Relationships between Clusters of Interchromatin Granules and Chromatin Fibres

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Abstract

Among nuclear compartments, interchromatin granule clusters (IGCs) are widely regarded as biomolecular condensates implicated in the regulation of gene expression leading to the production of distinct mRNA species. Nevertheless, their functional dynamics within the nuclear environment remain largely elusive. In this study, we employed multiple transmission electron microscopy approaches to investigate the spatial and structural relationships between IGCs and chromatin. Our observations in HeLa cells demonstrate that IGCs establish physical connections with chromatin fibers. Furthermore, we show that the periphery of IGCs is enriched in decondensed chromatin domains and transcriptional sites. Quantitative analyses reveal that, upon α-amanitin treatment, the number of decondensed chromatin sites in proximity to IGCs is significantly reduced compared to untreated cells. In untreated conditions, a positive correlation emerges between IGC size and the abundance of adjacent decondensed chromatin regions. Based on these findings, we propose a conceptual model of IGC organization that provides new mechanistic insights into their structural and functional dynamics within the cell nucleus.

Keywords : interchromatin granule cluster, nucleus, chromatin, transmission electron microscopy

Introduction

The nucleus of eukaryotic cells contains distinct functional compartments that are not delimited by membranes. These compartments appear within the nucleoplasm as biomolecular condensates, self-assembled through a thermodynamically driven liquid–liquid phase separation process [1-3]. The nucleolus, nuclear speckles, and Cajal bodies are well-known examples.

Nuclear speckles are highly dynamic structures [4, 5], whose number and size vary according to cell type and physiological state. Their size increases when transcription and/or splicing is inhibited [6-13]. Under transmission electron microscopy, speckles correspond to interchromatin granule clusters (IGCs), which are composed of interconnected granules measuring 20–25 nm in diameter [4, 14, 15]. Their chemical composition is characterised by an abundance of splicing factors [7, 16, 17]. However, numerous other molecules have also been identified within these speckles [18], including subunits of RNA polymerase II [19], transcription factors [20], pre-mRNA processing factors [21], and the 20S proteasome [22].

Currently, nuclear speckles are widely regarded as coordination platforms for mRNA regulation. They are thought to contain the molecular machinery required for transcription and pre-mRNA maturation, as well as for mRNA export and quality control [13, 21, 23-30]. Pathologies associated with speckle dysfunction have also been reported, including Nager syndrome and craniofacial microsomia [31].

Unlike nucleoli, which contain ribosomal DNA, speckles do not appear to harbour DNA [32]. Nevertheless, digestion experiments using DNase I and MNase suggest that maintaining speckle structure requires association with chromatin [33]. Furthermore, chromatin architectural factors such as cohesin and the transcriptional repressor CTCF are necessary to preserve proper speckle–chromatin interactions [34]. DNA, particularly in the form of decondensed chromatin and transcriptionally active regions, has been detected within the peripheral zone surrounding IGCs [32, 35-38]. This peripheral compartment is referred to as the perispeckle region [39].

In this work, we examined the interplay between chromatin and IGCs in HeLa cells treated or untreated with α-amanitin, an inhibitor of RNA polymerase II transcription. We first assessed the impact of transcriptional inhibition on the three-dimensional structure of IGCs using confocal microscopy, followed by three complementary transmission electron microscopy techniques. The cytochemical acetylation method was employed to enhance the contrast of condensed chromatin near IGCs. In addition, immunogold detection of modified nucleotides added to DNA termini by a specific transferase on ultrathin sections enabled the identification of decondensed DNA in the immediate vicinity of IGCs. Finally, we investigated RNA synthesis sites at the ultrastructural level by incorporating BrUTP into cells via a transfection vector. Our findings clearly indicate that chromatin fibres connect IGCs within the nucleus. Although chromatin clumps persist at the periphery of large IGCs in α-amanitin-treated cells, quantitative analysis reveals that the number of decondensed DNA

sites adjacent to IGCs is higher in untreated cells. In these cells, the larger the IGC, the greater the number of decondensed DNA sites. We also demonstrate that RNA synthesis sites are located in the periphery of IGCs, the perispeckle region. Ultimately, we propose a dynamic model of IGC organisation within the nucleus based on their interactions with chromatin.

Materials and Methods

Biological Materials

HeLa cells were cultured at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle Medium (Gibco-BRL, Life Technologies, Ghent, Belgium) supplemented with 10% fetal calf serum and 100 U/ml penicillin. For transcriptional inhibition, selected cultures were treated for 3 h with 10 µg/ml α-amanitin (Sigma, St. Louis, USA).

Immunofluorescence Procedures

Slides were fixed for 10 min at room temperature in 4% formaldehyde prepared in 0.1 M PBS (pH 7.4), rinsed twice for 10 min in PBS, and permeabilized for 6 min in 0.5% Triton X-100. Subsequently, they were washed in PBS containing 1% BSA (w/v) and normal rabbit serum (NRS) diluted 1:30, followed by incubation for 60 min at 37 °C with anti-mouse SC35 antibodies (Sigma) diluted 1:2000 in PBS supplemented with NRS (1:50) and 0.2% BSA. After three washes in PBS containing 1% BSA (10 min each), slides were incubated for 60 min at 37 °C with Alexa Fluor 594 rabbit anti-mouse antibody (Molecular Probes) diluted 1:2000 in PBS containing 0.2% BSA. Following rinsing, slides were counterstained for 15 min at room temperature with Hoechst 33258 (1:500), rinsed in distilled water, and mounted using CitiFluor™ AF1 (Agar Scientific, Stansted, UK).

Confocal Microscopy and Three-Dimensional Reconstructions

A Bio-Rad 1024ES system (Bio-Rad, Hercules, CA, USA) mounted on an inverted Olympus IX70 microscope was employed. Image acquisition was performed using a 60× plan-apochromat oil immersion objective (NA = 1.4). FITC was excited with the 488 nm line of a Krypton/Argon laser, and emission was collected through a 522 ± 16 nm band-pass filter. Phase-contrast images were simultaneously recorded on a dedicated detector. For 3D analysis, 30–50 optical sections were captured from the top of the cell at 0.5 µm z-steps and reconstructed as previously described [40]. Surface rendering was subsequently applied [41].

Transmission Electron Microscopy

Cells were fixed for 60 min at 4 °C in 1.6% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4) and acetylated as previously reported [42]. After washing in Sorensen's buffer, samples were dehydrated through graded ethanol series and embedded in Epon. Ultrathin sections were mounted on collodion-coated grids and stained with uranyl acetate and lead citrate prior to examination in a JEM 1400 transmission electron microscope operating at 80 kV. Random fields were photographed using an 11-megapixel camera system (Quemesa, Olympus).

Detection of DNA: In Situ Terminal Deoxynucleotidyl Transferase (TdT) Immunogold Method

The TdT-based immunogold technique was applied as previously described [43]. Acetylated ultrathin sections were incubated for 30 min at 37 °C in a reaction medium containing 100 mM sodium cacodylate (pH 6.5), 10 mM β-mercaptoethanol, 2 mM $MnCl_2$, 50 µg/ml BSA, 20 µM 5-bromo-2′-deoxyuridine triphosphate (BUdR), 4 µM each of dCTP, dGTP, and dATP, and 125 U/ml TdT. After rinsing, sections were blocked with normal goat serum (NGS) and incubated for 4 h at room temperature with mouse anti-BUdR antibodies (Roche) diluted 1:50 in PBS containing 0.2% BSA and NGS. Detection was achieved using rabbit anti-mouse IgG conjugated to 10 nm colloidal gold particles (Amersham Life Science) diluted 1:40. Control experiments omitting either TdT or the primary antibody yielded no detectable labelling.

Between 22 and 42 micrographs per sample were acquired at magnifications ranging from 10,000× to 15,000× in randomly selected nuclear regions. Morphometric analyses were performed using iTEM 5.2 software (Olympus SIS). Statistical significance of labelling differences between nuclear compartments and background (resin or cytoplasm) was assessed using Student’s t-test. Pearson correlation analysis was applied to evaluate the relationship between IGC size and peripheral labelling density.

Detection of Newly Synthesised RNA

BrUTP incorporation was carried out using FuGene-6 transfection reagent following the protocol of Thiry et al. [44], with 15 min pulse labelling. After incorporation, cells were fixed in 2% formaldehyde in PBS (pH 7.4) for 10 min, permeabilised with 0.3% Triton X-100, and blocked with NGS. Immunolabelling was performed using mouse anti-BUdR antibodies (Roche) followed by secondary goat anti-mouse antibodies conjugated to 10 nm gold particles. Control experiments omitting BrUTP or the primary antibody confirmed the specificity of the labelling.

Results

Confocal Microscopy

To confirm that α-amanitin treatment induces the formation of enlarged speckles in HeLa cells, we first performed confocal microscopy using SC35, a well-established marker of nuclear speckles. Analysis of optical sections revealed that, in α-amanitin-treated cells, certain SC35-labelled foci were markedly larger than those observed in untreated cells. In addition, an alignment of multiple SC35 clusters forming chain-like structures was occasionally detected. Surface rendering of SC35-labelled speckles in untreated cells showed that these clusters were generally spherical (Fig. 1A), although their surfaces appeared irregular and spiculated. The clusters were spatially separated and located in regions exhibiting weak Hoechst staining. Both the size and number of SC35 foci per nucleus varied among cells. In contrast, surface rendering of α-amanitin-treated cells revealed that some clusters had lost their spherical morphology, adopting elongated shapes (Fig. 1B), possibly resulting from the fusion of multiple speckles. Their surfaces appeared more irregular and spiny compared to untreated cells.

Transmission Electron Microscopy

Cytochemical Acetylation Method

To investigate the spatial relationship between chromatin and IGCs, we first applied the cytochemical acetylation method to both untreated and α-amanitin-treated cells. This technique enhances the contrast of condensed chromatin within the nucleus, thereby improving its visualisation in RNP-rich compartments.

In untreated cells, IGCs appeared as rounded structures with an average area of approximately 0.33 ± 0.16 μm² (n = 42), composed of numerous small granules (Fig. 2A). The peripheral zone of IGCs contained clumps of condensed chromatin. These clumps did not establish direct contact with the IGCs; rather, only certain chromatin fibres extended from these clumps into the intervening space, occasionally reaching the IGC surface (Figs. 2A, 2B).

In α-amanitin-treated cells (Fig. 2C), enlarged IGCs were observed in 75% of the examined nuclei (n = 100), with an average area of approximately 0.62 ± 0.21 μm² (n = 25). A Student’s t-test confirmed that this difference in mean area between conditions was statistically significant ($p < 0.01$). These enlarged IGCs were also composed of numerous granules, but their density was higher in the central region than at the periphery, imparting a bipartite appearance (Fig. 2D).

Immunocytochemical Detection of DNA Using TdT

Because the acetylation method does not allow identification of decondensed chromatin, we next employed the TdT-based immunogold technique to localise DNA with high sensitivity, regardless of its condensation state [45]. This method was applied to ultrathin sections previously processed by acetylation.

In untreated cells (Fig. 3A), gold particles were predominantly associated with condensed chromatin blocks, with lighter labelling observed on decondensed chromatin within interchromatin spaces. Virtually no labelling was detected within IGCs. However, in the narrow spaces separating IGCs from adjacent chromatin clumps—particularly near the IGC surface—gold particles were clearly present on decondensed chromatin.

A similar distribution was observed in α-amanitin-treated cells (Fig. 3B), although the density of gold particles in the periphery of IGCs appeared reduced. Quantitatively, 93% of IGCs in untreated cells exhibited peripheral labelling, compared to 76% in treated cells.

Statistical analysis (Fig. 4) confirmed that labelling over condensed chromatin and in the periphery of IGCs was significantly higher than background (resin or cytoplasm) in both conditions, whereas labelling within IGCs was not significant. A Student's t-test further demonstrated that the difference in peripheral labelling between untreated and treated cells was highly significant ($p < 0.001$).

We also examined whether the number of gold particles in the periphery correlated with IGC size. In untreated cells (Fig. 5A), a positive correlation was observed (Pearson correlation coefficient: 0.6), indicating that larger IGCs were associated with more decondensed chromatin contacts. In contrast, no such correlation was detected in treated cells (Pearson coefficient: –0.2), and the trend even appeared reversed (Fig. 5B).

Localisation of RNA Synthesis Sites Using BrUTP Incorporation

To map RNA synthesis sites in the vicinity of IGCs, cells were transiently transfected with BrUTP–FuGene complexes, and incorporated BrUTP was detected by immunogold labelling.

In untreated cells, gold particles were localised within the fibrillar components of the nucleolus and in interchromatin spaces adjacent to condensed chromatin blocks (Fig. 6A). Notably, clear labelling was observed in the periphery of IGCs, whereas no signal was detected within the IGCs themselves.

In α-amanitin-treated cells (Fig. 6B), only a few gold particles were detected in nucleolar fibrillar components and interchromatin spaces, and no labelling was observed either within or around IGCs.

Discussion

Our ultrastructural analysis strongly supports preferential contacts between interchromatin granule clusters (IGCs) and chromatin. More specifically, we demonstrate that these interactions involve decondensed chromatin regions that establish direct contact with the IGC surface, whereas no DNA was detected within the IGCs themselves—a finding fully consistent with earlier ultrastructural observations [32].

The presence of multiple decondensed chromatin contacts surrounding IGCs strongly suggests that these interactions play a critical role in the functional organisation of nuclear speckles. This interpretation is reinforced by recent biochemical studies showing that speckle–chromatin associations are essential for maintaining speckle integrity [33] and that chromatin architectural proteins such as cohesin and CTCF are required to stabilise these associations [34]. Nevertheless, the precise molecular nature of these interactions remains poorly understood.

Our quantitative data further reveal that the number of chromatin contacts varies according to the transcriptional state of the cell. In untreated cells, a positive correlation exists between IGC size and the number of peripheral decondensed chromatin sites, suggesting that transcriptional activity promotes the recruitment of chromatin regions to IGCs. In contrast, this correlation disappears—and even reverses—under α-amanitin treatment, which inhibits RNA polymerase II transcription. These findings imply that transcriptional inhibition triggers the coalescence of IGCs into larger, non-functional aggregates, a phenomenon reminiscent of the giant IGCs observed during early mitosis when transcription is globally silenced [46]. Similar morphological changes have also been reported following treatment with camptothecin [47] and DRB [9].

We also observed that α-amanitin-treated IGCs frequently exhibit a bipartite organisation, with a denser central region and a less compact periphery. While this structural rearrangement has previously been described during early mitosis [46], it should not be confused with the subcompartmentalisation of speckles reported by super-resolution microscopy [48]. At the ultrastructural level, IGCs appear as highly homogeneous assemblies of numerous granules, and the two-zone organisation inferred from confocal imaging is not discernible [49]. Nevertheless, the presence of IGC extensions toward decondensed chromatin in untreated cells may represent the structural basis for specialised functional domains within speckles.

Based on these observations, we propose a dynamic model for IGC organisation within the nucleus (Fig. 7). Gene activation would drive the recruitment and clustering of the molecular machinery required for gene expression, including splicing factors, thereby giving rise to highly organised structures in which the IGC core serves as a central scaffold, while the surrounding chromatin represents transcriptionally active loci anchored to the IGC. Our previous work and the present study both demonstrate that this peripheral zone contains decondensed chromatin, and we now provide ultrastructural evidence that these chromatin regions establish direct physical contacts with IGCs.

This model aligns with optical microscopy studies suggesting that transcription occurs at the periphery of speckles [27, 37, 50-53] and corroborates earlier electron microscopy findings indicating that RNA synthesis sites are located around IGCs [35]. Our BrUTP incorporation experiments further confirm that the periphery of IGCs harbours active transcription sites under normal conditions, whereas these sites disappear upon transcriptional inhibition.

Moreover, the perispeckle region has been identified by correlative microscopy as a major assembly site for exon junction complexes [39]. The size of this functional domain likely reflects the number of active genes engaged in transcription. Consequently, a reduction in transcriptional activity—whether physiologically during mitotic entry or experimentally through chemical inhibitors such as α-amanitin—would release IGCs from their chromatin anchors, allowing them to coalesce into large, transcriptionally inert aggregates within the nucleus.

Collectively, these findings provide new mechanistic insights into the interplay between nuclear speckles and chromatin, highlighting their dynamic reorganisation in response to transcriptional cues and reinforcing the view that IGCs act as central hubs coordinating gene expression within the three-dimensional nuclear architecture.

Figure Legends

Figure 1. Three-dimensional reconstruction and surface rendering of a HeLa cell nucleus either untreated (A) or treated with α-amanitin (B). Optical sectioning through the DNA volume (blue) enabled visualisation of SC35-labelled speckles (red) within the nuclear space. Scale bar: 5 μm.

Figure 2. Visualisation of condensed chromatin (C) revealed by the cytochemical acetylation method in HeLa cell nuclei, either untreated (A) or treated with α-amanitin (B). IGCs are delineated by dashed lines. Small blocks of condensed chromatin are observed in close proximity to IGCs (large arrowheads), while fibres extending from these blocks occasionally reach the IGC surface (small arrowheads). In treated cells, the IGC core frequently appears denser (interrupted line, D). Scale bar: 0.2 μm.

Figure 3. Detection of DNA by the TdT immunogold technique in HeLa cell nuclei, either untreated (A) or treated with α-amanitin (B). IGCs are outlined by dashed lines. Gold particles associated with non-condensed chromatin (C) are detected in the immediate periphery of IGCs (arrows). Rare gold particles are present within IGCs (circle). Scale bar: 0.2 μm.

Figure 4. Mean densities of gold particles (number of particles per square micrometre) across different nuclear compartments in HeLa cells, untreated or treated with α-amanitin for 3 h. Student's t-test for nuclear compartments vs. resin or cytoplasm (: $p < 0.0001$).

Figure 5. Trend curves illustrating the relationship between IGC area and the number of gold particles located in their periphery in untreated (A) and α-amanitin-treated (B) HeLa cells.

Figure 6. Detection of newly synthesised RNA in HeLa cells transfected for 15 min with BrUTP–FuGene complexes prior to fixation. BrUTP-labelled RNA was visualised by indirect immunogold labelling. IGCs are outlined by dashed lines. In untreated cells (A), strong labelling is observed in the periphery of IGCs (arrows). In contrast, no peripheral labelling is detected in α-amanitin-treated cells (B). A few gold particles are present in the nucleolus (Nu). C: condensed chromatin; NE: nuclear envelope. Scale bar: 0.2 μm.

Figure 7. Dynamic model for IGC organization. Active (left) and inactive (right) IGC organization. See text for description.

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