Architecture and Organization of Chicken Microchromosome 16: Order of the NOR, MHC-Y, and MHC-B Subregions

MARY E. DELANY, CHARMAINE M. ROBINSON, RONALD M. GOTO, AND MARCIA M. MILLER

From the Department of Animal Science, University of California, One Shields Avenue, Davis, CA 95616 (Delany and Robinson); and the City of Hope Medical Center, Department of Molecular Biology, 1500 East Duarte Road, Duarte, CA 91010 (Goto and Miller).

Address correspondence to Mary E. Delany at the address above, or e-mail: medelany@ucdavis.edu.

Abstract

Here we present a high-resolution cytogenomic analysis of chicken microchromosome 16. We established the location of the major histocompatibility complex (MHC)-B and -Y subregions relative to each other and to the nucleolus organizer region (NOR) encoding the 18S–5.8S–28S ribosomal DNA. To do so, we employed multicolor fluorescence in situ hybridization using large-insert bacterial artificial chromosome clones with fully sequenced inserts or repetitive sequence probes specific for the subregion of interest. We show that the MHC-Y and -B regions are located on the same side of the NOR, rather than opposite ends, as previously proposed. On the q arm, the MHC-Y is closely adjacent to the NOR, whereas the MHC-B is distal near the q-terminus. A relatively large GC-rich region separates the 2 MHC subregions and includes a specialized structure, a secondary constriction. We propose that the GC-rich large physical distance is the basis for the lack of genetic linkage between the NOR and MHC-B and between the MHC-Y and -B. An integrated model for GGA 16 is presented that incorporates gene complex order in the context of key architectural features including p and q arms, primary (centromere) and secondary constrictions, telomeres, as well as AT- and GC-rich regions.

Key words: chicken, major histocompatibility complex, microchromosome 16, nucleolus organizer region

Bloom and Bacon (1985) described an elegant case of trisomy mapping in a higher vertebrate. In that work, the nucleolus organizer region (NOR), site of the tandem array of 18S–5.8S–28S ribosomal (r) RNA genes (aka rDNA) and the major histocompatibility complex (MHC) were proved to be linked on microchromosome 16 of the chicken (GGA 16). Chickens exhibiting 2, 3, or 4 nucleoli (disomic, trisomic, or tetrasomic for GGA 16) segregated from crosses between trisomic individuals and also exhibited 2, 3, or 4 MHC haplotypes, respectively. The chicken MHC (aka B complex) was well known at the time to encode the set of key proteins regulating aspects of the immune response system (for review, see Kaufman 2008). The BF (class I) and BL (class II) genes encode cell membrane proteins that control cellular communication ultimately influencing resistance and susceptibility to disease. The MHC also includes the BG (class IV) region encoding erythrocyte antigens, a system widely used for decades to determine MHC haplotypes by serological testing. It was also well known at the time of the Bloom and Bacon paper that the NOR directs the formation of the nucleolus, the intranuclear site for transcription and synthesis of the 18S, 5.8S, and 28S preribosomal subunits incorporating 5S rRNA and the r-proteins (for review, see Hadjiolov 1985). rRNA synthesis and ribosome biogenesis are directly related to cellular capacity to synthesize proteins. Thus, NOR size (gene copy number) and expression have a major impact on cell growth rate and differentiation at all stages of growth and development (Larson et al. 1991; Delany et al. 1994, 1995; Jacob 1995). Therefore, the Bloom and Bacon (1985) paper, in addition to showing MHC and NOR linkage, was pivotal in dispelling the argument that chicken microchromosomes were akin to supernumerary chromosomes in plants, which are not stably transmitted and are largely devoid of impact on the organism. The recent analysis of the chicken genome sequence provided further additional evidence that in fact the microchromosomes are gene dense relative to...
the macrochromosomes (ICGSC 2004). Unfortunately, due to the repetitive nature of the NOR and the relatedness of the MHC genes/gene families, the sequence assemblies (2004 and 2006) lack information in regard to the MHC and NOR sequences. The molecular details of the numerous genes encompassed by the chicken MHC are now being established via sequencing of large-insert bacterial artificial chromosomes (BACs) (Shiina et al. 2007; Hosomichi et al. 2008) originally developed to aid creation of a physical map (Lee et al. 2003) to support the sequencing project. No effort has been expended as yet to sequence an 18S–5.8S–28S rRNA gene repeat unit although the coding region sequences are expected to be highly conserved with variant spacer sequences, relative to other vertebrates (Gonzalez and Sylvester 1995).

The chicken rDNA and the MHC complexes have been extremely well studied at the genetic, molecular, cellular, and organismal levels which has contributed to our understanding of the role these loci have in development, growth, and immunity (Schmid et al. 2000, 2005). A key finding from analysis of MHC haplotypes in pedigree families was that there are 2 related MHC regions that segregate independently from one another (Briels et al. 1993; Miller et al. 1994; Afanassieff et al. 2001), the MHC-B and Rfp-Y (later termed MHC-Y). At the same time, studies showed that the NOR undergoes recombination at high frequency resulting in a highly conserved with variant spacer sequences, relative to the macrochromosomes (ICGSC 2004). Unfortunately, to date untested. The hypothesis was that the NOR resides between the MHC-B and MHC-Y complexes and that the high rate of recombination of the NOR serves as the underlying genetic mechanism for the lack of genetic linkage between the OR and the MHC-B.B. Further, we describe the unexpected finding of a large block of GC-rich DNA separating the MHC-Y and -B. We propose that it is the large GC-rich physical distance between MHC-Y and -B and between the NOR and MHC-B that serves as the underlying mechanism for their lack of genetic linkage, respectively.

Materials and Methods
Chromosomes were prepared from Red Jungle fowl (RJF, Gallus gallus gallus) or Single Comb White Leghorn chicken (SCWL, Gallus gallus domesticus) inbred lines UCD-001 (RJF) and UCD-003 (SCWL) or the UCD-trisomic (SCWL) line (Bloom and Bacon 1985; Apblanp 1992; Delany and Pisenti 1998; Pisenti et al. 1999). Mitotic chromosomes were harvested from embryos at 4.5 days of embryogenesis (UCD-001 and -trisomic) or from single-embryo–derived chicken embryo fibroblast cultures (UCD-003). Meiosis I pachytene stage chromosomes were harvested from adult male gonad (UCD-001). Chromosome harvest and FISH procedures were standard (Rodionov et al. 2002; Romanov et al. 2005; Delany et al. 2007). The MHC probes were from BamHI (TAM31) and HindIII (TAM33) large-insert BAC libraries (Lee et al. 2003; Genefinder Genomic Resources, Texas A&M University) prepared from UCD-001 RJF DNA (female #256, the sequenced genome, ICSGC 2004) and included TAM31-JF256-B1-44G24 (MHC-B) and TAM31-JF256-B1-66A9 or TAM33-JF256-H3-34J16 (MHC-Y). The NOR probe was a 3-kb fragment from the 5′ region of the 18S–5.8S–28S rRNA gene repeat, the external transcribed sequence (ETS) (Delany and Krupkin 1999). Probes were labeled by nick translation incorporating nucleotides conjugated with Spectrum Orange (MHC-B), Spectrum Red (MHC-Y) (Abbott Molecular Inc.), or digoxigenin (NOR). The latter was detected using an antidigoxigenin–fluorescein–conjugated antibody (Roche Applied Science).

Results and Discussion

Cytogenomic Analysis

Here we describe a high-resolution cytogenomic analysis of chicken microchromosome 16. Multicolor FISH was employed using BAC clones with fully sequenced inserts or
repetitive sequence probes specific for the regions of interest on mitotic (metaphase and prometaphase) or meiotic (prophase I pachytene) chromosomes (Figure 1) and interphase cells (Figure 2). Chromosomes were stained by DAPI that under nonsaturating conditions produces a banding pattern reflecting GC- and AT-rich regions because DAPI preferentially binds in the minor groove of AT-rich DNA (Kapuscinski 1995). The meiotic cell FISH experiments provided consistently high-quality results. The source tissue, male gonad, has intrinsic advantages in that it provides an unlimited supply of pachytene cells with extended-state (aka decondensed) bivalents. Each bivalent consists of 2 homologous chromosomes bound by the synaptonemal complex. The use of bivalents as chromosomal targets for FISH allowed for greater resolution between closely positioned genetic regions in conjunction with structural features. The results establish the location of the NOR position (Figure 1D,F, long arrows). Secondary constrictions, similar to primary constrictions (centromeres), signify differences in higher order chromatin structure as compared with bulk chromatin. Both primary and secondary constrictions typically exhibit features characteristic of constitutive heterochromatin, for example, remain in a condensed state throughout the cell cycle with unusual interphase staining properties, consist of high copy number tandem repeat sequences, and exhibit biochemical properties of satellite DNA (Luke et al. 1992). Another described feature of secondary constrictions includes interindividual length polymorphisms (Sigmund and Schwarz 1979). The association of NORs with secondary constrictions was first described by McClintock (1934) in her studies of maize, and this association has been extended to numerous eukaryotes (Matsui et al. 1986 and references therein). In human, such secondary constrictions have been implicated in the etiology of chromosomal aberrations (Ferguson-Smith et al. 1962). Although adjacent, the chicken NOR itself does not appear to be located within the secondary constriction (Figure 1C,E).

**MHC-B, -Y Subregions, and NOR Gene Complex Order**

The NOR as identified by the 5’ ETS-rDNA probe (green signal, Figure 1) was readily confirmed on the q arm in every cell type and stage used here for chromosome analysis, as first reported by Bloom and Bacon (1985). The MHC-Y (red signal, Figure 1) and MHC-B (yellow signal, Figure 1) were also consistently found on the q arm, in both condensed (mitotic metaphase) and extended (mitotic prometaphase, meiotic pachytene) chromosomes. Thus, not only are both MHC subregions located on the q arm but also both are on the q-terminal side of the NOR, as opposed to the centromere proximal side or on opposite ends of the NOR. The close physical adjacency of the NOR and MHC-Y is congruent with sequence analysis reported 20 years ago. Guilleminot et al. (1988) described a cosmide clone (III) indicating that 2 rRNA gene repeats were within 15 kb of a MHC class II beta gene; later, these MHC class II genes were shown by Miller et al. (1996) to be part of MHC-Y. This same juxtaposition is present in the MHC-Y haplotype in UCD-001 RfJ (Goto RM, Miller MM, unpublished data). A recent analysis of DF-1, an immortalized chicken embryo fibroblast line, supports the results presented here. The DF-1 cell line was found to be homozygous for a fusion involving GGA 16 wherein the gene order appears as reported here along with the GC-rich gap region being visible on the derived chromosome (O’Hare TH, Delany ME, in preparation; O’Hare and Delany 2009). Further, the spatial distance between the 2 MHC subregions is also apparent in interphase cells. During interphase, the chromosomes are in their maximally extended configuration (excepting heterochromatin which remains condensed). Therefore, interphase nuclei provide a complimentary view of the relative positions of MHC-B, -Y, and the NOR and also allows for a perspective...
Figure 1. Architectural features and gene complex order of chicken microchromosome 16. Probes (NOR green, MHC-Y red, and MHC-B yellow) were hybridized to mitotic metaphase (A), prometaphase (B), or meiosis I pachytene (C–F) chromosomes stained by DAPI (blue). The white or black arrowheads indicate the p arm, the short black arrows indicate the centromere, and the longer black arrows indicate the q-arm secondary constriction. All scale bars are 5 μm. (1A) Mitotic metaphase chromosomes. This stage coupled with use of Colcemid produces maximally condensed chromosomes and thus a very small GGA 16. Although the NOR signal was typically distinct from the MHC-Y and -B probe signals, these were not resolved from each other although they appeared to be on the same side of the NOR and q-terminal. This indicates the q-arm order: NOR–MHC (-Y and -B overlapping). (1B) Mitotic prometaphase chromosomes. At this stage, the chromosomes are less condensed allowing visualization of the DAPI-bright (AT rich) p arm. All probes localized to the q arm, with clear delineation of their order relative to each other. The NOR
relative to domain orientation with respect to the nucleolus, the site of rDNA transcription, and ribosome biogenesis. In interphase nuclei, the \textit{MHC-Y} probe signal (red) was typically found internal to the nucleolus; Figure 2 shows a representative cell. The highly dispersed green NOR signal is due to the decondensation of the rRNA genes for transcription. Also indicated is a merged yellow signal resulting from the overlap/colocalization of the individual green (NOR) and red (\textit{MHC-Y}) probes. The interphase views provided additional evidence that even during the most decondensed chromatin stage, there is close physical proximity of the \textit{MHC-Y} and rDNA regions on GGA 16. The \textit{MHC-B} probe signal was consistently external to the nucleoli and some distance away from the other signals.

\textbf{Integrated Model for GGA 16}

Based on prior and current molecular and cytogenetic data, a model is proposed for GGA 16 gene complex organization and chromosome architecture, see Figure 3 (Delany and Krupkin 1999; Shiina et al. 2007). The \textit{p} arm is AT rich, the centromere is GC rich, and the \textit{q} arm possesses both AT- and GC-rich blocks. The NOR is AT rich and encompasses 5–7 Mb consisting of ~150 copies of the 18S–5.8S–29S intergenic spacer rRNA gene repeat, previously predicted to encompass 0.4 Mb having 55% GC content based on BAC sequence data (Miller MM, Goto RM, Shiina T, unpublished data). The GC-rich region separating \textit{MHC-Y} from \textit{MHC-B} appears to be about half the size of the entire chromosome combined as per the extended view of pachytenic bivalents. Presently, the full size of the \textit{MHC-B} region is also difficult to estimate. A 242 kb region of \textit{MHC-B} was recently sequenced (Shiina et al. 2007) and indicates an overall GC content of 55.4% with one 69-kb subregion as high as 61.9%. This region does not include the bulk of the BG gene family thought to contain 10 more genes and the class II \textit{\alpha} locus which is separated by an unknown physical distance from the 242-kb sequenced region. Thus, we estimate the \textit{MHC-B} region, located on the other side of the GC region and proximal to the \textit{q}-terminal telomere, to be about 0.5 Mb. In terms of the telomeres, a recent analysis (O’Hare and Delany 2009; O’Hare TH and Delany ME, in preparation) indicates that GGA 16 in UCD-001 RJF has normal-sized telomeres at both ends in contrast to UCD-003 SCWL wherein the \textit{p} arm exhibits a mega-telomere although with some degree of interindividual variation (Delany et al. 2007). Standard telomeres are ~20 to 40 kb and mega-telomeres range from ~100 kb to 3–4 mb (e.g., the Wq telomere, Delany et al. 2007; O’Hare and Delany 2009). An overall size estimate for GGA 16 based on cytogenomic information would be between 10 and 15 Mb incorporating telomeres (p telomere maximum of 0.5 Mb if possessing a mega-telomere and \textit{q} telomere maximum of 0.04 Mb), a \textit{p} arm similar in dimension to other microchromosomes of similar size (~2–3 Mb), a centromere (0.5–1.0 Mb as a best estimate compared with other vertebrates), and \textit{q}-arm \textit{loci} (NOR of 5–7 Mb, \textit{MHC-Y} of 0.4 Mb, and \textit{MHC-B} of 0.5 Mb). The completely unknown constituent is the size of the GC-rich region, which is not apparent in condensed mitotic chromosomes but appears to be half the overall chromosome length in meiotic pachytenic chromosomes.

Previously, it was hypothesized that the lack of genetic linkage between the \textit{MHC-Y} and \textit{B} subregions was due to their location on opposite sides of the NOR coupled with enhanced recombination involving the tandem rRNA gene repeats of the NOR (Miller et al. 1996). Here we provide cytogenomic evidence that negates the hypothesis of the order \textit{B-NOR-Y} and supports the \textit{q}-arm order: NOR/\textit{MHC-Y}—GC-rich region—\textit{MHC-B}. We propose that it is the large physical distance and the GC-rich nature of this region separating \textit{MHC-B} from \textit{Y} and \textit{MHC-B} from the NOR that causes these \textit{loci} to segregate independently. High GC content and higher recombination rates are features of chicken microchromosomes in general (ICGSC 2004). Our results support points made previously that high GC content in combination with the rRNA gene tandem repeats and MHC sequence homologies contribute to the difficulty in developing an accurate and robust sequence assembly for GGA 16 (ICGSC 2004).
MHCs and NORs: Comparative Organization among Vertebrates

Although there exists evidence for overall synteny conservation and general karyotype stability among Galliforme genomes, it is becoming clear that there exist numerous intrachromosomal microrearrangements (e.g., Shibusawa et al. 2004; Kayang et al. 2006; Griffin et al. 2008). In regard to the gene complexes studied here, it appears that MHC and NOR organization and the macro and microscales can be quite variable. Although the 18S–5.8S–28S coding sequences are highly conserved, spacer regions between repeats and overall gene numbers can be variable even within a species (Su and Delany 1998; Delany and Krupkin 1999; Delany 2000 and references therein). In terms of MHC and NOR molecular and cytogenetic organization, in Japanese quail (*Coturnix japonica*, ~35 million years (My) divergence from chicken), the MHC appears to be expanded relative to chicken (Shiina et al. 2004; Hosomichi et al. 2006). Interestingly, there are 5 NOR loci (Sasaki and Nishida 1981), although it is not known whether any of the NOR loci are linked to the MHC in quail. Whereas, in another well-studied Galliforme, the turkey (*Meleagris gallopavo*, ~40 My divergence from chicken), the single NOR locus is linked on the same chromosome as the MHC, and as in chicken, the MHC-Y complex is adjacent to the NOR although the MHC-Y appears to be centromere proximal. The turkey MHC-B subregion is the more distal locus from the NOR and MHC-Y (as in chicken), but it appears that the MHC-B is on the p arm (thus across the centromere) and has an interstitially located mega-telomere indicated nearby (Chaves et al. 2007).

Kaufman (2008) argues that the arrangement of the classical MHC region (-B) with a “nearby” nonclassical MHC (-Y) may be the ancestral vertebrate condition. In *Xenopus*, another nonmammalian vertebrate model useful for studying ancestral MHC organization, there is 1 NOR locus (chromosome 12, Funaki et al. 1975; Schmid et al. 1987) as well as a single MHC (Ohta et al. 2006) although their linkage relationships are unknown. In the human genome, there are 5 NOR loci (HSA 13, 14, 15, 21, and 22), none of which are linked to the MHC (HSA 6). Just as among avian species, comparative genome analysis indicates that
variability also exists for MHC organization among mammals, for example, the bovine MHC exhibits a large physical distance separating class II regions that has been attributed to a single inversion event (Childers et al. 2006).

The GGA 16 organization results presented here provide new insight regarding the cytogenomic features of this interesting and well-studied chromosome that encodes loci important for growth, development, and immunity. We hope that the updated model for GGA 16 will promote novel research to explore the sequences within the large physical distance separating the elements of the chicken MHC, as well as contribute to research on comparative genome organization within and among avian and other vertebrate genomes.

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