Generic Features of Tertiary Chromatin Structure as Detected in Natural Chromosomes

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Received 12 May 2004/Returned for modification 21 June 2004/Accepted 13 August 2004

Knowledge of tertiary chromatin structure in mammalian interphase chromosomes is largely derived from artificial tandem arrays. In these model systems, light microscope images reveal fibers or beaded fibers after high-density targeting of transactivators to insertional domains spanning several megabases. These images of fibers have lent support to chromonema fiber models of tertiary structure. To assess the relevance of these studies to natural mammalian chromatin, we identified two different ~400-kb regions on human chromosomes 6 and 22 and then examined light microscope images of interphase tertiary chromatin structure when the regions were transcriptionally active and inactive. When transcriptionally active, these natural chromosomal regions elongated, yielding images characterized by a series of adjacent puncta or “beads”, referred to hereafter as beaded images. These elongated structures required transcription for their maintenance. Thus, despite marked differences in the density and the mode of transactivation, the natural and artificial systems showed similarities, suggesting that beaded images are generic features of transcriptionally active tertiary chromatin. We show here, however, that these images do not necessarily favor chromonema fiber models but can also be explained by a radial-loop model or even a simple nucleosome affinity, random-chain model. Thus, light microscope images of tertiary structure cannot distinguish among competing models, although they do impose key constraints: chromatin must be clustered to yield beaded images and then packaged within each cluster to enable decondensation into adjacent clusters.

Chromatin structure can be classified hierarchically (44). Primary structure refers to the organization of DNA on nucleosomes, for example, the 10-nm fiber. Secondary structures arise from interactions between nucleosomes, for example, the 30-nm fiber. Tertiary structures are formed by interactions between secondary structures, and quaternary structures are formed by interactions between tertiary structures. Thus, tertiary and quaternary structures can involve the organized compaction of the chromatin fiber over domains of hundreds of kilobases (16). Here we use the term “tertiary structure” to refer generally to all higher-order chromatin structures beyond the level of the 30-nm fiber.

A knowledge of this tertiary structure and the molecules involved in regulating it will be critical for a complete understanding of the molecular processes involving DNA, including replication, repair, and transcription (26). Only the primary structure of chromatin packaging and, to some extent, the secondary structure are now known with certainty (5). However, these levels of packaging account for only a fraction of the compaction required to fit the cellular content of DNA into a nucleus (15). Thus, there is much to be learned about higher levels of chromatin packaging. A critical first step is to define

the higher-order structures in a natural mammalian interphase chromosome. To date, most knowledge has come instead from several model systems that may or may not be representative of natural interphase chromatin.

Extracted metaphase chromosomes reveal a series of DNA loops attached to a protein scaffold (28). These observations have led to a radial-loop, protein scaffold model for metaphase chromosomes that has been extrapolated to interphase chromatin (14, 24, 27). Some meiotic chromosomes, namely, oocyte lampbrush chromosomes, also exhibit DNA loop structures (33). During interphase, the only direct structural evidence for DNA loops comes from the interpretation of electron microscope images of the Balbiani ring genes in polytene chromosome puffs of the fly *Chironomus* (1, 20). At present, it is not certain whether these model systems, namely, mitotic, meiotic, and polytene chromosomes, are representative of normal interphase chromatin and, if so, whether the structures observed after extraction and fixation are genuine.

To address these concerns, tandem array systems have been constructed and then visualized during interphase in living mammalian cells. The tandem arrays are based on green fluorescent protein (GFP)-tagged proteins that bind to a repeated series of target sites that can be transactivated (17, 23, 25, 30, 39, 40, 47). The resultant light microscope images of tertiary chromatin have been interpreted as fibers that elongate in response to transactivation. These interpretations support a chromonema fiber model in which progressive folding of nucleosomal DNA generates a series of thicker fibers: first, secondary structures, corresponding to fibers 30 nm thick; sec-

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ond, tertiary structures, corresponding to fibers 60 to 80 nm thick; and third, quaternary structures, corresponding to fibers 100 to 130 nm thick (4). Rather than a scaffold to provide structural support, it is thought that chromonema fibers are held together by fiber-fiber interactions, which could be regulated by different histone variants or tail modifications (16).

Tandem array systems, however, are also subject to questions about their general applicability to natural chromatin structure, since they are artificial. The arrays are composed of very densely packed head-to-tail repeats that recruit strong activating factors to very high densities along at least several megabases of chromatin. In contrast, natural chromosomes are often characterized by large intergenic regions and promoters that are typically much weaker than targeting the potent VP16 activation domain (32) used in *lac* operator arrays (40) or the binding of the glucocorticoid receptor to its cognate promoter, the mouse mammary tumor virus (MMTV) promoter, used in MMTV tandem arrays (23). Therefore, it is not known to what extent these systems are relevant to natural chromatin.

The different approaches to studying tertiary chromatin structure have yielded apparently conflicting models based on loops and scaffolds or on chromonema fibers. At present, it is not clear whether the results from these different systems can be reconciled at all or whether the differences arise because the systems studied are not representative or natural. Our goals in this study therefore were (i) to investigate tertiary chromatin structure in natural human chromosomes during interphase and (ii) to consider which models are consistent with structures observed in natural chromosomes.

The results obtained in the present study show that natural chromosomal domains of ~400-kb yield images in which a single puncta or "bead" decondenses into a series of adjacent beads dependent upon transcription. These features seen in images of tertiary chromatin are likely to be quite common, since they arise in both natural and artificial systems, despite significant functional differences among these various systems. We also show here how these images of tertiary structures can be explained by adapting a loop-scaffold model, a chromonema model, or even a random-chain model. Therefore, the conclusion that tandem array systems favor chromonema models (16, 23, 40), although potentially still correct, is premature.

MATERIALS AND METHODS

Cell culture. The MMTV array cell line (3617) was grown as previously described (23). MRC-5, Raji, and Jurkat cells were obtained from the American Type Culture Collection (Manassas, Va.) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine or RPMI medium supplemented with 10% FBS. For microscopy experiments, cells were grown on coverslips. These were coated with poly-L-lysine for Raji and Jurkat cells. For interferon induction of MRC-5 cells, cells were incubated with 200 U of gamma interferon/ml in fresh DMEM for 2 or 5 h.

DNA probes. Bacterial artificial chromosome (BAC) probes RP11-36N5, CTA-433F6, and CTA-526G4 for chromosomal region 22q11.21-22a and RP11-10A19 and P1-derived artificial chromosome (PAC) RP1-93N13 for the major histocompatibility complex (MHC) class II (MHCII) locus were obtained from Research Genetics, Huntsville, Ala. End sequences for each probe were obtained by primer extension sequencing with SP6 and T7 primers and then were compared to the entire chromosome 22q sequence or to the MHC sequence by using BLAST.

Plasmid probes 45.1DR α 120 and 45.1DR β 008 (38) for the MHC locus were kind gifts from Eric Long (National Institute of Allergy and Infectious Diseases).

DNA FISH. Cells were fixed for 20 to 30 min with 3.5% paraformaldehyde in phosphate-buffered saline (PBS). DNA fluorescence in situ hybridization (FISH)

for 3617 cells was performed essentially as described previously (23) but with the following modifications. (i) Dehydration through ethanol was done immediately after denaturation, omitting a 5-min incubation in 50% formamide-2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) on ice. (ii) Hybridization buffer contained 5% rather than 10% dextran sulfate. (iii) The DNA probe was prepared from the entire plasmid pM18, not a restriction fragment. (iv) The biotinylated probe was detected by immunofluorescence with a primary anti-biotin antibody and a fluorescent secondary antibody. The DNA FISH procedure for MRC-5 cells was the same, except that digoxigenin was sometimes used to label the probe before detection with appropriate primary and secondary antibodies. The DNA FISH procedure for Raji and Jurkat cells differed from that for 3617 cells as follows. (i) Denaturation of cellular DNA was performed at either 90 or 95°C. (ii) Hybridization buffer contained 50% formamide instead of 25%, 1.4 mg of salmon sperm DNA/ml and 0.6 mg of human Cot-1 DNA/ml instead of tRNA, and 25 μ g of DNA probe/ml instead of 5 to 10 μ g/ml. (iii) The DNA probe was denatured at 85°C for 10 min and then incubated for 15 to 30 min at 37°C.

For colocalization of known MHC plasmid probes with the BAC and PAC probes for the MHC, the following procedure was used. Cells were fixed with 3.7% formaldehyde in PEM buffer [90 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), 4.5 mM EGTA, 1.8 mM MgCl₂, 0.18% Triton X-100 (pH 6.8)]. The DNA FISH protocol was modified slightly from that used for Raji and Jurkat cells. Cellular DNA was denatured at 88°C in 70% formamide-2 $\times$ SSC. Nick translation was used to tag the BAC and PAC probes with biotin and the plasmid probes with digoxigenin. For hybridization, the two probes were mixed at concentrations of 20 μ g/ml for the BAC and PAC probes and 15 μ g/ml for the plasmid probes. The tags were detected with fluorescein-conjugated antidigoxigenin antibody or rhodamine-conjugated avidin.

Bioinformatics. Levels of transcription from the q arm of human chromosome 22 (hereafter referred to as 22q) were extracted from published microarray data in which mRNA levels were compared between 69 different pairs of cell lines at all known and predicted exons on 22q (36). These data were originally used to confirm gene annotation on 22q by defining full-length transcripts based on coregulated exons. We reordered the exon expression data to match the known positioning of exons on 22q and then searched for domains along 22q with high levels of transcription. Crucial for our analysis was the availability of a finished sequence for 22q, enabling the unambiguous determination of gene order, and the complete coverage of 22q exons from microarray data, enabling an accurate measure of transcriptional output.

To identify active domains, we divided 22q into segments ~1 to 2 Mb wide. Segment boundaries were defined by a series of BAC clones that had been mapped by DNA FISH to cytogenetic locations on 22q (18). These BAC clones provided established markers for measuring by DNA FISH the degree of decondensation across a domain. Within each domain, relative transcriptional levels were estimated for each of the 69 pairs of cell lines. Exons which showed at least a twofold difference between cell lines were considered significant. The relative intensity difference for these exons was multiplied by the absolute microarray spot intensity to provide at each exon a measure of the magnitude of the difference in transcription between two cell lines. For each gene, the scores from all exons were averaged and then multiplied by the annotated length of the gene, including introns. This process yielded a measure of transcription proportional to the length of the transcribed region, a factor likely to be important for tertiary chromatin structure (23). Finally, the total score for a domain was the sum of scores for all genes contained in the domain.

Deconvolution microscopy. For high-resolution images, deconvolution microscopy was performed essentially as described previously (23), except that the focal plane spacing was 0.07 μ m and the maximum-likelihood algorithm was run for 100 to 200 iterations for DNA FISH and 500 iterations for images of live or fixed cells containing the MMTV array.

Measurements of decondensation. Cells were imaged primarily on a Leica DMRA upright microscope with a Leica $\times$ 100/1.3 N.A. oil immersion objective. Images were obtained with a Sensys charge-coupled device camera (Roper, Trenton, N.J.). Length measurements of DNA FISH signals were performed with Metamorph software (Universal Imaging, Downingtown, Pa.). For nearly contiguous BAC or PAC pairs (the green and blue probes in Fig. 1b and 2c), two signals were typically observed in different focal planes of the same nucleus, consistent with the diploid karyotypes of the cells. For measurements, the larger of the two signals was always selected. This image was zoomed with smoothing to avoid pixilation. A curve was drawn to delineate the longest axis of the FISH signal, and then its length was recorded. For the "end-point" BACs on chromosome 22 (blue and red BACs in Fig. 2c), two pairs of signals were typically observed in both Raji and Jurkat cells. In most cells, it was easy to identify isolated signal pairs. In such unambiguous cells, the straight-line distance between one randomly selected pair of signals was measured.

FIG. 1. Induction of MHCII by interferon and characterization of DNA probes for this region. (a) Interferon induction leads to expression of cell surface HLA antigens, as determined by immunofluorescence with antibodies against HLA-DP, HLA-DQ, and HLA-DR antigens. As determined with a *t* test, significant increases in intensity were observed after 2 and 5 h of interferon induction ($P < 0.01$). Standard errors are shown. (b) Two probes, PAC RP1-93N13 and BAC RP11-10A19, were used in DNA FISH to detect the MHCII region. These probes were end sequenced and found to align with the MHCII locus. Genes within the MHCII region are shown below the black line representing the locus; orange letters indicate genes induced by interferon. (c) Confirmation of probe specificity in DNA FISH by colocalization with known MHCII-specific probes.

Transcriptional inhibition. For MRC-5 cells, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) was used at 100 μ g/ml for 0.5 h. For Raji and Jurkat cells, DRB was used for 1 h at 100 to 200 μ g/ml. For 3617 cells, DRB was used at 100 μ g/ml for 1.5 h. Actinomycin D was used at 10 μ g/ml for 2 h for MRC-5 cells and 1.5 h for Jurkat cells.

Immunofluorescence. For anti-HLA staining of MRC-5 cells, cells were fixed for 20 min with 3.5% paraformaldehyde in PBS and then washed three times in PBS for a total of 15 min. Cells were incubated overnight at 4°C with an anti-HLA antibody (DakoCytomation, Carpinteria, Calif.) diluted 1:20 in PBS containing 4% bovine serum albumin and 0.1% Tween 20. After being washed in

FIG. 2. Transcriptionally active domain on chromosome 22 and characterization of probes for this region. (a) Based on an analysis of microarray expression data (see Materials and Methods), a transcriptional score was calculated on the q arm of human chromosome 22 (chr 22q) for the expression of Jurkat cells relative to Raji cells. A peak was found in the domain from 4.3 to 6.1 Mb (encircled). (b) A finer-scale analysis of transcriptional levels within the 4.3- to 6.1-Mb domain revealed that transcriptional activity there peaked at between 5.3 and 5.8 Mb. (c) To probe tertiary chromatin structure in this domain, three probes were used. Their alignment with the 4.3- to 6.1-Mb domain was determined by end sequencing. Each probe was previously mapped by FISH (18), confirming its specificity. The red and blue BACs mark the end points of the 4.3- to 6.1-Mb domain and were used to measure the separation between these two locations. They span a 1,680-kb domain. The green and blue BACs were used to examine tertiary structure in the most transcriptionally active subdomain of the 4.3- to 6.1-Mb domain. These probes span a 440-kb domain.

PBS, cells were incubated with a Texas red-conjugated anti-mouse secondary antibody for 45 min at room temperature. Cells were washed once more in PBS and examined. Intensity measurements were made by computing the average intensity within a 3- μ m-diameter spot positioned at the brightest location in the cell. Background intensity was determined from cells not exposed to interferon, and this intensity measurement was subtracted from the measurements at 2 and 5 h.

Nucleosome affinity, random-chain (NARC) model and simulated micros-

copy. To simulate a 400-kb chromatin domain, we used a biased, self-avoiding three-dimensional (3D) random walk in which nucleosomes exclusively occupy a certain position in space, whereas segments of linker DNA can occupy the same position. The chain was calculated at a resolution of 11.5 nm.

Each nucleosome in the chain accounted for 146 bp of DNA and occupied a single voxel element (11.5 nm³). Nucleosomes were connected by linker DNA. Different average linker lengths were evaluated, namely, 50, 68, 85, 155, and 200

bp. This range was chosen in part based on measured linker lengths reported for a variety of organisms (~ 50 to 130 bp) (41) but also more generally to test the sensitivity of the simulation to this parameter. For each average linker length, 260 random chains were generated. For each chain, linker lengths varied around the average (L_{avg}) over the following ranges: 34 to 66 bp for an L_{avg} of 50 bp, 34 to 102 bp for an L_{avg} of 68 bp, 34 to 136 bp for an L_{avg} of 85 bp, 34 to 272 bp for an L_{avg} of 155 bp, and 34 to 378 bp for an L_{avg} of 200 bp. Linkers were presumed to be decondensed, double-helical DNA at 0.34 nm/bp. Given the resolution of the simulation (11.5 nm³), linkers therefore occupied from 1 to 12 voxels, depending on their lengths. For linkers longer than one voxel, a random path was generated. Nucleosomes and linkers were added iteratively to the chain, with new nucleosome voxels also being selected at random locations with respect to the preceding voxel. Although overlapping linker DNA was permitted, neither linker DNA nor nucleosomes were allowed to overlap preexisting nucleosomes. When such an overlap occurred, a new random location was selected. This process was iterated until a 2,000-nucleosome chain corresponding to 400 kb was constructed.

To generate various degrees of decondensation of chains with a given average linker length, we allowed for different average distances between nucleosomes in the chain by setting a threshold distance between new elements added to a given chain and their nearest nucleosome. When the randomly selected location for either a new nucleosome or a linker segment exceeded the threshold distance from the nearest nucleosome, a new random location was selected until the distance to the nearest nucleosome was smaller than the threshold distance. By varying this threshold distance from one chain to the next over a range from 16 to 46 nm, we generated an assortment of random chains with different degrees of decondensation. This range of threshold distances was comparable to the range of measured nucleosome spacings in H1-depleted nucleosome chains (~ 15 to 60 nm) (46).

In some instances, this random-chain algorithm became "trapped," with no new position available for the next element given the constraints of the algorithm. In practice, trapping occurred for high degrees of condensation, corresponding almost exclusively to single-bead structures in the simulations. To avoid trapping of the chain, we implemented a simple escape mechanism. If no free position was available, then additional linker DNA was added until a free position was found (typically a single additional voxel, corresponding to an additional 34 bp, was sufficient).

The average computation time to produce a 2,000-nucleosome chain was 10 min with an Intel XEON 2.6-GHz processor. Using a 50 CPU Linux cluster, we generated a total of 1,300 different chains at different degrees of decondensation to yield a representative assortment.

To simulate light microscope images from these chains, we assigned a uniform intensity of 255 to each nucleosome (corresponding to 146 bp). Linkers were assigned an intensity of 44 per voxel. When linker DNA overlapped with other linker DNA, the intensity of that voxel was increased accordingly. As another test of the robustness of the simulation, a separate set of 260 chains in which linkers were assigned a lower intensity of 22 per voxel was used, such that the intensities of nucleosomes relative to those of linkers were altered. In all instances, simulated images were generated by convolving the nucleosome chain data with a theoretically generated point spread function for a $\times 100/1.35$ N.A. Olympus objective at a spatial resolution of 11.5 nm³. XCOSM software was used to generate the point spread function, and the program Image3dm was used to perform the 3D convolution (a kind gift from Jose Conchello, Oklahoma Medical Research Foundation, Oklahoma City). The convolved data at a resolution of 11.5 nm³ then were downsampled to a resolution of 70 nm³ by using the program Reduction (a kind gift from Jose Conchello) in which the intensities from 6 by 6 adjacent pixels were averaged and the average was assigned to a single pixel. Finally, these blurred and downsampled images were deconvolved by using the maximum-likelihood algorithm in the Huygen's Essential software package. The point spread function parameters were identical to those used during deconvolution of the real images from the natural systems. The algorithm was run for 10 to 50 iterations with a background estimate set to zero.

RESULTS

Identification of natural systems with extended domains of transcriptional activation. We identified in natural chromosomes two different regions each containing extended domains (~ 400 kb) of transcriptional activation.

The first natural system was the classical MHCII locus on human chromosome 6, for which interferon induces 13 genes

spanning ~ 700 kb. Earlier studies showed that after interferon induction, the MHCII region is more often external to the chromosome territory (42). However, the tertiary chromatin structure of MHCII and its dependence on transcription have not been investigated. We verified that under our conditions, interferon induced the expression of class II antigens (Fig. 1a), and then we identified a BAC and a PAC probe that together span 375 kb of the MHCII region (Fig. 1b). The specificity of these probes for the MHCII region was confirmed by DNA FISH colocalization with established probes for two loci in this region (Fig. 1c).

In a complementary approach, we used bioinformatics to identify another transcriptionally active domain in human cells. Several previous bioinformatic analyses demonstrated that co-regulated genes tend to be clustered along the chromosome (8, 10, 31, 37), but in none of these studies has the corresponding tertiary chromatin structure been examined. To identify such a domain in cells amenable to light microscopy, we analyzed microarray expression data for a number of cultured cell lines (36). The microarray data represented relative expression levels in cell lines at all known and predicted exons on the q arm of human chromosome 22. We reordered the expression data to match the known positioning of exons on 22q and then searched for domains along 22q with high levels of transcription (see Materials and Methods). We identified a subdomain of 440 kb in which expression in one cell line (Jurkat) was markedly higher than that in another cell line (Raji) (Fig. 2b). Then we identified two BAC probes that span much of this domain (Fig. 2c).

Images of tertiary structure in natural chromosomes are beaded. We used the probes for the chromosome 6 and 22 domains to examine tertiary structure in these natural chromosomes by DNA FISH. In the transcriptionally active state, namely, in interferon-treated cells (chromosome 6) or in Jurkat cells (chromosome 22), the relevant probes for these domains typically yielded images characterized by a series of adjacent puncta or "beads" (hereafter referred to as "beaded images") (Fig. 3a and c). As detected by light microscopy, the number of adjacent beads in these images normally ranged from two to four, but occasionally as few as one bead or as many as six beads were observed. Bead diameters varied but typically ranged from 0.4 to 0.8 μm . In most images, beads were closely spaced, with dim intervening strands sometimes visible between them. Occasionally, larger spaces between beads were observed, and in such instances connecting regions were typically not visible. In contrast, before induction with interferon or in the transcriptionally less active Raji cell line, the same probes revealed single beads in most images (Fig. 3b and d).

As a control to test whether DNA FISH preserved tertiary structure at light microscopic resolution, we compared images of the MMTV tandem array after DNA FISH with those for live cells (Fig. 3e and f). Both types of images were similar, suggesting that DNA FISH preserves the basic features of tertiary structure, as detected by the light microscopy methods used.

Transcriptionally active states yield longer structures than inactive states. In tandem array systems, images of transcriptionally active states yield longer structures (23, 39, 40). To test the analog of this scenario in natural systems, we measured the

FIG. 3. Beaded images of tertiary structure arise in transcriptionally active regions in natural chromosomes. Projections of 3D deconvolved images of tertiary chromatin structure are shown. (a and c) Images of tertiary structure typically exhibit two to four beads in either the MHCII region on chromosome 6 after induction by interferon (a) or in a transcriptionally active domain on chromosome 22q in Jurkat cells (c). An assortment of decondensed states is shown, reflecting the natural variability in the cell population. Beads are always present; dimmer connecting strands sometimes are detected between them. In most images, the beads are nearly in contact, but wider separations sometimes occur. (b and d) In contrast, when these same natural domains are less transcriptionally active, images exhibit predominantly single beads. Single beads are detected when the MHCII region is examined before induction by interferon (b) or when chromosomal region 22q-11.21-22a is examined in Raji cells (d), which transcribe significantly less from this region than do Jurkat cells. (e and f) As a control for the preservation of structure by DNA FISH, we examined cells harboring the MMTV array after transcription was induced by hormone addition. (e) For live cells, images of the array containing up to ~15 beads are observed with a GFP-tagged glucocorticoid receptor. (f) Similar images are seen for cells examined after DNA FISH with a probe for MMTV DNA. (The higher background in panel e is due to binding of the GFP-tagged glucocorticoid receptor throughout the nucleoplasm, while in panel f a DNA probe specific for the tandem array was used.)

FIG. 4. Transcriptionally active states yield longer structures. Upper panels show examples of nondeconvolved images used for measurements of tertiary chromatin structures for the three different domains examined. Both the 440-kb domain on chromosome 22q and the 375-kb domain for MHCII were spanned by two nearly contiguous BAC or PAC probes. In contrast, the 1,680-kb domain on chromosome 22q was marked by a BAC at each end point, giving rise to images in which two separate structures could be discerned (b). Lower panels (bar charts) show the mean lengths of the structures measured in the different cell types in the presence (white bars) or absence (gray bars) of the transcriptional inhibitor DRB. The means are from 50 to 300 measurements, and each experiment was repeated at least three times with similar results; standard errors are shown. The brackets indicate that significant differences between means were found with a *t* test (*P* values are shown).

total lengths of the structures revealed by DNA FISH. In a comparison of Jurkat and Raji cells, we found that the same 440-kb domain yielded structures that were longer in Jurkat cells than in Raji cells (Fig. 4a).

To test whether this behavior extended to larger domains, we used our bioinformatics analysis to identify a 1,680-kb domain encompassing the 440-kb subdomain. This larger domain was also more transcriptionally active in Jurkat cells than in Raji cells (Fig. 2a). To estimate the extension of this larger domain in the two cell lines, we measured the separation between two BACs that marked the end points of the domain (red and blue BACs in Fig. 2c). This distance was consistently greater in Jurkat cells than in Raji cells (Fig. 4b). Therefore, these data also show that the tertiary chromatin structure of a transcriptionally active domain is longer.

We then investigated whether this was also true for the MHCII region on chromosome 6 in MRC-5 fibroblasts. In this analysis, we used the two probes specific for this region and found that the MHCII region became longer in cells after transcription was induced by interferon (Fig. 4c). We conclude that transcriptional activation also induces changes in the tertiary structure of the MHCII region, resulting in its elongation.

Natural systems require transcription to maintain the elongated state. Since transcriptionally active regions were consistently longer, we examined whether they required transcription for their maintenance. In such analyses, contradictory results have been obtained with tandem array systems: inhibition of transcription had little effect on VP16 tandem arrays (40) but induced the recondensation of MMTV arrays (23). In classical systems, transcription is required for the decondensation of lampbrush chromosomes (22) and for normal puffing of genes in polytene chromosomes (2, 6).

To test the role of transcription in the chromosome 6 and 22

domains, cells were treated with the transcriptional inhibitor DRB (9), and then the lengths of the tertiary structures were measured. Results similar to those described below were also obtained with another transcriptional inhibitor, actinomycin D (data not shown). After transcriptional inhibition of Jurkat cells, the lengths of tertiary structures decreased significantly (Fig. 4a). Similarly, for the larger, 1,680-kb domain in Jurkat cells, transcriptional inhibition caused a decrease in the distance between the BACs marking the end points of this domain (Fig. 4b). Thus, transcription in Jurkat cells is necessary to maintain the extended chromatin conformation. In contrast, transcriptional inhibition of Raji cells yielded no significant reduction in length for either the 440-kb domain or the 1,680-kb domain (Fig. 4a and b), consistent with microarray data indicating that Raji cells showed relatively little transcriptional activity in these domains compared to Jurkat cells. To test the effects of transcriptional inhibition on the transcriptionally active MHCII region in MRC-5 cells, cells were pre-treated with interferon and, 1.5 h later, transcription was inhibited for 0.5 h. As in Jurkat cells, this treatment resulted in a contraction of the MHCII region compared to the results obtained with the controls (Fig. 4c).

A NARC model yields beaded images. One goal of the present study was to determine which models are consistent with the principal features of tertiary chromatin in natural systems, namely, images that yield beads which elongate into a series of adjacent beads upon transcriptional activation. In the Discussion, we consider the applicability of chromonema and loop-scaffold models, but here we investigated a simpler possibility, namely, random folding of a chromatin fiber. Random walk models are widely used to describe the folding of polymers (13) and have been specifically applied in several studies to the modeling of secondary and tertiary chromatin structures

(3, 19, 29, 43). A characteristic feature of a random walk is its tendency to extensively explore a given locale and then jump to a new locale, where the exploration process is repeated (see Fig. 1.4 in reference 7). This local exploration process gives rise to local clusters that have the potential to explain the beaded images that we detected.

To explicitly test the applicability of a random walk model, we adapted and extended earlier models (21, 45) that introduced irregularity to account for the secondary structures of 30-nm fibers. These models incorporated randomness by varying the angle between successive nucleosomes. The resultant random chain then was blurred (21) based on the expected resolution of the atomic force microscope, and the predicted images were found to be quite similar to those obtained by atomic force microscopy of actual nucleosomal fibers.

We adopted a very similar approach here. We assume a randomly oriented linker DNA whose average length is 50 bp (linkers used in the earlier studies ranged from 44 to 64 bp). For simplicity, in our model, each nucleosome is 11.5 nm^3 and accounts for 146 bp of DNA, yielding ~ 200 bp per repeat element in the chain. Sufficient elements are then added to yield a 400-kb chain. Simulated light microscope images of this chain are produced by blurring the chain by using the optical characteristics of a high-numerical-aperture objective lens.

To account for the fact that transcriptional activation should lead to elongation of the chain, we made a simple, general assumption, namely, that the average spacing between nucleosomes in a random chain can increase upon transcriptional activation. This assumption is meant to incorporate in a simple way the many complex and unknown factors that could, in principle, regulate the decompaction of a nucleosome chain. In the model, this average spacing rule is enforced by setting a threshold limit for the maximal spacing between nucleosomes in a particular chain. If the randomly chosen location for a new nucleosome or linker is at a distance greater than the threshold, then that position is disallowed, and a new random location is sought and accepted only when its distance to the next closest nucleosome is less than the threshold. Our range of threshold values (16 to 46 nm) mirrors those measured by atomic force microscopy for H1-depleted nucleosome chains (~ 15 to 60 nm) (46). We refer to this feature of the model as nucleosome affinity, hence, the NARC model.

Random chains produced by this NARC model indeed produced the clustering characteristic of random walks (Fig. 5a and d). Simulated light microscope images of these clusters yielded a series of adjacent fluorescent spots (Fig. 5b, c, e, and f). By reducing the magnification of these simulated images to that used for real images, we found considerable similarity: both real and simulated images revealed beads with occasional dimmer connecting segments (compare Fig. 5g and Fig. 3). Very similar images were obtained when we changed some of the underlying assumptions in the model, such as the mean length of linker DNA (lengths of 68, 85, 155, and 200 bp were tested) or the relative fluorescence intensities assigned to nucleosomes versus linkers (data not shown). These results suggest that the model is robust and that its success is largely due to the tendency of random walks to cluster.

As a further test of the applicability of the NARC model, we performed a simple analysis to examine whether the structures it predicted could easily extend into longer structures. In both

the natural and the MMTV tandem array systems, transcriptional activation yielded multiple beads from what were originally single beads in the transcriptionally inactive state (Fig. 3). For such a transition to occur, the underlying nucleosome chain would need to have been reasonably ordered to reduce entanglements that would arise upon separation into a series of adjacent local clusters of the chain.

To investigate this requirement, we used colors to mark positions along the nucleosome chain. We colored the first third of the chain blue, the second third green, and the last third red. Using this coloring scheme, we examined a number of random chains and found that colored domains remained largely distinct, with little overlap between them either for longer chains (256 kb) or for shorter segments (64 kb) within them (Fig. 5h). These results imply that on a macroscopic scale, the chain remains largely ordered and therefore could, in principle, split into separate domains without major entanglements.

DISCUSSION

Light microscopy images of tertiary chromatin structure are beaded. We identified transcriptionally active domains in natural human chromosomes and then examined their tertiary structure by light microscopy. The characteristic features of these images were a series of puncta which we have called beads. When transcriptionally inactive, a 400-kb region typically appeared as a single bead. When transcriptionally active, an image of the same chromosomal region typically exhibited two to six adjacent beads. Sometimes in these images we detected larger intervening regions between beads, and occasionally a strand connecting the beads could be discerned.

Beaded features can be recognized in images of several tandem array systems proposed previously as models for tertiary chromatin. Both the 2-Mb MMTV tandem array (23) and the 2-Mb *tet*-regulated *lac* operator arrays (see Fig. 6 in reference 39) exhibit such features. Since these are all live-cell systems, the beaded features cannot be artifacts of fixation or hybridization. To some extent, beads are also discernible in images of the first tandem array examined in live cells, the 90-Mb *lac* operator array activated by VP16 targeting. However, the latter array exhibited a much more pronounced ribbon-like fiber structure (see Fig. 2 in reference 40). The impact of this first study led to the prevailing view that chromonema fiber models best explain the images of tertiary chromatin seen in tandem arrays (16, 23, 40); therefore, despite the presence of beaded features in the other tandem array systems, they have not been emphasized.

Our examination of natural systems here shifts the emphasis from fibers to beads, which are in fact the common feature in images from all of these systems and the predominant feature in natural systems. This distinction is important. Describing these images of tertiary chromatin as beaded rather than as fibers not only is a more accurate description but also is a characterization that by its name favors no particular class of model. As we discuss below, beaded images can indeed be explained by a chromonema fiber model but also by a loop-scaffold model or by a self-avoiding random walk model; therefore, higher-resolution studies will be necessary to distinguish among these possibilities.

FIG. 5. A NARC model can explain beaded images. (a and d) Examples of 400-kb simulated nucleosome chains. (b and e) High-magnification views of the simulated images reveal the presence of fluorescence puncta; most details of the nucleosome chain are lost. (c and f) Overlays show that less densely packed regions of the chain may extend beyond the puncta and may be invisible. (g) Sampling of simulated projected images following deconvolution for 400-kb chains with different degrees of decondensation. Note that these images exhibit beads resembling those in the actual images (compare to Fig. 3). (h) When the first third of the chain is colored blue, the middle third is colored green, and the last third is colored red, distinct largely nonoverlapping regions are obtained either for long chains of 256 kb (left panel) or for shorter chains of 64 kb (middle and right panels). These data indicate that, on a macroscopic scale, neighbor-neighbor relations are preserved, and so the chain could unravel without significant entanglement.

FIG. 6. Chromonema models and radial-loop, protein scaffold models can be adapted to account for beaded images. (a) Chromonema models propose a hierarchically folded chromatin fiber that yields a series of different fiber thicknesses (4, 35, 40). Here, a thinner fiber is helically wound to yield a thicker fiber that would appear as a bead (green haze) when viewed in the light microscope. Yellow struts represent fiber-fiber interactions, for example, between chromatin cross-linking proteins, that could stabilize the folded structure (16). The diameter of the bead detected by the light microscope would depend on the thickness of the underlying fiber and the size of the hollow core. If the underlying fiber were 30 nm in diameter, then the hollow helical core would need to be ~ 500 nm in diameter to match the measured bead diameter of 0.4 to 0.8 μm . However, if the underlying fiber were thicker, for example, 100 nm (4), then the hollow core would need to be only twice as large as the fiber diameter, namely, ~ 200 nm. (b) Local disruption of fiber-fiber interactions would lead to unraveling of the fibers and would yield a light microscope image of two adjacent beads. (c) Radial-loop models (14, 27) propose that the chromatin fiber forms loops attached to a protein scaffold (yellow spheres). A cluster of loops would be imaged as a bead by light microscopy. If a typical loop were 80 kb, then the radius of the bead would correspond to 40 kb. Assuming that loops are formed from 30-nm fibers which are ~ 40 -fold compacted, the bead radius would be $40 \text{ kb} \times (1/40) \times (0.34 \text{ nm/bp})$, or 0.34 μm , in reasonable agreement with the measured bead diameter of 0.4 to 0.8 μm . (d) The structure shown in panel c could decondense into two beads by local detachment of a loop from the scaffold, but in order to elongate into two beads, the scaffold would have to either fragment (as shown here) or stretch.

Transcription induces longer structures that require continued transcription for their maintenance. In both of the natural systems examined here, tertiary structures were longer after transcriptional activation. This finding demonstrates that elongation is a characteristic feature of transcriptionally active tertiary structures, as light microscope images of all of the artificial tandem arrays examined showed longer structures upon activation (17, 23, 39, 40).

The ability to elongate imposes constraints on the underlying folding of chromatin within tertiary structures. First, the fiber must be reasonably organized so that it can be easily unraveled to produce more extended structures. Second, if the

fiber is attached to a structural scaffold of some form, then elongation requires that the scaffold either stretch or fragment. Explicit examples are discussed below and are shown in Fig. 6.

In order to maintain their elongated state, the two natural systems examined here required transcription. This is likely to be the norm, since it is also seen in the MMTV tandem array that uses a natural promoter (23) as well as with most genes examined in puffs or lampbrush chromosomes (2, 6). The one clear exception to this rule are *lac* tandem arrays, which do not require transcription for maintaining the decondensed state (40). These *lac* arrays do not use a natural promoter but rather target the potent VP16 activation domain to very high densities. This design may override the normal transcriptional control of the elongated state and therefore produce an unnatural result. Alternatively, it is possible that some natural systems not yet examined will also reveal no dependence of the elongated state on transcription.

Models of tertiary structure. To understand tertiary structure, one must ultimately determine the patterns of chromatin fiber folding. Visualization of folding patterns on the scale of a 30-nm fiber clearly requires a resolution higher than that afforded by conventional light microscopy. However, our light microscope images do provide an envelope into which higher-order folding patterns must fit.

The envelope described here is a bead. A beaded image will arise in the light microscope when chromatin is more densely packed in a set of relatively discrete clusters. Packing within a cluster need not be space filling, simply more dense than in surrounding regions, where fluorescence is found by light microscopy to be much dimmer or absent. The ability of clusters to split, yielding images with adjacent clusters, places an additional constraint on the patterns of chromatin folding within each cluster. We now consider below how these constraints can be realized with three explicit examples.

We found that a randomly folded fiber calculated according to the NARC model could explain our observations. Here, we were inspired by models previously developed to explain images of secondary chromatin structure, namely, atomic force microscopy images of 30-nm fibers. These images revealed irregular packing of nucleosomes into fibers whose diameters varied but were, on average, ~ 30 nm. These irregular secondary chromatin structures were successfully modeled by allowing for variable linker lengths, which in turn produced random variations in the angle between successive nucleosomes in the chain. Interestingly, we found that adaptation and extension of this concept of random angles in the form of our NARC model could also explain the tertiary structures that we observed by light microscopy in natural chromosomes. In addition, this NARC model could account for the ability of beads to easily separate into a series of adjacent beads. We found that within a bead, fiber folding was sufficiently organized so that local domains were preserved. This property would enable separation into two beads without major entanglements of the underlying fiber.

Although a simple NARC model can account for our observations, other models with more organized folding of the chromatin fiber can also be entertained. Indeed, the congruence between the predicted random-chain and real images does not ensure that the underlying chromatin structure is random. Diffusion, for example, can be modeled as a random walk, despite

the fact that individual particles undergo complex trajectories precisely defined by Newton's laws. Similarly, we cannot rule out a more complex organization of chromatin with a precise and varied sequence of bending angles. The average properties of such complex folding could, by analogy with the process of diffusion, yield macroscopic behavior at the resolution of conventional light microscopy that can be captured by a random-chain model.

While a macroscopic average over an assortment of complex, organized folding patterns could give rise to the images that we have observed, it is also possible to imagine more regular, repeated patterns of folding that could likewise explain our data. For example, chromonema fiber models can be adapted by assuming that beads correspond to locally thicker segments of the chromonema arising from higher-order folding of an underlying, thinner fiber (Fig. 6a). Although for the purposes of illustration we show here helical folding of the thinner fiber (35), any reasonably ordered fiber packing within the bead envelope would satisfy the requirement that major entanglements should be avoided upon separation into a series of adjacent beads. Chromonema models posit that tertiary structures are maintained by some form of interaction between adjacent fibers (16), shown in Fig. 6a and b as yellow cross-links. Breaking these cross-links at a specific location would lead to local unraveling of the fiber, yielding an image with two adjacent beads (Fig. 6b).

A radial loop-protein scaffold model could also be adapted to explain a beaded image, if a bead arises from a cluster of chromatin loops (Fig. 6c). Separation into a pair of adjacent beads would proceed by unraveling of one or several adjacent loops, generating two adjacent looped clusters (Fig. 6d). The radial looping of fibers in this model naturally preserves the sequence along the axis of the chromosome; therefore, this model easily accounts for the splitting of chromatin clusters without major entanglements. However, in this model, the observed elongation yielding a pair of adjacent beads requires that the protein scaffold either stretch or fragment at the site where the loops unravel. Therefore, in this model, elongation does not occur as simply as in a chromonema model, where unraveling requires only the loss of local fiber-fiber interactions.

In summary, a variety of models can be adapted to explain the characteristic features of tertiary structures seen by light microscopy. The chief requirement for any model is that chromatin be clustered into denser subdomains that yield the beaded images seen by light microscopy. Distinguishing among different models of bead substructure will require higher-resolution imaging of chromatin fiber folding to determine whether it is random, hierarchically folded, or looped. These questions may soon be addressed with new fluorescence microscopy techniques that provide improved resolution, such as 4Pi microscopy (34), spatially modulated illumination microscopy (12) and spectral precision distance microscopy (11).

Generic features of tertiary structures. Overall, our images of tertiary chromatin structures and the dependence of these structures on transcription largely support what has been found for artificial tandem arrays. This situation is remarkable, given the differences between tandem arrays and natural systems. The 440-kb chromosome 22 domain contains 6 genes and 60% coding sequence, while the 375-kb chromosome 6 domain

contains 10 genes and 20% coding sequence. In contrast, some tandem arrays contain close to 100% coding sequence and/or recruit an abnormally high density of activators over large domains of at least 2 Mb. In addition, the methods of activation vary drastically across these systems, ranging from natural transcription factors recruited to their target promoters to chimeric transactivating factors recruited to viral promoters or simply tethered to chromatin in the absence of any promoter. The fact that these very disparate systems form similar light microscopy images suggests generic features of the underlying tertiary structures of activated chromatin, irrespective of the specific underlying sequences and functionality.

ACKNOWLEDGMENTS

We are indebted to both the National Cancer Institute and GEN-AU project BIN, Austrian Ministry for Education, Science, and Culture, for financial support.

We are grateful to the Graz University of Technology for computational support for the modeling studies. We thank Gordon Hager for 3617 cells, Eric Long for MHCII plasmid clones, and Jose Conchello for software used in the simulated microscopy. We are grateful to David Clark, Mike Kruhlak, Tatiana Karpova, Richard Leapman, and Andre Nussenzweig for comments on the manuscript. Microscopy was performed at the National Cancer Institute Fluorescence Imaging Facility, and we gratefully acknowledge Tatiana Karpova for expert assistance.

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