Localization of centromeric satellite and telomeric DNA sequences in dorsal root ganglion neurons, in vitro

FILIO BILLIA* and UMBERTO DE BONI†
Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada
*Present address: Department of Medical Biophysics, University of Toronto, Ontario Cancer Institute, Toronto, Ontario M4X 1K9, Canada
†Author for correspondence

Summary

Chromatin domains of interphase nuclei are organized in a tissue-specific, non-random manner. In the present work, the spatial arrangement of satellite (sDNA) and telomeric (tDNA) DNA was examined in nuclei of murine Dorsal Root Ganglion (DRG) cells, maintained in vitro. In situ hybridization in conjunction with three-dimensional reconstruction was employed. A mean number of 8.02±0.40 sDNA signals/nucleus was detected, of which 41.65±0.59% were associated with the nucleolus. The remaining fraction of signals was localized between the nucleolus and the nuclear membrane. sDNA signals were reproducibly localized at a mean distance of 3.15±0.06 μm from the nuclear center and measured 1–2 μm in diameter. Given a centromere complement of 40 per murine nucleus, the relatively low number of signals detected and their large signal volumes were interpreted to reflect clustering of centromeres, a phenomenon common in mammalian cells. An average of 37.00±1.52 tDNA signals was detected per nucleus. Of these, and in contrast to sDNA signals, only 18.45±0.41% of these signals were associated with the nucleolus while the remainder was distributed between the nucleolus and the nuclear membrane. Both centromeric and telomeric signals often occurred in pairs and were distributed throughout the nucleoplasm. No evidence for a classical Rabl configuration was found.

Key words: cell nucleus, neurons, centromere, telomeres, nuclear organization.

Introduction

The spatial organization of chromatin in interphase nuclei is currently of much interest. Early work by Rabl (1885) indicated that interphase chromatin may retain a telophase-like arrangement of chromosomes with centromeres and telomeres associated with opposite poles of the nucleus. Agard and Sedat (1983), Hochstrasser et al. (1986) and Hochstrasser and Sedat (1987a, b) found evidence for this model in the specialized, polytene nuclei (Hiraoaka et al. 1990). It is possible, however, to identify specific, well-defined chromosome regions such as centromeres and telomeres. The three-dimensional topology of satellite DNA (sDNA) sequences, localized to centromeres (Jones, 1970; Pardue and Gall, 1970; Joseph et al. 1989; Matsumoto et al. 1989), has been extensively studied. This was achieved either by indirect immunofluorescence, which detects kinetochore proteins associated with sDNA sequences (Moroi et al. 1981; Earnshaw et al. 1984; Hadlaczyky et al. 1986; Chauly and Brown, 1988), or, alternatively, by in situ hybridization (Jones, 1970; Pardue and Gall, 1970; Rae and Franke, 1972; Manuelidis, 1982, 1984b, 1985a, b; Joseph et al. 1989). While a considerable body of evidence shows that telomeres are often attached to the nuclear membrane in polytene nuclei and in some plant nuclei (Mathog et al. 1984; Hochstrasser et al. 1986; Katsumata and Lo, 1988; Rawlins and Shaw, 1990a,b), little is known regarding the spatial arrangement of telomeric DNA (tDNA) in mammalian interphase nuclei.
DNA localized to centromeric and telomeric regions is enriched in highly repetitive DNA sequences (Horz and Altenburger, 1981; Manuelidis, 1981; Moyzis et al. 1988). Therefore, these sequences can be effectively probed to determine their spatial, intranuclear positions (Hsu et al. 1971; Rae and Franke, 1982; Manuelidis, 1982, 1984; Moyzis et al. 1985a,b; Manuelidis and Borden, 1988; Moyzis et al. 1988; Rawlins and Shaw, 1990a,b).

In a test of the hypothesis that centromeric and telomeric sequences are reproducibly and nonrandomly compartmentalized in Dorsal Root Ganglion (DRG) neurons in vitro, the arrangement of these domains was examined by in situ hybridization and computer-assisted three-dimensional reconstruction. DRG neurons are highly differentiated and, as they are permanently arrested in interphase, the spatial topology of chromosomes is not subject to events related to the cell cycle.

Materials and methods

Tissue preparation

DRG neurons were cultured as previously described (Fung and De Boni, 1988). Briefly, DRGs were aseptically removed from neonatal mice, collected in a drop of cold Hank's Balanced Salt Solution (HBSS) and then transferred to 0.25% trypsin (GIBCO, 10 min, 37°C). Upon inactivation of the latter with fetal bovine serum, DRG neurons were dissociated by trituration, centrifuged (500 g, 5 min) and then resuspended in a few drops of culture medium containing 58% (v/v) Minimum Essential Medium with Hank's Salts (GIBCO), 10% (v/v) fetal calf serum (GIBCO), 1% (v/v) glacial acetic acid, which was permitted to dry before seeding of cells. Neurons attached to the collagen substratum (3x5 min) to inhibit further Pronase activity, coverslips and 37°C for 1 h. After washing with 2 mg/ml glycine in PBS (5 min), cells were seeded onto 22 mm x 22 mm Corning no. 1 glass coverslips contained within a 22 mm x 22 mm Corning no. 1 glass coverslips contained within the center of plastic Petri dishes with tight-fitting lids (Falcon no. 1096). Coverslips had been previously coated with approximately 0.3 ml of calf skin collagen (Calbiochem; 0.5 mg/ml in 0.1% (v/v) glacial acetic acid), which was permitted to dry before seeding of cells. Neurons attached to the collagen substratum within 2–3 h after which 0.5 ml of culture medium was added. Cultures were maintained for 5–6 days with the culture medium replaced every 3 days.

For use in experiments, cultures were washed with HBSS (prewarmed to room temperature (RT)) and fixed (4% (w/v) paraformaldehyde in PBS, 30 min). After washing in PBS (3x5 min), the fixed cells were exposed to a series of pretreatments. Cultures were incubated in 0.1% HCl (5–20 min, RT) time depended on cell type, see Results). After washing in PBS (3x5 min), the cells were incubated with 1% Triton X in PBS (1 h, RT) and incubated in 0.2 mg/ml1 RNase A (Boehringer Mannheim, 37°C, 1 h), previously boiled (10 min) to eliminate endogenous DNAses. Following washing in PBS (3x5 min), nuclear DNA was further digested for 10–15 min, with 0.6–0.8 mg/ml-1 Pronase (Boehringer Mannheim; in 0.05 M Tris–HCl, 5 mM EDTA, pH 7.6; concentration and time depended on cell type, see Results). Pronase was nontargeted before use at 37°C for 1 h. After washing with 2 mg/ml-1 glycine in PBS (3x5 min) to inhibit further Pronase activity, coverslips and attached cells were dehydrated in a graded ethanol series and air dried.

Probe preparation

pMSAT5, a gift from J. Rossant (University of Toronto, Toronto) contains a 1.9 kb (kilobase) repeat of mouse centromeric sDNA subcloned in the vector pTZ194 between EcoRI and HindIII restriction sites. The methods of isolation of murine sDNA (Manuelidis, 1981b) and characterization of its consensus sequence have been described by Manuelidis (1981a) and Horz and Altenburger (1981).

For tDNA, a 246 bp (base pair) repeat was subcloned in the vector pBR322 at the PstI restriction site and designated pHUR93 (Moyzis et al. 1988). To use 'vector-tails' as an aid in amplification of the hybridization signal (Singer et al. 1986), the 1.8 kb TaqI digest fragment containing the insert was used for in situ hybridization. A 200 ng sample of probe was random-primed with a biotin-16-deoxyUTP nucleotide (Boehringer Mannheim) and E. coli DNA were added to labelled inserts of pMSAT5 and pHUR93, respectively. After ethanol precipitation, the labelled probes were resuspended in 50 µl of deionized formamide.

In situ hybridization

Cellular DNA was denatured in 70% formamide in 2×SSC (90°C, 3 min; SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) immediately after which the coverslips were immersed in a cold ethanol series (80%, 95%, 100% ethanol; 5 min each) and placed back on ice. A 10 µl sample of probe-hybridization mixture was then added to each coverslip, which was placed, cell-side down, onto parafilm in a plastic Petri dish. The humidity in the dish was maintained by including a paper tissue saturated with spent denaturation solution. Dishes were sealed with parafilm and incubated at 37°C, for ≥16 h.

Detection of hybridized sequences

Following washing (5x5 min, 2×SSC, RT), bound probe was reacted with 1:100 rabbit anti-biotin antibody (Enzo) in 2×SSC, 0.1% Triton X (2 h, RT), washed again and incubated with 1:50 fluorescein-conjugated goat anti-rabbit antibody (Jackson) in 2×SSC, 0.1% Triton X (2 h, RT), followed by washing as before. Coverslips were mounted on glass microscope slides using antifading mountant (Johnson and Noguera Aguino, 1981). Neurons exhibiting circular nuclei were photographed with phase-contrast optics (x100, oil-immersion) and fluorescent signals were visualized in a standard fluorescent microscope (l excit. 495 nm, bandpass 540 nm) and photographed on Kodak TMAX p3200 film. Nuclei were optically sectioned at 1 µm intervals, 6 µm above and below the nuclear midplane, as previously described (De Boni and Mintz, 1986). The film was push-developed to 6400 ASA using D-76 developer (Kodak).

Three-dimensional analysis of the spatial position of intranuclear loci

Negatives were projected onto paper and the outlines of nuclei and nucleoli derived from phase-contrast micrographs, as well as fluorescent sDNA and tDNA signals, were traced for each frame. Nuclei of these cells, previously shown to be spherical (De Boni and Mintz, 1986), had their center assigned as the origin of a three-dimensional XYZ coordinate system. The projections were digitized, using an in-house computer program, and the distance from the nuclear center for each detected locus was calculated. Distances are expressed as mean±standard error of the mean (S.E.M.). Signals were compartmentalized according to criteria previously reported (Borden and Manuelidis, 1988), so that signals found to be >1 µm from the nucleolus or the nuclear membrane were defined to be nucleoplasmic signals. In contrast, signals clearly opposed to the nucleolus were counted as nucleolus-associated signals. Computer-assisted three-dimensional reconstructions of some nuclei illustrated the arrangement of signals within the nucleus.

Results

Morphology

DRG neurons routinely exhibited a large vesicular nucleus with a clearly defined nuclear envelope and nucleolus. DRG neurons were readily distinguishable.
from other cell types on the basis of size and cell morphology. As neuronal cells dedifferentiate in culture, they typically exhibit three to four nucleoli. However, as the culture matures and cells redifferentiate, nucleoli are observed to fuse into one central, large nucleolus. Only DRG neurons with a single, large nucleolus were analyzed in the present study.

Despite the extensive pretreatments and denaturation conditions employed, nuclear morphology was sufficiently preserved to permit clear identification of cellular and nuclear outlines and of nucleoli. Only nuclei that did not overlap with other nuclei of DRG neurons or of background, non-neuronal cells were chosen. Ambiguous, faint signals were not included in the analysis.

Probe penetration was improved by prolonged pretreatments with 0.1 M HCl and Pronase and by use of increased denaturation times. This resulted in poor nuclear morphology. Therefore, conditions were optimized to permit adequate probe penetration while maintaining nuclear morphology. Optimal conditions for in situ hybridization for non-neuronal, fibroblast-like nuclei resulted from 10 min incubations with 0.6 mg ml⁻¹ Pronase, in the absence of HCl treatment. For detection of sDNA in nuclei of DRG neurons, cultures were incubated with 0.1 M HCl for 5 min and exposed to 0.8 mg ml⁻¹ Pronase for 10 min. In contrast, more prolonged pretreatment was required for DRG neurons probed for tDNA sequences. These included incubation with 0.1 M HCl for 20 min and 0.8 mg ml⁻¹ Pronase for 15 min. It should be noted that treatment with Pronase had previously been shown not to result in a reorganization of chromatin (Manuelidis, 19846).

The average nuclear diameter in all DRG neurons examined (n=73) was determined to be 10.08±0.17 μm (range 7.00–14.54 μm). In vitro studies have previously established the average diameter of an unfixed DRG neuronal nucleus as 10.72±0.32 μm (Fung and De Boni, unpublished). The fixation protocol, dehydration and pretreatments thus altered the dimensions of the nucleus by a mean factor of 0.94. Owing to the range of nuclear diameters encountered in the DRG nuclei examined, all nuclear dimensions were normalized to a nuclear diameter of 10.72 μm.

**Control experiments for in situ hybridization**

Several control experiments were carried out to eliminate the possibility that the observed signals were due to non-specific hybridization of probe to endogenous denatured DNA and to exclude the possibility that no unincorporated, biotinylated nucleotides were detected. In a typical control, 5–10 μl of hybridization buffer was used instead of denatured probe, while in others, the primary antibody was omitted. In both of these controls only low levels of cytoplasmic autofluorescence were detected, with the nucleus clearly outlined as a dark, circular area, without any fluorescent signals (not shown).

**Arrangement of tDNA sequences**

The spatial arrangement of tDNA sequences in neurons was examined in 40 cells (319 signals; Fig. 1A,B). A mean number of 8.02±0.40 signals/nucleus (range 5–13 signals/nucleus) was observed. The hybridization signals from tDNA sequences measured 1–2 μm in width and were clearly in focus in more than one optical section (1 μm). Of all tDNA signals assayed, 41.65±0.59 % were found closely associated with the nucleolus, ranging from 2 to 5 signals/nucleus (Figs 2, Fig. 3A-C). The remaining fraction of 57.23±0.56 % or 3–8 signals/nucleus was detected between the nucleolus and the nuclear periphery (Figs 3C, 4). A small fraction of these signals, approximately 10 %, was found in close proximity to the nuclear membrane. Overall, sDNA signals were found at a mean distance of 3.15±0.06 μm from the center of the nucleus, representing 63.81±0.07 % of the nuclear radius (Fig. 3A).

In non-neuronal interphase nuclei of background, fibroblast-like cells, 37–40 sDNA signals/nucleus were detected (n=10; Fig. 1C,D). These sDNA signals were found throughout the nucleus, were often observed occurring in pairs, were smaller in size, approximately 0.5 μm in width, and in focus only within one optical section of 1 μm.

**Arrangement of sDNA sequences**

The spatial pattern of telomeric DNA sequences was examined in 33 cells (1219 signals; Fig. 5). In contrast to sDNA sequences, an average of only 18.45±0.41 % of the total number of signals was associated with the nucleolus (Figs 3B,C, 4B). The remaining fraction of 81.79±0.41 % of signals was either associated with the nuclear periphery (12.81±0.50 %) or observed at positions between the nucleolus and the nuclear membrane (Figs 3B, 6). Signals were found at a mean distance of 3.57±0.03 μm from the nuclear center, corresponding to 66.59±0.61 % of the nuclear radius (Fig. 3B). Although this mean is not significantly different from the mean distance of sDNA signals from the nuclear center, the frequency distributions differ significantly from that found for sDNA (Fig. 3) as determined by the non-parametric Kolmogrov-Smirnov two-group test (P=0.0126). This statistical test is ideal for determining the level of significance of a frequency profile (Young, 1977; van Dekken et al. 1990). In contrast to sDNA signals, tDNA signals were much
Discussion

Chromosomes occupy distinct territories within the interphase nucleus of many cell types. Several investigators have shown that chromatin arrangement is cell type-
Specific, an arrangement that may be influenced by differentiation, pathological conditions or gene expression (Barr and Bertram, 1949; Manuelidis, 1985a; Blobel, 1985; Borden and Manuelidis, 1988; Arnoldus et al. 1989). The finding reported here that sDNA signals in DRG neuronal nuclei are found to be associated primarily with the nucleolus and at positions intermediate between the nucleolus and the nuclear membrane, is similar to the arrangement of sDNA sequences reported for interphase nuclei in human cancer cell lines (Haaf and Schmid, 1989; van Dekken et al. 1990), in Chinese Hamster Ovarian and B-lymphoid cell lines (Moroi et al. 1981), in mouse liver cells (Katsumata and Lo, 1988), in mouse Sertoli cells (Rae and Franke, 1972) and in mouse and human central nervous system cells (Manuelidis, 1984a, 1985a; Manuelidis and Borden, 1988).

Non-neuronal 'fibroblast'-like cells examined in the present work exhibited a number of sDNA signals close to the diploid complement of the mouse genome, 40 signals/nucleus. This confirmed that all centromeric sDNA targets were detected under the conditions employed, and stands in contrast to the reduced number of signals/nucleus arrangement of sDNA sequences reported for interphase nuclei in human cancer cell lines (Haaf and Schmid, 1989; van Dekken et al. 1990), in Chinese Hamster Ovarian and B-lymphoid cell lines (Moroi et al. 1981), in mouse liver cells (Katsumata and Lo, 1988), in mouse Sertoli cells (Rae and Franke, 1972) and in mouse and human central nervous system cells (Manuelidis, 1984a, 1985a; Manuelidis and Borden, 1988).

Fig. 4. Representative, three-dimensional reconstructions of neurons showing spatial positions of sDNA (A) and tDNA (B), relative to nucleoli (filled) and nuclear periphery. Reconstructions are of neurons shown in Fig. 1 and in Fig. 5. Halves are shown rotated -35 degrees and +150 degrees, respectively. Note large size and lower number of sDNA signals (A) compared to tDNA (B).

Fig. 5. Micrographs showing labelled tDNA signals (B) and corresponding phase-contrast image (A) of representative neuron. Note 5 clearly defined signals in this focal plane, others out of focus. Bar, 5 μm.

Fig. 6. Diagrammatic representation of the spatial positions of nucleoli and corresponding tDNA signals (triangles) in 26 neurons. See legend to Fig. 2 for details, with the exception that tDNA signals are small and their actual size is contained within the filled triangle. Note spread of signal positions between nucleolus and the nuclear periphery.
observed in DRG neuronal nuclei. This is likely to be due to clustering of sDNA signals, resulting in the large signal volumes observed in neurons. Given an ultrastructurally determined average width of centromeric regions of approximately 0.2–0.3 μm (Sedat and Manuelidis, 1978; Manuelidis, 1982), it may be considered that centromeric regions of several chromosomes cluster to form the signals observed, with the signal size potentially proportional to the number of centromeres contained in the cluster. In contrast, the larger number and smaller size of sDNA signals observed in nuclei of the ‘fibroblast-like’ cells, suggest that little or no clustering occurred in this cell type. Clustering of centromeres, as reported here for neuronal nuclei in DRGs, is a common, well-documented phenomenon in neurons (Manuelidis, 1984a,b; Arnoldus et al. 1989). It has also been described in plant cell nuclei (Fussel, 1975; Church and Moens, 1976; Evans and Filion, 1982). The mechanism that underlies the condensation of centromeres into clusters remains unclear. It is thought, however, that interactions between kinetochore proteins could mediate such clustering (Haaf and Schmid, 1989). This is supported by results of ongoing work in our laboratory that have clearly shown that kinetochore proteins are present in post-mitotic DRG neurons. Moreover, these proteins present as clusters, are quantitatively similar in distribution to sDNA signals (Holowacz and De Boni, 1991).

In contrast to the extensive body of work carried out on sDNA patterns, relatively little work has addressed the topology of tDNA in interphase nuclei. In contrast to results of work by Rawlins and Shaw (1990a,b), who recently reported an association of telomeres with nucleoli and the nuclear membrane in Pisum sativum, the work shown here indicates that approximately 80% of tDNA signals are not localized to these loci but, rather, are found in the nucleoplasm, intermediate between the nucleolus and the nuclear membrane.

The observation that the average number of telomere signals detected was half that expected for a diploid complement may result either from an inconsistency in labelling or, alternatively, may be due to a distinct labelling pattern of mouse acrocentric chromosomes (Narayanswami et al. 1989). In that work, in situ hybridization to telomeres also resulted in labelling of only half of the potential labelling sites. This was interpreted to indicate that labelling occurred to only those telomeres associated with the short segment of mouse acrocentric chromosomes and that either hybridization at the other chromosome end was less intense or tDNA was possibly inaccessible to the probe. On this basis, it could be predicted that the distances for sDNA and tDNA signals would be similar. This is supported by results reported here. In addition, the observation that signals of both centromeres and telomeres often occur in pairs may be interpreted as an indication of pairing of homologous chromosomes, as reported by Brinkley et al. (1986), Hadlaczyk et al. (1986) and Arnoldus et al. (1989).

The finding that satellite and telomeric DNA sequences are distributed throughout the nucleoplasm and not associated with any particular pole of the nucleus stands in contrast to the topology of chromosomes exhibiting a classical Rabl configuration, as seen in polytene nuclei (Agard and Sedat, 1983; Hochstrasser et al. 1986; Hochstrasser and Sedat, 1987a,b) and in nuclei of several plant species (Fussel, 1975; Church and Moens, 1976; Avivi and Feldman, 1980). This is in keeping with evidence from other mammalian systems where Rabl configurations are not generally found (Pardue and Gall, 1970; Rae and Franke, 1972; Manuelidis, 1982, 1984b, 1985a,b; Schardin et al. 1985; Manuelidis and Borden, 1988; Arnoldus, 1989).

Order within the interphase nucleus is thought to result from attachment of specific chromosome segments, i.e. centromeres, telomeres and heterochromatic regions, to the nuclear envelope, to the nuclear matrix and to the nucleolus. The observation that 47% of the sDNA signals in neuronal nuclei are associated with the nucleolus indicated that nucleolar-organizing region (NOR)-bearing chromosomes may be contained within these sDNA clusters. The nucleolus is a major structure in the nucleus wherein its spatial position is tissue-specific (Bourgeois et al. 1979; Hubert and Bourgeois, 1986; Wachtler et al. 1986; Hochstrasser et al. 1987a; Bourgeois and Hubert, 1988). In interphase, it represents the position of active NORs (Hsu et al. 1971; Bourgeois et al. 1984; Wachtler et al. 1986; Rawlins and Shaw, 1990a) and is thought to be the most striking example of nonrandom organization of chromatin domains (Comings, 1980). Moreover, Haaf and Schmid (1989) and Schardin et al. (1985) proposed such models in which repetitive sequences could act as a structural center for the extension and condensation of chromatin to and from a transcriptionally competent nuclear compartment. It is possible that this organization reflects the physiological state of a given cell (Manuelidis and Borden, 1988; Borden and Manuelidis, 1988) and is thought to remain dynamic in highly differentiated cells. Rearrangement of specific chromatin domains has been repeatedly observed in vitro, represented by a phenomenon termed Nuclear Rotation (Pomerat et al. 1967; De Boni and Mintz, 1986; De Boni, 1988; Fung and De Boni, 1988; Hay and De Boni, 1991; Park et al. 1991; Park and De Boni, unpublished data). In addition, rearrangement of chromatin domains has been implied to occur in vivo (Manuelidis, 1985a; Borden and Manuelidis, 1988; Haaf and Schmid, 1991). It remains to be shown in which manner the tissue-specific, non-random pattern of chromatin organization is established. Nuclear Rotation, the motion of chromatin domains, has been proposed to play such a role during differentiation. In fact, recent observations indicate that Nuclear Rotation is most pronounced during differentiation of cells in vitro and is less pronounced as cells assume their fully differentiated state (Park and De Boni, unpublished data; Park et al. 1991).

In summary, the results presented here confirm that specific chromatin domains are reproducibly compartmentalized. While this has been postulated to represent one level of control of gene expression (Comings, 1980), it remains to be shown whether the nuclear location of a gene effects its expression.
References


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