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Organization of the Amplified Type I Interferon Gene Cluster and Associated Chromosome Regions in the Interphase Nucleus of Human Osteosarcoma Cells

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Abstract

The organization of the amplified type I interferon (IFN) gene cluster and surrounding chromosomal regions was studied in the interphase cell nucleus of the human osteosarcoma cell line MG63. Rather than being arranged in a linear ladder-like array as in mitotic chromosomes, a cluster of approximately 15 foci was detected that was preferentially associated along the periphery of both the cell nucleus and a chromosome territory containing components of chromosomes 4, 8 and 9. Interspersed within the IFN gene foci were corresponding foci derived from amplified centromere 4 and 8 sequences. Other copies of chromosome 4 and 8 were frequently detected in pairs or higher order arrays lacking discrete borders between the chromosomes. In contrast, while chromosomes 4 and 8 in normal WI38 human fibroblast and osteoblast cells were occasionally found to closely associate, discrete boundaries were always detected between the two. DNA replication timing of the IFN-gene cluster in early- to mid-S phase of WI38 cells was conserved in the amplified IFN gene cluster of MG63. Quantitative RT-PCR demonstrated a ~3 fold increase in IFN β transcripts in MG63 compared with WI38 and RNA/DNA FISH experiments revealed 1-5 foci of IFN β transcripts per cell with only approximately 5% of the cells showing foci within the highly amplified IFN gene cluster.

Keywords

human osteosarcoma cells; interferon (IFN) gene cluster; gene amplification; chromosome territories; IFN chromosome; fluorescence in situ hybridization (FISH); quantitative RT-PCR

Introduction

The cell nucleus is both the repository for the genome and the complex processes of replication, transcription, and RNA processing (Bartova and Kozubek, 2006; Parada et al., 2004; Pombo and Branco, 2007). How these functions are precisely coordinated within the nuclear landscape

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has long been a topic of interest (Gilbert, 2002; Razin et al., 2007). Recent advances in microscopic imaging of the cell nucleus have led to an emerging view of a highly compartmentalized organelle with both spatial and temporal functional domains (Berezney, 2002; Dundr and Misteli, 2001; Lanctot et al., 2007; Wei et al., 1998). These domains are comprised of multiple protein and nucleic acid complexes working together to achieve genomic regulation (Schneider and Grosschedl, 2007; Stein et al., 2003; Zaidi et al., 2007).

The application of chromatin labeling techniques to the interphase nucleus has further changed our view of genomic organization. Pulse labeling of replicating DNA with thymidine analogs illustrates that not only does replication follow a precise order but the labeled chromatin domains that makes up a replication site represent a fundamental higher order chromatin structure termed the *1 mbp chromatin domain* (Jackson and Pombo, 1998; Ma et al., 1998). These 1 mbp domains are organized into chromosomes occupying discrete territories without extensive overlapping of boundaries at the level of light microscopy (Albiez et al., 2006; Cremer and Cremer, 2001; Visser and Aten, 1999).

The chromosome specific labeling method termed *chromosome painting* is widely used to evaluate karyotypes of clinical samples during metaphase (Garcia-Sagredo, 2008). An application of this technique termed spectral karyotyping (SKY) has been valuable in its ability to discern genome wide chromosomal rearrangements in disease states (Patel et al., 2000; Schrock et al., 1997). In particular, the karyotypic arrangement of the genome in cancer cells is typically highly altered including changes in chromosome numbers, translocations, deletions and amplifications (Frohling and Dohner, 2008; Storchova and Pellman, 2004). Although much progress has been made in identifying common disease specific chromosomal aberrations (Frohling and Dohner, 2008; Zaidi et al., 2007), little has been done to investigate these alterations at the level of chromatin in the functional interphase nucleus. A fundamental issue is: "To what extent are these aberrations in chromosome organization in cancer cells affecting the normal three-dimensional nuclear landscape and resulting in defects in highly regulated genomic processes such as transcription and replication?"

As a step in this direction we are investigating the metaphase and interphase organization of the amplified type I interferon (IFN) gene cluster and surrounding chromosomal regions in the human MG63 osteosarcoma cell line. Osteosarcoma cells are characterized by particularly large alterations in chromosome number and organization (Bridge et al., 1997; Ragland et al., 2002). The MG63 genome, for example, is hypotriploid and contains numerous alterations including chromosome duplications, deletions, additions, translocations and gene amplifications (Lim et al., 2005; Lim et al., 2004). The type I IFN gene cluster resides on the short arm of chromosome 9 in normal diploid cells and is composed of 13 alpha genes, 1 omega gene, 11 pseudogenes and a single beta gene contained within an approximately 450 kb chromatin region (Diaz et al., 1994). Owing to its particular architecture, this multi-member gene cluster participates in multiple deletion and translocation events (Bode et al., 2000). We have recently found a high degree of amplification of the IFN gene cluster in the MG63 osteosarcoma cell line (Marella et al., 2008). Moreover, the amplified IFN gene cluster was arranged in a ladder-like array of ~six repeating bands that spanned from one end to the other on a chromosome that contained numerous complex rearrangements. These rearrangements included segments of chromosome 4, 8 and 9 and also contained amplified centromeric DNA repeats from chromosomes 4 and 9 (Marella et al., 2008).

In this report we study the arrangement of this amplified IFN gene cluster and its associated segments of chromosomes and centromeric DNA repeats within the three dimensional structure of the interphase cell nucleus. The amplified IFN gene cluster is readily identified in the MG63 cell nucleus by fluorescence in situ hybridization (FISH) as a cluster of multiple spots with the number of spots being consistent with the 5-6 fold amplification of this gene cluster (Marella

et al., 2008). Chromosome 4, 8 and 9 territory regions along with multiple spots for centromeric 4 and 9 DNA were also found in close association. Virtually all chromosome 4 and 8 copies were closely associated as pairs or higher arrays suggesting a high degree of structural arrangement. Moreover, the chromosome 4/8 pairs typically interdigitate forming a mosaic-like organization which was absent in normal WI38 human fibroblast and osteoblast cells. Despite the arrangement of the amplified IFN gene cluster across the entire chromosome, its replication timing was maintained in early- to mid-S phase when compared to WI38 cells. Analysis of the transcriptional level following activation of the IFN β gene in diploid WI38 cells versus the amplified genes in MG63 cells revealed a similar temporal pattern of activation (2 h peak) with a several fold higher level of transcription in the MG63 cells. Consistent with these measurements, we detected several intense foci of newly transcribed IFN β RNA in the MG63 cell nucleus. Signals over the region containing the amplified IFN gene cluster, however, were found in only ~5% of the cells.

Materials and Methods

Cell culture and synchronization

WI38 normal human diploid fibroblasts (ATCC, Rockville, MD) and MG63 osteosarcoma (ATCC) cell lines were cultured in advanced DMEM containing 3% FBS, 1% penicillin and streptomycin in a 37°C incubator at 5% CO₂. HOB normal human diploid hipbone osteoblasts (PromoCell) were grown in osteoblast growth media (PromoCell) supplemented with 10% FBS. MG63 human osteosarcoma cells were synchronized at the G₁/S border by serum deprivation (0.1% FBS for 48 h) followed by release into normal media containing aphidicolin (2 µg/ml) for 14 h.

Interferon gene cluster probes

A series of BAC and cosmid probes that spanned the IFN gene cluster were used in this study. The BAC probes were obtained from the human RP-11 BAC library at Roswell Park Cancer Institute and consisted of: P8 (RP11-1P8) which covers the first one-third of the gene cluster beginning at the 5'-end, G20 (RP11-95G20) which encompasses the second one-third of the cluster and I7 (RP11-158I17) which spans the last third of the cluster (Marella et al., 2008). The cosmid probes used were: C9 and 133D4 in the first one-third of the gene cluster beginning at the 5'-end, 49D4 and F5 in the second-third of the cluster and C12 near the end of the gene cluster (Marella et al., 2008). The BAC and cosmid probes were labeled with either biotin-dUTP or digoxigenin-dUTP by nick translation (Roche Applied Science, Indianapolis, IN) as per manufacturer's protocol. Probe fragments were purified by passage through a PCR purification column from Qiagen (Valencia, CA). Single stranded DNA probes for IFN β mRNA detection were constructed as in (Chakalova et al., 2004) with minor modifications. Briefly, IFN β cDNA was cloned into pGEM-T Easy (Promega, Madison, WI) and in vitro transcribed with T7 polymerase. IFN β RNA was then reverse transcribed using random hexamer primers in the presence of biotin-dCTP.

Fluorescent in situ Hybridization

For standard DNA FISH, cells were fixed in 3:1 methanol/acetic acid for at least 1 h at 20°C. Cells were dehydrated through a 70% and 100% ethanol series. Cell denaturation was at 75°C in 70% formamide/2X SSC for 1 min. BAC, cosmid and chromosome paint probes in 50% formamide/2XSSC/10% dextran sulfate were denatured for 8-10 min at 75°C. DNA Probes were cooled on ice, hybridized overnight at 37°C, followed by three post-hybridization washes with 50% formamide/2XSSC/0.05% Tween 20, 2XSSC/0.05% Tween 20 and 1X SSC for 30 min each at 37°C.

For 3D DNA FISH, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, incubated in 20% glycerol/1XPBS 40 min, freeze-thawed in liquid nitrogen four times and treated with 0.1N HCl for 5 min. The cover slips were then stored in 50% formamide/2X SSC at 4°C until denaturation at 75°C for 5 min in 70% formamide/2XSSC followed by 50% formamide/2XSSC for 2 min. Aquarius paint probes (Cytocell) were denatured for 8 min at 75°C. Probe solution was applied to cover slips and sealed with rubber cement. Hybridization was carried out at 37°C for 48 h. Three post-hybridization washes were 50% formamide/2XSSC/0.05% Tween 20; 2XSSC/0.05% Tween 20 and 1XSSC for 30 min each at 37°C. Cover slips were mounted in Vectashield.

For RNA FISH, cells were fixed with 4% PFA for 10 min, permeabilized with 0.5% Triton X-100 for 10 min and incubated in 20% glycerol/1XPBS for 25 min followed by 4 freeze thaw cycles in liquid nitrogen. Cells were rinsed in 50% formamide/2XSSC and biotin labeled single stranded DNA probes were hybridized to cells overnight at 37°C. Post-hybridization washes consisted of 40% formamide/2XSSC/0.5% Tween 20; 2XSSC/0.05% Tween 20 and 1X SSC for 15 min each at 37°C. RNA signals were detected by indirect immunofluorescence.

For combined RNA-DNA FISH, cells were fixed with 4% PFA/10% acetic acid for 10 min, permeabilized with 0.5% Triton X-100 for 10 min. and incubated in 20% glycerol/1XPBS for 25 min followed by 4 freeze thaw cycles in liquid nitrogen. 10 ng ssDNA probes for RNA FISH, diluted in 50% formamide/2XSSC/10% dextran sulfate, 0.5µg Cot-1 DNA, 1 µg tRNA, were hybridized overnight as in DNA FISH. Post-hybridization washes consisted of 40% formamide/2XSSC/0.5% Tween 20; 2XSSC/0.05% Tween 20 and 1X SSC for 15 min each at 37°C. To increase sensitivity and enhance stability of RNA foci, RNA signals were detected with a tyramide-Alexa488 signal amplification kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Briefly, a streptavidin HRP conjugate was bound to the hybridized biotin-ssDNA IFNβ probe. Tyramide-Alexa488 is then activated by HRP and becomes covalently attached to proximal nucleophilic residues. For subsequent DNA FISH, RNA was first digested with 100 µg/ml RNase A for 45 min at 37°C. Cells were dehydrated through a 70% and 100% ethanol series and denatured for 8 min at 75°C in 70% formamide/2X SSC. DNA FISH probes were denatured and hybridized as above.

Replication timing analysis

WI38 fibroblasts growing on coverslips were pulsed with 10 µM BrdU for 15 min followed by immediate incubation in 0.075M KCl for 10 min. Cells were then fixed overnight in 3:1 methanol, acetic acid at -20°C. DNA FISH was performed and replication timing was determined based on the percentage of doublet signals counted in BrdU positive cells (Selig et al, 1992) and an approximately 11 hour S phase estimated by the CldU/IdU pulse-chase-pulse method (Panning and Gilbert, 2005). For replication timing of the amplified IFN gene cluster in MG63, the cells were synchronized at the G₁/S border by serum deprivation followed by aphidicolin (see above). The G₁/S arrested cells were released into normal media and incubated with 10 µM BrdU for 15 min at successive time points followed by immediate incubation in 0.075M KCl for 10 min. DNA FISH with probe 49D4 was performed and the number of FISH signals was tallied in BrdU positive nuclei.

Immunofluorescence microscopy of FISH and replication sites

Detection of interferon probes for DNA and separate RNA FISH was performed by reaction with by rabbit anti-biotin (Enzo, Farmingdale, NY) and/or mouse anti-digoxigenin antibodies (Roche, Indianapolis, IN) diluted (1:50) and secondary anti-rabbit and anti-mouse antibodies conjugated to Alexa594 and/or Alexa488 (Invitrogen, Carlsbad, CA). Replicated DNA was labeled with mouse anti-BrdU (Roswell Park Cancer Institute, Buffalo, NY) and the secondary antibody, anti-mouse Alexa488 (Invitrogen, Carlsbad, CA).

Following labeling, indirect immunofluorescence was detected with Chroma filter sets using an Olympus BX51 upright microscope (100 X plan-*apo*, oil, 1.4 NA) equipped with a Sensicam QE (Cooke Corporation, USA) digital CCD (charge-coupled device) camera, motorized z-axis controller (Prior) and Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO). 0.5 μ m optical sections were typically collected, deconvolved using a NoNeighbor algorithm operating within Slidebook 4.0 and exported as 16 bit tiff intensity files for further analysis. For some studies the 3-D image sets were transformed into maximum pixel intensity 2-D projections.

To verify the accuracy of our co-localization studies, we imaged 0.5 μ m diameter Fluoresbrite beads (Polysciences, Inc. Warrington, PA) in both the red and green channels and examined the merged images. Observation of completely yellow beads confirmed the overall validity of our results. We further determined that the average sub-pixel shift between the red and green channels of the imaged beads was 0.54 pixels ($\sim$ 0.03 microns) under the microscopic conditions used in our experiments (Malyavantham et al., 2008).

IFN β induction and real time PCR

WI38 and MG63 cells were treated with 50 μ g/ml poly (I:C). Total RNA was isolated using RNAqueous-4PCR (Ambion, Austin, Texas). 0.5 μ g RNA was reverse transcribed with StrataScript QPCR cDNA Synthesis kit using oligo(dT) primers (Stratagene, La Jolla, CA). Primers P2F: 5'-GCTTGGATTCTACAAAGAAGCAGC-3', P2R: 5'-CAA AGT TCA TCC TGT CCT TGA GGC-3' amplified a 101 bp fragment of the IFN β gene detected with SYBR green. MXpro software (Stratagene, La Jolla, CA) was used to analyze standard curves for a serial diluted IFN β -containing plasmid and GAPDH product to determine amplification efficiencies of target and control genes, respectively. The differences in IFN β gene expression are compared to pre-induced cells following normalization to GAPDH and adjustment for reaction efficiency.

Results

Organization of the IFN gene cluster in the cell nucleus of the human osteosarcoma cell line MG63

Using array comparative genomic hybridization (aCGH) we have found that the type 1 interferon (IFN) gene cluster is approximately 5-6 fold amplified as an entire unit in human osteosarcoma MG63 cells (Marella et al., 2008). Detailed FISH studies using BAC and cosmid probes that span the entire gene cluster confirmed this amplification and revealed a ladder-like arrangement that encompassed the entire chromosome [(Marella et al., 2008) and (Fig 1)]. The chromosome containing the amplified IFN cluster is not a normal chromosome 9 but rather a highly altered chromosome composed of portions of chromosomes 4, 8, 9 and amplified centromeric sequences from chromosomes 4 and 9 (Marella et al., 2008). Intriguingly, the amplified centromeres 4 and 9 were also arranged in a ladder-like array that interdigitated with the IFN-clusters (Marella et al., 2008). A schematic model summarizing the arranged of these components in this highly altered chromosome, termed the *IFN chromosome*, is presented in Figure 1.

With this as a basis, we have investigated the arrangement of the amplified IFN gene cluster and the associated chromosomal and centromeric elements within the interphase cell nucleus of the MG63 cell line. Using WI38 fibroblast cells as a normal diploid control and our series of BAC and cosmid probes that span the entire gene cluster, we consistently found that the IFN gene probes appeared as single spots over the chromosome 9 territories with no associations with either chromosome 4 or 8. This is shown for a representative cosmid gene probe 49D4 in Figure 2A-C. Performing the same FISH experiments for the IFN gene cluster and chromosome

territories in the MG63 cells, reveals the aberrant nature of the IFN gene cluster and its associated chromosomal components in this human osteosarcoma cell line (Fig 2 D-F). Unlike the very discrete chromosome territories labeled in the normal diploid WI38 cells, all the chromosomes labeled in the MG63 osteosarcoma cells (chromosomes 4, 8 and 9) showed multiple copies and significantly increased amounts of more diffuse staining throughout the cell nucleus (Fig 2 D-F). To exclude experimental variation we grew both MG63 and WI38 cells on the same coverslip and performed FISH with chromosome 9. We observed a consistently higher diffuse signal in the MG63 nuclei compared to WI38 cells. (data not shown). The highly amplified IFN cluster in MG63 appears as a cluster of FISH spots that localize to a region containing one of the copies of chromosomes 4, 8 and a diffusely stained portion of chromosome 9 (Fig 2 D-F).

Centromere 4 and 9 sequences were also found associated in repeating arrays that alternated with the amplified IFN gene cluster on the IFN chromosome (Marella et al., 2008). We, therefore, examined the arrangement of these two centromeric DNA sequences in relationship to the amplified IFN gene cluster in the MG63 cell nucleus. In both cases the centromeric DNA appeared as a similar number of fluorescent spots interspersed within the cluster of multiple spots that denote the amplified IFN genes (Fig 2 G, H). There was also a significant degree of overlap (as judged by the yellow spot color) between the repeating centromeric 4 and 9 sequences found in association with the amplified IFN gene cluster (Fig 2 I).

SKY analysis demonstrated an average of 3 copies of chromosome 4 and 5 copies of chromosome 8 in the MG63 genome (Marella et al., 2008). We detected a very high degree of association of the multiple chromosome 4 territories with chromosome 8 territories in the interphase cell nucleus of MG63 cells. This occurs as pair-wise or higher array associations of chromosome 4 and 8 and is illustrated in Figure 3A where all discrete staining regions for chromosome 4 that are large enough to be considered as chromosome territories, or significant parts of territories, are in close association with chromosome 8 regions. Rather than being discrete territories with well separated borders, the chromosome 4/8 pairings and higher arrays appear to be intermingled into a mosaic of closely associating chromosome 4 and 8 regions that we term *mega-territories* (see arrows in Fig 3A). This interdigitation of chromosome 4 and 8 territorial regions is more readily discerned at higher magnification (Fig 3B) and can be observed in a surface rendering of a 3-D optical stack of images (Fig 3C).

While chromosome territories 4 and 8 in normal WI38 cells do show associations between each other ($28.7\% \pm 4.3$), only $11.7\% \pm 5.3$ of the observed nuclei ($n=94$), demonstrated association of both copies of chromosomes (Table 1). In contrast, $74\% \pm 4$ ($n=26$) of the multiple chromosome 4 copies in MG63 cells were directly associating with copies of chromosome 8 in mega-territories. The much more limited close associations of chromosomes 4 and 8 observed in WI38 fibroblast and osteoblast cells had well defined territorial borders with no indications of significant intermingling (Fig 3D, Fig S1-C and D). These findings were reproducible using a 3-D FISH technique (see Materials and Methods) and line profile analysis confirmed a significant overlap along the borders of chromosome 4/8 in MG63 but not in the corresponding normal osteoblast cells (Fig S1).

Since SKY and other analysis has demonstrated a high level of chromosomal alterations in MG63 osteosarcoma cells, it is possible that the observed pairing is due to a high level of translocations between chromosomes 4 and 8 in this cell line. We have, therefore, performed chromosome painting for both chromosomes 4 and 8 in metaphase spreads. Aside from the presence of chromosome 4 and 8 in the IFN chromosome (Fig 3E and F, yellow arrows), one chromosome translocation between 4 and 8 is observed in 75% ($n=42$) of the spreads (Fig 3E, white arrow) with 25% showing a second translocation involving only a very small region of

chromosome 4 (Fig 3F, white arrows). Typically there are several other copies of chromosome 8 and 4 that show no associations with each other (Fig 3E and F).

Thus the striking association of chromosomes 4 and 8 in the cell nucleus of the MG63 osteosarcoma cells is only partially explained by the presence of translocations between chromosomes 4 and 8. Preferential pair-wise and higher array associations of separate chromosomes 4 and 8 territories are also present with potential intermingling to form chromosome 4/8 mega-territories.

Amplification of the IFN gene Cluster

Amplification of genomic regions is a common feature of osteosarcomas (Lau et al., 2004; Man et al., 2004; Squire et al., 2003). To assess the homogeneity of the IFN cluster amplification, two adjacent BAC probes P8 and G20 covering over 2/3 of the cluster were hybridized simultaneously in both WI38 and MG63 cells. In WI38 nuclei the two probes displayed partial co-localization and the normal 2 alleles of each IFN region were detected (Fig 4A). In MG63 the signals for the P8 and G20 probes are found in an approximately equal number of spots that alternate within the amplified IFN gene cluster (Fig 4B). To further evaluate the degree of IFN gene cluster amplification, three IFN specific probes IFN β , F5 and probe 49D4, covering the beginning and extending into approximately two-thirds of the gene cluster, were hybridized in MG63. The different probes tallied over multiple experiments gave a similar range of 21-24 signals (n= 50) in cycling MG63 cells with an average of 22.5. This suggests equal amplification across the entire IFN gene cluster. Probe C12 which showed a similar number of spots and is located at the centromeric end of the cluster provides further support for this conclusion (data not shown). Moreover, aCGH analysis of the genomic region covering the IFN gene cluster demonstrated that the level of amplification is similar throughout the cluster and abruptly ends within ~100 kb of both ends of the gene cluster (Marella et al., 2008).

We previously determined an average of 15 spots of the IFN-gene cluster from metaphase spreads of MG63 which agrees well with the ~ 5-fold amplification of the IFN-gene cluster in this hypotriploid cell line (Marella et al., 2008). Our present finding of ~ 22 spots per nucleus is consistent with these results since a significant number of the cycling cells are in S phase (30-35%) which results in an increase in spot number due to DNA replication.

Replication Timing Analysis of IFN Gene Cluster

Abnormalities in cancer may lead to alterations in the highly coordinated activity of DNA replication. One way to assay possible defects is to study the replication timing of a given region of chromatin. To visually determine the replication timing of the IFN gene cluster in WI38 fibroblasts, the single/doublet foci counting method was used (Selig et al., 1992). As controls we confirmed the established late replication timing of PPFIA2 and observed consistent early replication for the highly transcribed PTEN and ATM (A. Fritz, S. Sinha and R. Berezney, manuscript in preparation). Unsynchronized cells were pulsed with BrdU for 15 minutes and prepared for FISH. Probes P8, G20 and 49D4 were hybridized separately in multiple FISH experiments and scored as single or doublet foci in actively replicating cells (BrdU positive, Fig 4C). Each IFN probe displayed doublet signals in over 60 percent of the cells with an average of ~ 65% for the three separate probes (Fig 4E). We have estimated an approximately 11 h S-phase for WI38 using the CdU/IdU double labeling method (Dimitrova and Berezney, 2002; Dimitrova and Gilbert, 1999; Panning and Gilbert, 2005). We, therefore, determine an average replication timing for the IFN gene cluster to be ~ 4 h into S phase or in the early to mid S phase of WI38 cells.

Next we examined replication timing of the amplified IFN gene cluster in MG63 cells (Fig 4D). Using the IFN gene probe 49D4, FISH was performed and the number of spots was counted following synchronization of the cells at the G₁/S border with aphidicolin (Fig 4F). The cells were then released from the G₁/S block and pulsed for 15 min with BrdU at 2 and 6 h. FISH labeling was performed using the 49D4 probe and the number of foci was counted in BrdU positive cells. Importantly, the number of FISH spots counted in the cells at the G₁/S border (sixteen) is close to the predicted number for MG63 cells that have yet to enter S-phase (Marella et al., 2008). The number of labeled FISH foci increased to 25 after a two hour chase and completely doubled (32 foci) after 6 h (Fig 4F). Thus the highly amplified IFN gene cluster in MG63 cells maintains a replication timing window (2-6 h) that is similar to the average replication timing estimated in normal WI38 fibroblast cells (4 h).

IFN β gene expression analysis

Gene amplification associated with cancer has been observed to affect expression levels with reports of both over and under expression of transcripts relative to gene amplifications/deletions (Hyman et al., 2002; Sugimoto et al., 1987). Early studies demonstrated an increased production of IFN β protein by MG63 cells in comparison to fibroblast cell lines following induction (Billiau et al., 1977). We, therefore, compared the spatial expression pattern and expression levels of the amplified IFN β genes in MG63 with its normal diploid state. Type I IFN production is stimulated by the innate immune system in response to viral pathogens (Sen, 2001; Stetson and Medzhitov, 2006). We used this feature to induce IFN β expression with the synthetic dsRNA polyriboinosinic:polyribocytidylic acid (poly I:C). To assess the functionality of the amplified IFN cluster in MG63 cells and its effect on IFN β expression, we performed real time RT-PCR and RNA/DNA FISH analysis. Both MG63 and WI38 cell lines exhibited markedly similar temporal responses to poly (I:C) treatment, with maximum levels of IFN β transcripts at 2h post induction (Fig 5A). The amount of transcript then rapidly decreased to background levels by 8 h. The maximum RNA levels at 2 h post-induction in MG63 cells, however, were ~ 3 fold higher than in WI38 (Fig 5A).

The nuclear localization of IFN β transcripts in MG63 and WI38 cells was then examined by RNA FISH 2 h post induction. In WI38 cells, 1-2 sites of IFN β RNA foci were detected in the nucleus (Fig 5C) compared to 1-5 more sites in MG63 (Fig 5E). This is significantly less than the total number of IFN β gene foci estimated at 16 in G₁ arrested cells. To determine whether the highly amplified IFN β genes encompassing the *IFN chromosome* were functional, an approach was developed using RNA/DNA FISH. RNA FISH signal detection was enhanced using tyramide signal amplification, RNA was then digested with RNase A and DNA FISH was performed with the P8 BAC probe. As before, IFN β RNA was observed in the nucleus as 1-5 foci with most cells containing 2 foci. The vast majority of the IFN β RNA foci were detected in close proximity or partially co-localizing with IFN gene clusters outside of the amplified clusters of the IFN chromosome (Fig 5 I-K). In many cases the RNA foci were in proximity to two or more different IFN gene cluster foci (Fig 5 L-M). Although the amplified IFN β genes found on the IFN chromosome were predominantly silent in gene expression, ~5% of nuclei analyzed did contain expression emanating from sites within the cluster (Fig 5 G, H).

Discussion

Chromosome aberrations are a hallmark of cancer cells (Bode et al., 2000; Frohling and Dohner, 2008). Recent advances in chromosomal analysis such as SKY (Macville et al., 1997; Schrock et al., 1997) and aCGH (Pinkel et al., 1998; Vissers et al., 2003) are enabling investigators to define the details of these complex chromosomal abnormalities and DNA copy number changes in a variety of human cancers. Similarly, aberrations in the structural organization of the interphase cell nucleus and its chromatin have long been studied as a

characteristic feature of cancer cells (Bernard, 1963; Pienta et al., 1989; Stein et al., 2000). Of fundamental significance for future research in this area is defining how alterations in the genome at the chromosomal level are translated into the abnormalities visible in the interphase nucleus of the cancer cell.

With this in mind we are investigating the organizational and functional properties of the type I interferon (IFN) gene cluster in the interphase cell nuclei of the human osteosarcoma cell line MG63. We previously reported a ladder-like amplification of the IFN gene cluster in MG63 cells along a highly rearranged chromosome termed the *IFN chromosome* (Marella et al., 2008). 5-6 bands of IFN-genes were observed spanning this chromosome which alternated with corresponding bands containing centromeric 4 and 9 sequences and other chromosome 4 components [Fig 1, and (Marella et al., 2008)]. The IFN chromosome also stained faintly for chromosome 8 and 9 specific paints [Fig 1 and (Marella et al., 2008)].

Using a combination of chromosomes paints and IFN gene probes in multi-labeling experiments, we have visualized the organization of this highly disarranged chromosome in the interphase cell nucleus of MG63. We find that the IFN chromosome consistently resides along the periphery of the osteosarcoma nucleus and is composed of diffusely staining chromosome 4, 8 and 9 components. Rather than being arranged in a ladder-like linear array, the amplified IFN-gene clusters are organized into three-dimensional clusters of FISH foci that often form arrays along the periphery of the aberrant IFN chromosome. Similarly, we found that the IFN gene cluster is always located along the periphery of chromosome 9 in diploid WI38 cells. The chromatin surrounding the amplified IFN gene cluster was also rich in amplified centromeric sequences. These centromeric sequences specific for chromosome 4 and chromosome 9 were interspersed between the IFN gene clusters and partially co-localized with one another.

A strikingly high level of association of chromosome 4 with chromosome 8 territories was also detected in the MG63 cells. ~75% of the multiple chromosome 4 copies were directly associated with chromosome 8 copies. These associations occurred as simple pair-wise arrays or higher combinations involving two or more copies of chromosomes 4 and/or 8 that formed a continuous mega-territory. Moreover, there were significant levels of apparent intermingling of these chromosomes in these larger mega-territories (Fig 3B). In contrast, close association of both copies of chromosome 4 with chromosome 8 territories in WI38 fibroblast or osteoblast cells was a rare event (~12%), and the territories maintained discrete borders at their sites of association (Fig 3D, Fig S1- C and D).

The co-localization of portions of chromosome 4, 8 and 9 that compose the IFN chromosome within the same chromosome territory, and the close associations and apparent intermingling of chromosomes 4 and 8, have major implications for our understanding of the role of spatial positioning in gene regulation. Recent findings demonstrate that actively transcribed genes from distally located regions along the same or different chromosomes are assembled at common sites for transcription termed *transcription factories* (Osborne et al., 2004; Osborne et al., 2007; Xu and Cook, 2008). Thus global changes in the positioning of the chromosomes can have a profound impact on transcriptional programming in the cell whereby portions of different chromosomes find themselves within the same chromosome territories and can establish different spatial relationships among their genes for transcriptional activation and regulation at shared transcription factories.

In this investigation, we have studied the well-known transcriptional activation of the IFN β gene in fibroblast and osteosarcoma cells following treatment with poly (I:C) (Billiau et al., 1977; Whittemore and Maniatis, 1990b). Levels of IFN β mRNA in response to poly (I:C) was approximately three-fold higher in MG63 compared to WI38 cells (Fig 5A). Although the

levels of mRNA differed, the response in WI38 and MG63 cells conforms to the well documented rapid expression and transient nature of IFN β transcripts following induction. In both cell lines a maximum level of IFN β transcript was measured at 2 h post-induction followed by a rapid decrease to minimal levels by 8 h. (Fig. 5A). Negative regulation of IFN β has been shown to occur at the promoter region and postranscriptionally through IFN β mRNA degradation (Raj and Pitha, 1993; Whittemore and Maniatis, 1990b). Early studies examining the regulation of IFN β observed a decrease in transcripts in MG63 after approximately 8 h post induction with Sendai virus (Whittemore and Maniatis, 1990a; Whittemore and Maniatis, 1990b).

Since the IFN cluster is highly amplified in the MG63 cell line, we investigated whether the amplified IFN β genes were functional and if this amplification contributed to the increased levels of transcripts in comparison to WI38 fibroblasts. RNA FISH analysis 2 h post-induction demonstrated a range of 1-5 deleted very bright foci per MG63 cell nucleus with most nuclei containing 2 foci. Combined RNA-DNA FISH revealed that only a small percentage of nuclei (~5%) displayed sites of expression from within the highly amplified IFN clusters which contained the great majority of the IFN genes. Instead, most of the sites of expression were localized to secondary sites of amplification. The highly amplified IFN gene cluster within the repeating ladder-like array is, therefore, functioning at a very low rate following induction with poly (I:C), compared to other chromosomal locations for the IFN gene cluster in MG63 cells. We further observed that the RNA foci for IFN β gene expression are often proximal to two or more distal IFN gene sites (Fig 5 L-M). This raises the possibility that multiple IFN gene sites may access a shared transcription factory and that the actual number of genes active in transcription following activation with poly (I:C) may be much higher than the observed 1-5 RNA FISH foci.

Previously, global replication patterns of replication site organization were observed to be identical in normal, transformed and tumor cell lines (Dimitrova and Berezney, 2002). Here we show that this may hold true for individual genes as well. The amplified IFN gene cluster had a similar replication timing compared to the normal diploid IFN gene cluster in WI38 fibroblasts. In both cell lines the IFN gene cluster was observed to replicate in early to mid S-phase (Fig 4E, F). Since the amplified IFN gene cluster is arranged as bands that transverse from one end of the chromosome to the other [Fig 1, (Marella et al., 2008)], it will be of interest to determine how this replication timing is transmitted across the entire chromosome territory. For example, this may suggest the presence of an inherent signal within or adjacent to the IFN gene cluster. Cues such as GC content and local gene density have previously been shown to correlate with replication timing (Tenzen et al., 1997; Woodfine et al., 2004).

Transcriptional competence of the IFN region may also contribute to the conserved replication timing. Although there are exceptions, genome and chromosome wide studies indicate that the majority of actively expressed genes replicate in early S-phase (Chakalova et al., 2005; Schubeler et al., 2002; White et al., 2004; Woodfine et al., 2004). Genes poised for rapid activation such as the interferons might, therefore, be maintained in a microenvironment conducive for early-S phase replication. Indeed, several studies have demonstrated that genes poised or engaged in transcription are typically located near their territorial peripheries (Chambeyron and Bickmore, 2004; Christova et al., 2007; Volpi et al., 2000) as is the case for the IFN genes in WI38 cells and in their amplified counterpart in MG63 cells. This is also the case for the primary diploid fibroblast line FS4 (Winkelmann, 2007). While these arguments may apply to the transcriptionally active IFN gene clusters it is somewhat unclear how they pertain to the inactive IFN gene clusters. It has been shown, however, that genes residing in GC rich regions often do not switch replication timing regardless of changes in transcriptional activity during differentiation (Hiratani et al., 2004). More work is needed to define the DNA sequences immediately adjacent to the translocated IFN genes in MG63 to determine if this

can explain the conservation of replication timing over the rarely expressed IFN gene clusters which are amplified in a ladder-like array.

Finally, it is interesting to speculate that the heterochromatin centromeric sequences that alternate with the IFN gene clusters convey a repressive state of gene silencing to a portion of the amplified IFN genes. Recent evidence suggests that replication timing may be controlled independently of transcriptional activity through the use of histone modifications (Aladjem, 2007; Goren et al., 2008). The high concentration of heterochromatic centromeric sequences proximal to the amplified IFN gene cluster in MG63 cells presents an opportunity to assess the influence of repressive chromatin modifications from amplified centromeric DNA on both transcriptional activation and replication timing. In addition, the spatial positioning of the IFN chromosome along the nuclear periphery where it could potentially interact with the perinuclear heterochromatin (Feuerbach et al., 2002; M. Bouteille, 1974; Misteli, 2004) could provide further insight into the mechanism of gene repression of the amplified IFN gene cluster.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

IFN, interferon; FISH, fluorescent in situ hybridization; SKY, spectral karyotyping; aCGH, array Comparative genomic hybridization; BAC, bacterial artificial chromosome; CCD, charge coupled device; poly (I:C) polyribonucleic acid.

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Figure 1. A model of the amplified IFN gene cluster, centromeres 4, 9 and chromosomes 4, 8 and 9 on the IFN chromosome of MG63 cells

The IFN gene cluster (blue) is present in 5-6 ladder-like bands encompassing the entire length of the IFN chromosome. The chromosome also contains repeating bands of amplified centromere 4, centromere 9 and chromosome 4 DNA (yellow) adjacent to the IFN gene cluster. Chromosome 8 and chromosome 9 (grey) are distributed faintly across the length of the chromosome. Centromere 9 (red) was identified as the functioning centromere.

Figure 2. Interphase organization of the interferon gene cluster and associated chromosomal regions in MG63 and WI38 cells

A-C: Representative images of the IFN gene cluster in WI38 labeled with cosmid probe 49D4 (red) and (A), Chr4 (green); (B), Chr8 (green); (C), Chr9 (green). D-H: Representative images of the amplified IFN gene cluster in MG63 labeled with probe 49D4 (red) and (D), Chr4 (green); (E), Chr8 (green); (F), Chr9 (green). G-I: IFN gene cluster association with amplified centromeric DNA in MG63 (G), BAC probe P8 (green) and centromere 9 DNA (red); (H), P8 (red) and centromere 9 DNA (green); (I), centromere 4 DNA (green) and centromere 9 DNA (red). Arrows indicate the regions containing the repeating IFN gene and centromeric foci. Scale bars indicate 10 μ m.

Figure 3. Organization of chromosomes 4, 8 and IFN gene cluster

(A), Triple labeling of Chr4 (green), Chr8 (blue) and BAC probe P8 (red) in MG63 denoted by yellow arrow. White arrows indicate additional associations between Chr4 and Chr8. (B), Enlargement of a Chr4 and Chr8 association denoted by the white box from (A). (C), 3-D surface rendering of MG63 nucleus showing association of Chr4 (red) with Chr8 (green). (D), WI38 showing one association between Chr4 (green) and Chr8 (red) indicated by a white arrow, DAPI DNA stain (blue). (E, F): Metaphase spreads of MG63 chromosomes with Chr8 (red) and Chr9 (green). (E), Translocation of Chr4 and Chr8 denoted by a white Arrow. Chromosomes are stained with DAPI (blue). (F), Two translocations of Chr8 and Chr4 indicated by white arrows. Chromosomes are stained with DAPI (blue). In both cases yellow arrows indicate the

chromosome containing the ladder-like amplified IFN gene cluster. White scale bars indicate 10 μm ; yellow scale bars indicate 2 μm .

Figure 4. Replication timing analysis of IFN gene cluster

A,B: Double labeling across the IFN gene cluster with adjacent BAC probes P8 (red) and G20 (green). DAPI DNA stain (blue) in (A), WI38 and (B), MG63. Arrow points to the array of foci for the amplified IFN gene cluster which is enlarged in the inset. C, D: Representative images of BrdU replication labeled DNA (green) and BAC probe P8 (red) in (C), WI38 and (D), MG63. (E) The percentage of replicated (doublet FISH signals) scored in BrdU positive WI38. (F) Average number of foci for IFN gene cluster probe 49D4 in BrdU positive MG63 cells following release from G1/S. White scale bars indicate 10 μ m.

Figure 5. Quantitative and spatial IFN β expression

Quantitative RT-PCR: (A), Relative levels of IFN β RNA post-induction with poly(I:C) in WI38 and MG63 cells. B-E: IFN β RNA FISH (green), DAPI DNA stain (blue) in (B), non-induced WI38 cells; (C), WI38 cells 2 h post poly(I:C) induction; (D), Non-induced MG63 cells; (E), MG63 cells 2 h post poly(I:C) induction. (F), IFN β RNA FISH (green) in RNase A treated MG63 nucleus. G-K: Combined RNA/DNA FISH with IFN β RNA (green) BAC probe P8 (red). (G, H), IFN β gene expression from sites within the amplified IFN gene cluster. (I-K), IFN β gene expression from sites outside of the amplified IFN cluster. White and yellow arrows point to regions enlarged in corresponding white and yellow boxes above each image. L-M: High resolution images of IFN β RNA FISH foci (green) and BAC probe P8 (red) show examples where more than one IFN gene foci is proximal to a common IFN β gene expression site. Scale bars indicate 10 μ m.

Table 1Association values of chromosome 4 and chromosome 8^a

chr4 and chr8 association (%)	WI38 fibroblasts	HOB osteoblasts	MG63 osteosarcoma
1 copy of 4 proximal to chr8	28.7	29.1	Na
All chr4 copies proximal to chr8	11.7	10.9	74.0

^aPercent associations were determined in 94 WI38, 55 HOB and 26 MG63 cells^bChr4 and chr8 were scored as being associated if the minimal distance between their borders was less than 300 nm.