely condensed chromosome fragments.

**MATERIALS AND METHODS**

A simian virus 40-transformed cell line of the Indian muntjac, Muntjac muntiacus kindly supplied by Dr Karl Sperling, was used; many of the cells in the population are near-diploid though some are aneuploid, with a variety of chromosome re-arrangements. They were grown in monolayer in Eagle's Minimal Essential Medium (MEM), supplemented with non-essential amino acids and 10 % calf serum (Gibco Bio-Cult Ltd). HeLa cells were grown and synchronized in mitosis as described previously (Johnson, Mullinger & Downes, 1978).

Mitotic HeLa cells (2 × 10⁶) were fused with 10⁶ randomly growing Muntjac cells, using inactivated Sendai virus, as described previously (Johnson & Rao, 1970). Cells from the fusion mixture were treated with Hanks' hypotonic solution (1 part Hanks' balanced salt solution: 4 parts distilled water) for 20 min at 37°C, fixed in methanol: acetic acid (3: 1) and, for light microscopy, dropped onto glass slides, air-dried and stained with 3 % Giemsa. For electron microscopy, fixed chromosomes were dropped onto glass coverslips that had previously been sputter-coated with a gold layer, about 100 nm thick. After air-drying and storage for 2–3 days, chromosomes on coverslips were treated, as for G-banding, with 0·006 % trypsin (Koch-Light, twice re-crystallized) in 0·85 % saline, for between 20 and 60 s, rinsed in phosphate buffer and prepared for electron microscopy according to the method of Harrison et al. (1981). Material was fixed in 3 % glutaraldehyde for 1 h and then in 1 % osmium tetroxide for 30 min, both in 0·1 M-Sorensen's phosphate buffer (pH 7·4) at 4°C. Following this, it was treated three times according to the following schedule at room temperature: three changes of distilled water for a total of 5 min; freshly prepared saturated sodium thiocarbohydrazide in distilled water for 30 min; three changes of distilled water for a total of 5 min; and 30 min in 1 % osmium tetroxide in distilled water. Material was rinsed briefly in distilled water, dehydrated in a graded series of acetones ending with absolute acetone, and finally critical-point-dried from liquid carbon dioxide. Fragments of coverslips were examined in a Jeol 200 CX electron microscope operated at 80 kV in the secondary emission mode (or, in the case of Fig. 8, in a Cambridge S4 scanning electron microscope operated at 20 kV); stereo pairs were taken before and after tilting the specimen by 8°.
For autoradiography 1·5 μCi/ml [methyl-3H]thymidine (49 Ci/mmol, Radiochemical Centre, Amersham) was added to the fusion mixture for the last 20 min of incubation at 37 °C, immediately prior to hypotonic treatment. Chromosome preparations from this material were extracted with 5% trichloracetic acid at 4 °C and coated with Ilford K2 nuclear emulsion diluted 1:1·8 with 0·002% sodium lauryl sulphate. After exposure for 7 days they were developed in Ilford D19 developer for 3 min at 20 °C.

Fig. 1. Light micrographs of Giemsa-stained chromosome preparations derived from the fusion products of mitotic HeLa and S-phase Indian muntjac cells. In each case the densely staining mitotic HeLa chromosomes can be distinguished from the more lightly staining S-phase PCC. A, Early S-phase PCC consisting of greatly attenuated chromosomes; B, mid-S-phase PCC with apparent fragmentation and a mixture of single and duplicated blocks; C, late S-phase PCC with large G2-like elements; D, autoradiograph of S-phase muntjac PCC showing incorporated [3H]thymidine mainly associated with unstained regions. A–C, ×1000; D, ×1200.
RESULTS

The structure of S-phase PCC

Fusion between mitotic HeLa and random Muntjac cells results in the induction of PCC of $G_1$, $G_2$ and S-phase types; in this study we have concentrated primarily on S-phase PCC.

Fig. 2. Scanning electron micrograph of chromosome preparation derived from the fusion product of three cells: mitotic HeLa, mitotic muntjac and S-phase muntjac. The large muntjac metaphase chromosomes, smaller HeLa metaphase chromosomes and S-phase PCC are visible. Material was prepared by an osmium impregnation technique as described in Materials and Methods. ×1300.

Fig. 3A, B. Enlargements of regions of the S-phase PCC shown in Fig. 2 illustrating the heterogeneity of fibre aggregates. Chains of single (s) and paired (p) aggregates are visible. ×7800.
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Fig. 3
S-phase *Muntjac* PCC in conventionally spread, Giesma-stained preparations viewed in the light microscope appear as an irregular array of condensed, stained fragments separated by non-stained regions (Röhme, 1974, 1975; Fig. 1A–D). Early S-phase PCC appear as a mixture of more or less intensely stained regions (Fig. 1A); the size of the intensely staining (duplicated) regions increases throughout S phase and the linear arrangement of the fragments becomes clearer (Fig. 1B, C). Autoradiographs show that DNA synthesis is usually associated with the lightly or non-staining regions (Sperling & Rao, 1974; Röhme, 1975; Fig. 1D).

Fig. 2 shows a low-power scanning electron micrograph of a trypsin-treated chromosome preparation derived from fusion of three parents: mitotic HeLa, mitotic *Muntjac* and interphase *Muntjac*. The large metaphase *Muntjac* chromosomes are readily distinguished from the small and more numerous HeLa chromosomes and from the PCC. Superficially, the PCC appear as a scattered array of fragments of different size and shape, typical of S-phase PCC from this organism (Röhme, 1974; Sperling, 1982). At higher power the heterogeneous S-phase structures are resolved into roughly spherical or ovoid aggregates varying in diameter between 0.25 and 1.6 μm (Fig. 3A, B). Each aggregate is made up of tightly packed fibres seen best in stereo (Fig. 4A–C). While the bulk of the fibres are arranged in aggregates, at a number of sites in the PCC they are more widely dispersed. In these regions the fibre disposition is varied: some fibres are grouped loosely together, others are more scattered (Fig. 4B, C). We believe that these dispersed areas, which grade into the smaller aggregates, together correspond to the unstained, or poorly stained, apparently uncondensed regions seen in the light microscope.

There are about 1000 aggregates in the PCC shown in Fig. 2. They show a continuum of size between 0.25 and 1.6 μm, with a mode at 0.75 μm (Fig. 5A) and no obvious categories of size or shape. Though it appears random, the spatial distribution of the aggregates shows signs of order, especially if one allows for some distortion during preparation. The most conspicuous patterns are linear chains, illustrated in Fig. 3. Chains are loosely or tightly packed and contain aggregates of similar or varied sizes. Pairs of similarly sized aggregates are quite common (Figs 3, 4A) and occasional laterally paired chains running for up to six aggregates can be traced.

Although different in detail, the appearance of S-phase PCC from other cells resembles that shown in Fig. 2. The fibre aggregates are of much the same size range and the total number per spread varies from about 500 to 1000. However, the overall disposition of the aggregates and their size distribution vary; for example, in the spread shown in Fig. 7A the aggregates are generally larger than those in Fig. 2, and are mostly arranged in extended chains (Figs 5, 7). By analogy with light-microscopic preparations (Fig. 1) such structural variations in the appearance of S-phase PCC probably reflect the position of the cells in S phase.

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Fig. 4A–C. Stereo pairs of enlargements of the S-phase PCC shown in Fig. 2. These should be inspected with a stereo magnifying viewer in order to see details to full effect. A. Chains of aggregates, either single or in matched pairs (p). ×12,000; B, aggregates of various sizes, including groups of small aggregates (arrow). ×9000; C, fibre aggregates (a) and more widely dispersed fibres (d). In stereo, fibre loops (l) are visible. ×23,000.
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Average diameter of aggregates in arbitrary units

Fig. 5. Histograms showing the size distribution of aggregates in two different PCC spreads. Measurements were made of all aggregates in a selected area of each spread. For each aggregate the diameter shown represents the average of the maximum and minimum measured diameters, since most aggregates are not perfectly symmetrical. On the abscissa, 10 arbitrary units correspond to 1.4 μm. A. Region of Fig. 2 spread containing over 500 aggregates; B, region of Fig. 7 spread containing over 300 aggregates.

Fibre arrangement in S-phase PCC

The precise arrangement of fibres in the aggregates is not easy to see, even in stereo pairs. In the larger aggregates many of the fibres seem to radiate from the centre (Figs 4c, 6), but in less condensed regions the orientation is more varied (Figs 4c, 6A). Fibres from adjacent aggregates are often intermingled (Fig. 6).

Fibres have a variable thickness and are usually irregular (Fig. 6); generally the diameter falls within the range 20–40 nm, although some are thinner and are particularly visible as loops at the edges of aggregates (Figs 4c, 6B), or at the junction between aggregate and substrate (Fig. 6A). These thinner fibres also have a variable diameter and are often beaded (Fig. 6A); they may be as thin as 12 nm.

The structure of metaphase chromosomes

Prepared in the same way, Muntjac metaphase chromosomes are seen to be constructed of closely juxtaposed, large fibre blocks lying in linear array along each chromatid (Figs 8, 9). The blocks vary in size (1.3–2.2 μm across the chromatid width.

Fig. 6A, B. Stereo pairs of aggregate chains showing variability of their fibre composition. A. Enlargement of Fig. 2. ×25 000; B, enlargement of Fig. 7B. ×28 000.
Fig. 7
Fig. 8. Scanning electron micrograph of metaphase muntjac chromosomes prepared in the same manner as the PCC shown in previous figures. Chromosomes are subdivided into a series of discrete blocks. ×2900.

and 0.5–2 µm along the length) and each chromosome has a characteristic longitudinal pattern, which matches rather well in sister chromatids (Figs 8, 9). In some preparations the boundary between blocks is obscured, a clear distinction depending on optimal trypsinization. As with PCC, fibres of about 20–40 nm diameter emerge from the body of the chromosome (Fig. 10); there are some loops, particularly at the edges of the chromatids where thinner fibres appear again (Fig. 10B).

Factors affecting ultrastructural appearance

The appearance in the SEM of both metaphase and prematurely condensed chromosomes shows considerable variation, depending partly on known factors such as

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Fig. 7. A. Muntjac S-phase PCC with aggregates arranged mostly in extended chains. ×1600. B. Enlargement of A. ×12 000.

Fig. 9A, B. Higher-power SEMs of metaphase muntjac chromosomes showing the blocks in more detail and their fibre composition. A, ×11 000; B, ×7300.
Fig. 9. For legend see p. 187.
as the degree of spreading of the chromosomes and the length of trypsin treatment but also on other factors, which we have not so far been able to identify and control. When the trypsin step is either omitted or too short, very little surface detail is visible (Fig. 11), whereas prolonged exposure to trypsin leads to loss of material; even chromosomes from a single cell can show variation in these respects. In this paper we have confined our attention to those sporadic preparations judged to be both generally well preserved and also reasonably spread and trypsinized.

DISCUSSION

In this paper we have shown that the osmium impregnation technique developed by Harrison et al. (1981) for the examination of metaphase human chromosomes is a useful, though at present still capricious, method for the examination of S-phase PCC at levels both close to and beyond the resolution of the light microscope. At first sight the technique is surprising as a basis for electron microscopy, since it involves

Fig. 11. SEM of sub-optimally prepared metaphase chromosomes. A layer of material obscures much of the fibre detail. X23 000.
first fixing cells in methanol/acetic acid, a procedure that produces cell distortion and may also extract chromosomal proteins (Sumner, Evans & Buckland, 1973; Burkholder & Duczek, 1982). Nevertheless, it is interesting that the overall appearance of metaphase chromosomes prepared by this method is similar to that in scanning electron micrographs of metaphase chromosomes prepared from isolated chromosomes without prior treatment in methanol/acetic acid, e.g. chromosomes microsurgically isolated from Muntjac cells (Korf & Diacumakos, 1978); or isolated from HeLa and Chinese hamster cells in hexylene glycol (Daskal, Mace, Wray & Busch, 1976; Marsden & Laemmli, 1979). In terms of detailed surface structure the osmium impregnation method allows greater resolution of fine details, since the specimen is not hidden by a thick metal surface coat.

The trypsinization step, before glutaraldehyde fixation, is an essential stage in the preparative method used in this paper. Without this, very little surface detail is visible either in metaphase chromosomes or PCC. Chromosomes when initially dropped on coverslips are coated with material (Fig. 11), presumably of cellular origin, which may protect them from damage during the initial air-drying step. After trypsinization, drying takes place only by the critical-point method. A rational basis for the use of a trypsin step in G-banding has not yet been advanced (Sumner, 1982).

As seen in our electron micrographs, S-phase PCC and mitotic chromosomes of Muntjac contain chromatin fibres with similar dimensions. Since the composition of the fibre is uncertain (Sumner et al. 1973; Burkholder & Duczek, 1982) the correspondence between the dimensions of the fibres seen in this paper (generally 20–40 nm, but sometimes thinner) and those observed elsewhere for chromatin may be fortuitous. In transmission electron micrographs of isolated chromatin and thin sections of chromosomes a basic 10 nm chromatin fibre is assembled into higher-order fibres of 20–30 nm diameter (e.g. see Marsden & Laemmli, 1979; Thoma, Koller & Klug, 1979).

The scanning electron micrographs presented in this paper go some way towards clarifying the structure of S-phase PCC and additionally provide preliminary information about the functional organization of the replicating nucleus. At the light-microscope level the discontinuous nature of S-phase PCC is probably more apparent than real. Viewing these structures under phase-contrast or bright-field conditions suggests that the condensed fragments may be linked in chains by intervening material, though usually this is difficult to see and the conclusion about continuity is often subjective and based on the occurrence of suggestive linear groups (Sperling & Rao, 1974; Röhme, 1975). DNA synthesis is, however, associated with the unstained or poorly stained, presumably uncondensed, regions. Towards the end of S phase the PCC are essentially G1-like in structure, condensed and duplicated. Replication occurring late in S phase is associated solely with the remaining few regions of decondensation interspersed between G2 blocks; [3H]thymidine incorporated into DNA at this time can be chased into the G2-like structures (Sperling, 1982). These observations suggest that chromatin engaged in DNA replication is not susceptible to mitotic condensing factors until synthesis is complete. The mutual exclusivity of DNA synthesis and chromosome condensation is further supported by the decondensation of both PCC
and metaphase chromosomes associated with repair synthesis after ultraviolet radiation (Schor et al. 1975; Johnson, Collins & Waldren, 1982), and by the decondensation of G1 chromosomes as they prematurely synthesize DNA some hours after PCC is induced (Hanks & Rao, 1980).

By scanning electron microscopy we have shown that the heterogeneous appearance of S-phase PCC results from the varied association of fibre aggregates interspersed with more loosely packed fibres. Though the localization of replication sites remains uncertain in this material we suggest that it is likely to occur in those regions of relative fibre dispersion that would be considered uncondensed in light microscope preparations and often intervene between the larger fibre aggregates. The latter represent the condensed blocks of S-phase PCC seen in the light microscope. Even in early S-phase nuclei some chromosome regions would be replicated and pairs or duplicated chains of aggregates should occur unless the structure is severely disturbed during preparation. Such paired structures can be seen, though they are quite rare in the preparation shown in Fig. 2. Questions about sites of replication and the generation and packing of duplicated regions can only be resolved by studying PCC in cells in known stages of S phase, combined with autoradiography. Such work is in progress.

If there is indeed a distinction between large fibre aggregates that are not undergoing DNA replication and the dispersed aggregates that are, we can ask whether the structures described here can give any information about the spatial and temporal organization of replication. In eukaryotes DNA replication is initiated at many sites; the individual units of replication are of variable size, but in mammalian cells they mostly fall within the range 5–100 μm (Hand, 1978). Thus, assuming an average size of 50 μm (150 × 10^3 base-pairs), a genome with 5 × 10^9 base-pairs of DNA would be replicated via a total of about 3 × 10^4 units of replication. Although synthesis is initiated at different times in different regions of the chromosome, it is synchronous in clusters estimated to contain between 2 and 100 replication units (Hand, 1978). This would mean that for a genome with a total of 3 × 10^4 units and an average of 50 units per cluster the total number of clusters would be about 0.5 × 10^3. Thus, many of the fibre aggregates seen in S-phase PCC may represent replication clusters.

In addition to the possibility that fibre aggregates reflect the functional organization of the replicating nucleus, they may also reveal something about the way the highly condensed chromosomes of metaphase are assembled. Aggregates in S-phase PCC occur in a wide range of sizes and it is tempting to consider them as a hierarchical series of packing units. For example, longitudinal metaphase fibre blocks with the dimensions taken from our micrographs have an estimated volume equivalent to about 3–30 S-phase PCC fibre aggregates with a diameter of 0.75 μm, and the metacentric chromosome shown in Fig. 9B has about 200 such aggregates per chromatid. In the karyotype (Ved Brat, Verma & Dosik, 1979) this chromosome is likely to be number one, which contains approximately one quarter of the DNA. An unduplicated genome would, by these calculations, consist of about 800 S-phase PCC aggregates, a figure close to that found for PCC spreads examined in this study. The relationship between the aggregates and the construction of the large fibre blocks that make up the metaphase chromosome awaits further work, but the fact that S-phase PCC often
contain regions of distinct linear chains consisting of uniformly sized aggregates is suggestive of chromosome assembly. The more tightly packed of these chains begin to resemble the elongated chromosomes of G1 PCC. Reduction in the length of the G1 or G2 PCC and simultaneous increase in the diameter of the chromatid(s) to form the metaphase structure is presumably achieved both by amalgamation of aggregates to produce the typical metaphase fibre blocks and by further changes in packing, such as gyration of the chromatid axis (Mullinger & Johnson, 1980). The relationship between the blocks in metaphase chromosomes and the longitudinal banding patterns revealed by standard staining procedures has been elucidated by the work of Harrison et al. (1981, 1982), grooves between the blocks corresponding to G bands. In this connection it is interesting that G-band frequency is significantly greater in the elongated G2 PCC, with presumably smaller assemblies of aggregate fibre blocks, than in their metaphase counterparts (Unakul, Johnson, Rao & Hsu, 1973).

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REFERENCES


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