The Barr body is a looped X chromosome formed by telomere association
(chromosome structure/X-inactivation/centromere/interphase cytogenetics/in situ hybridization)

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ABSTRACT We examined Barr bodies formed by isodicentric human X chromosomes in cultured human cells and in mouse–human hybrids using confocal microscopy and DNA probes for centromere and subtelomere regions. At interphase, the two ends of these chromosomes are only a micron apart, indicating that these inactive X chromosomes are in a nonlinear configuration. Additional studies of normal X chromosomes reveal the same telomere association for the inactive X but not for the active X chromosome. This nonlinear configuration is maintained during mitosis and in a murine environment.

Barr bodies are unique chromatin structures formed in nuclei of the mammalian female as a means of sex chromosome dosage compensation. First identified as a nucleolar satellite present only in female cells (1), the Barr body represents a single inactive X chromosome. In cultured human cells, it is most easily identified at the periphery of the interphase nucleus, when other chromosomes are not condensed. Because the Barr body is difficult to see among clumped heterochromatin in interphase mouse fibroblasts, and because the silent human X reactives more frequently in rodent than human cells, Dyer et al. (2, 3) suggested that mouse cells may not form proper Barr bodies. Analysis of cultured human cells by electron microscopy (4) or in situ hybridization (2) places the Barr body adjacent to the nuclear envelope in 75–80% of interphase cells. Comings (5) suggested that inactive X chromosomes attach randomly to the nuclear membrane, and the multiple Barr bodies in aneuploid cells are widely distributed (6, 7). Nuclear matrix attachment sites are similar for the active and inactive X chromosomes (8). Yet, the configuration of the Barr body has been relatively unexplored. DNA hybridization, in situ (9), has provided a powerful method to examine chromosomes during interphase, revealing an orderly arrangement of chromosomes in the interphase nucleus (10–13) and tissue-specific variation (14, 15). Using such methods to explore the human inactive X chromosome, we find that the Barr body consists of a condensed X chromosome in a nonlinear configuration, with telomeres in close proximity.

We examined the Barr body in interphase and mitotic cells using fluorescent probes for centromere and telomere regions of human X chromosomes. In addition to normal X chromosomes, we studied isodicentric X chromosomes (16), which form bipartite Barr bodies (16). Always inactive, they are mirror image duplications with two centromeres (one non-functional) and with two identical telomeres (see Fig. 1). The duplicate centromeres as well as common telomeres and their longer length facilitate structural analysis. To compare distance between hybridization signals with relative physical length we examined three isodicentrics, two joined by their long arms (3935 and 7213) and the third attached at the short arms (411). We isolated these dicentric chromosomes from their normal homologue in hybrid cells so that all signals would come from the dicentric X chromosome and to examine the human Barr body in a mouse cell environment. Finally, we simultaneously hybridized centromere and subtelomere probes using differential labels and confocal microscopy.

MATERIALS AND METHODS

Cell Lines. These are characterized in Table 1. The hybrids derived from A9 mouse fibroblasts were selected in hypoxanthine/aminopterin/thymidine medium, back selected in 6-thioguanine to eliminate the active X; to retain the inactive X, the silent HPRT locus was reactivated by 5-azacytidine. Inactive X hybrids derived from tsA159T mouse cells were selected directly at 39°C for activity of the A1SRT locus at Xp11 (17).

Preparation of Slides. Interphase cells. Confluent cells in LabTek slide chambers were fixed in methanol/acetone (3:1) and air dried.

Mitotic cells. Logarithmic-phase cells were treated with colchicine (1 hr), and mitotic cells were detached by shake-off, fixed in methanol/acetate acid, dropped onto slides, and air dried.

Probes. For simplicity, the human X-specific probes used to mark the centromeres and ends of the short and long arms of the chromosome are called Xcen, Xptel, and Xqtel, respectively. Fig. 1 shows the location of sequences homologous to these probes (labeled XC, 29C1, and F8).

Xcen. The XC probe for the centromere region is a 2-kilobase (kb) BamHI fragment (19) homologous to alphoid DNA; under stringent conditions XC hybridizes specifically with the X chromosome (19).

Xptel. The 29C1 probe used to mark the short arm (Xp) telomere is a 1.8-kb Pst I fragment hybridizing to a subtelomeric sequence located about 20 kb from the end of the short arms of X and Y chromosomes (20); each X chromosome has 3–10 tandem copies.

Xqtel. The F8c probe used to mark the long arm (Xq) telomere is a 1.4-kb EcoRI fragment containing exon 26 of the blood-clotting factor VIII locus (21), which hybridizes exclusively to the locus in Xq28, near the telomere.

Nick-Translation. XC and 29C1 inserts and the entire F8c plasmid were labeled with Biotin-11-dUTP using the BRL nick-translation kit. The probes were purified in a spin column containing 50 mM Tris, 10 mM EDTA, and 0.1% SDS (pH 7.4) and stored at 4°C for up to 1 month.

Abbreviations: DNP, dinitrophenyl; DAPI, 4',6-diamidino-2-phenylindole.

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In Situ Hybridization. Biotinylated probes. Slides were immersed for 2 min in 70% formamide/2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate), dehydrated in a series of 70–100% ethanol, and air dried. Hybridization was as described by Devilee et al. (22) (37°C overnight) using 80–90 ng of probe per slide in formamide (60% for XC, 50% for 29C1, and F8c). For hybridizations with XC, the slides were washed (30 min, 20°C) in 2× SSC, 0.1% SDS and then washed in 0.1× SSC/0.1% SDS (30 min, 42°C). The third wash was like the first. Hybridizations with 29C1 and F8c were as for XC, except all washes were in 2× SSC/0.1% SDS. Slides were washed in a fluorescein buffer of 3 M NaCl/20 mM Tris, pH 8. The biotin label was detected as described by Pinkel et al. (23), except that fluorescein buffer was substituted for BN buffer in all washes and solutions.

Dinitrophenyl (DNP)-labeled probes. XC was labeled with DNP by reaction with 2,4-dinitrophenylbenzaldehyde (DNBAL, Aldrich) as described by Shroyer et al. (24) and purified in a spin column with TE buffer. The hybridization mixture (50% formamide, 0.4 mg of salmon sperm DNA per ml, 2× SSC, and 60 ng of biotinylated 29C1 and 60 ng of DNP-labeled XC per slide) was heated to 70°C, iced, and placed under coverslips. For double labels, slides were incubated, rinsed, and labeled with fluorescein as for single probes. After the last wash in fluorescein buffer, 100 μl of 1:50 rabbit anti-DNP/5% bovine serum albumin and 0.04% goat serum was applied (overnight, 4°C). Slides were rinsed three times with phosphate-buffered saline, treated with 100 μl of anti-rabbit IgG conjugated to a Texas red label in PBS, and counterstained with propidium iodide in phenylenediamine antifade solution.

4′,6-Diamidino-2-phenylindole (DAPI) Staining. Cells fixed on slides, pretreated with RNase, were stained (10 min) with DAPI in 150 mM NaCl/10 mM Tris/1% Triton X-100/0.1% bovine serum albumin, and Mowiol solution was placed under the coverslip before sealing.

Analysis. Single label. Slides were examined microscopically for number and position of signals. The two signals from the two ends (or two centromeres) of isodicentric chromosomes were usually in the same focal plane (those that were not could not be measured). In contrast, if also present, the signal from the normal X chromosome was discrete. Cells after focal plane. The distance between signals was determined from photomicrographs (1200× magnification); distance was measured from the center of one signal to the center of the other. In some cases, measurements were made directly from superimposed confocal images, using the "length" program, and were similar to those obtained from photomicrographs.

Double label. Signals obtained by labeling Xp<sup>tel</sup> with biotin and X<sup>cen</sup> with DNP were analyzed using a Nikon Optiphot microscope mounted to a laser-scanning confocal imaging system (Bio-Rad MRC 500). We obtained z and xz series from computer-assisted images taken simultaneously from two channels. The images were subtracted, one from the other to eliminate cross signals, and then merged.

RESULTS

Signals in Control Cells. The mean percent of cells with at least one signal was 37 (range, 17–70) with X<sup>cen</sup>, 25 (range, 16–34) with Xp<sup>tel</sup>, and 13 with Xq<sup>tel</sup> probes. For all probes, and in all cell lines, the signals were discrete. Cells from normal males and females labeled with X<sup>cen</sup> showed the expected X dosage. The number of Xp<sup>tel</sup> signals was similar in both sexes reflecting the locus on two X chromosomes in females and on X and Y chromosomes in males; most often the two signals were in separate parts of the cell, consistent with separate domains for the two X chromosomes in females and the X and Y chromosomes in males (25, 26). Hybrids with only a normal human active X chromosome (DB1214-tsal09) had only a single signal with either X<sup>cen</sup> (Fig. 2A) or Xp<sup>tel</sup> in >90% of labeled cells.

Signals in Interphase Cells with Isodicentric Chromosomes. Cells with three discrete X<sup>cen</sup> signals were seen in all three dicentric cell lines. The signal for the normal X is not always seen in photographs as it may not be in the same focal plane as the dicentric signals. Fig. 2D shows a cell with three signals, including two that are close. This characteristic close double signal (in a single focal plane) was seen in 41% of cells with an X<sup>cen</sup> signal (shown in Figs. 2 C and E and 3: X<sup>cen</sup>). Often peripheral, the double signal resembles the bipartite Barr body formed by these chromosomes in interphase nuclei (16) (visualized with DAPI in Fig. 2B). Two close Xp<sup>tel</sup> signals were also seen in 37% of labeled cells (Fig. 3: Xp<sup>tel</sup>) and in cells labeled with Xq<sup>tel</sup> (Fig. 2G).

Double Signals Originate from Isodicentric Chromosomes. As it is absent in control fibroblasts, this characteristic double signal is not due to replicated or diffuse signals. Double signals were seen in hybrid cells with a dicentric chromosome but no normal X chromosome (66% of cells labeled with X<sup>cen</sup> and 37% of cells labeled with Xp<sup>tel</sup>). In cells with two signals, the two were invariably close. As expected, two sets of paired (four) signals were common in mitotic cell hybrids (Fig. 4). That the close double signals were rare in cells lacking dicentric chromosomes and frequent in cells with only dicentric chromosomes indicates they come from...
the two centromeres (or the two telomeres) of the dicentric chromosomes.

**Interphase Position of Isodicentric X Centromeres.** The distance between the two centromeres varied among cell lines, ranging in fibroblasts from 0.9 to 2.2 μm (Table 2). Unexpectedly, it was greater in the 411 chromosome, joined by the short arms, than in 3935 and 7213 chromosomes with centromeres separated by the long arms (Fig. 1).

**Interphase Position of Isodicentric X Telomeres.** The two signals from chromosomes labeled with Xp<sub>del</sub> were surprisingly close; the distance was like that between centromeres, both about 1 μm (Table 2). When both telomeres of 411 were labeled with Xq<sub>del</sub>, the distance between them (1.0 ± 0.3 μm) was less than expected for a chromosome of its size and was considerably less than between centromeres (2.3 ± 0.7 μm) (Table 2).

**Isodicentric Human X Chromosomes in Hybrid Cells.** Hybrid cells also let us examine the human inactive X chromosome in a foreign environment. In hybrids derived from mouse A9 cells by 5-azacytidine treatment, the two X<sup>cen</sup> signals were as close as in human parent cells (2.2 vs. 2.3 μm and 1.3 vs. 1.3 μm for 411 and 3935, respectively) (Table 2 and Fig. 3: X<sup>cen</sup>). However, the centromeres were significantly further apart in the 7213 hybrid (derived from mouse tsA1 cells) than in parent human cells (2.6 μm vs. 0.9 μm, P < 0.0005). The Xp<sub>del</sub> signals were only slightly farther apart in hybrids than in parent cells (1.5 vs. 1.1, P < 0.005, and 1.6 vs. 1.1, P < 0.0005, for 3935 and 7213, respectively) (Table 2 and Fig. 3: Xp<sub>del</sub>).

**Mitotic Cells.** To examine the inactive X chromosome in mitosis, cells from mitotic shake-offs were fixed without hypotonic treatment. One caveat is that if sister chromatids are still joined, signals due to replication of the sequence at one telomere (signal on each sister chromatid) are not easily distinguished from those at two telomeres. However, this ambiguity can be resolved when the two chromatids disjoin in anaphase, seen in cells with four signals (i.e., Fig. 4). For all three isodicentrics, the mean distance between double signals, whether telomeric or centromeric, was consistently about 1 μm. Therefore, at mitosis, when chromosomes are most condensed, the two ends (which may be as close as the replicated chromatids—i.e., Fig. 4C) are only slightly closer than in interphase. The distance between centromeres changed little from interphase to mitosis for the 3935 chromosome (1.3 vs. 1.1 μm, P = 0.05) but decreased significantly for the 411 chromosome with centromeres separated by the short arms (2.3 vs. 1.1, P = 0.0005).

**Relationship of Centromere and Telomere Analyzed by Simultaneous Hybridization with X<sup>cen</sup> and Xp<sub>del</sub> Probes.** When the 3935 chromosome was doubly labeled [DNP-labeled X<sup>cen</sup> conjugated with Texas red and biotin-labeled Xp<sub>del</sub> conjugated with fluorescein isothiocyanate (FITC)-avidin] the four signals obtained were close but difficult to resolve with the compound microscope. Using confocal microscopy, we could superimpose Texas red and FITC-avidin signals and optically section the cell from top to bottom along the z axis by 0.5-μm intervals to examine the three-dimensional relationships. Signals were often at the periphery of the nucleus and most often near the top, perhaps an ascertainment bias favoring brighter signals. Fig. 5A shows that all four signals were adjacent. Although cells were acid fixed, we could detect differences in the depth of signals. Telomere signals (green) came into view before those from the centromeres (red), suggesting that telomeres were closer to the nuclear membrane; this was supported by an xz image (optical section in the vertical plane), which also revealed a greater distance from centromere to telomere than between the two centromeres or the two telomeres (Fig. 5B). However, the span
between Xcen and Xpet signals was not large compared to the widely separated signals seen in hybrids with the normal human active X chromosome (data not shown).

Table 2. Distance between double signals resulting from in situ hybridization of biotinylated probes.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Probe</th>
<th>Phase of cell cycle</th>
<th>Distance,* μm</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB hybrid</td>
<td>Xpet + Xqtel</td>
<td>Interphase</td>
<td>10.4 ± 5.3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactive normal X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 hybrid</td>
<td>Xpet + Xqtel</td>
<td>Interphase</td>
<td>1.5 ± 0.3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactive dicentric X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F411</td>
<td>Xcen</td>
<td>Interphase</td>
<td>2.2 ± 1.7</td>
<td>24</td>
</tr>
<tr>
<td>411-hybrid</td>
<td>Xcen</td>
<td>Interphase</td>
<td>2.3 ± 0.7</td>
<td>28</td>
</tr>
<tr>
<td>411-hybrid</td>
<td>Xcen</td>
<td>Mitosis</td>
<td>1.1 ± 0.3</td>
<td>35</td>
</tr>
<tr>
<td>GM3935</td>
<td>Xcen</td>
<td>Interphase</td>
<td>1.3 ± 0.5</td>
<td>23</td>
</tr>
<tr>
<td>3935-hybrid</td>
<td>Xcen</td>
<td>Interphase</td>
<td>1.3 ± 0.5</td>
<td>16</td>
</tr>
<tr>
<td>3935-hybrid</td>
<td>Xcen</td>
<td>Mitosis</td>
<td>1.1 ± 0.3</td>
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<td>GM3935</td>
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<tr>
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<td>Xpet</td>
<td>Mitosis</td>
<td>1.1 ± 0.3</td>
<td>36</td>
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<tr>
<td>GM7213</td>
<td>Xcen</td>
<td>Interphase</td>
<td>0.9 ± 0.3</td>
<td>21</td>
</tr>
<tr>
<td>7213-hybrid</td>
<td>Xcen</td>
<td>Interphase</td>
<td>2.6 ± 0.4</td>
<td>41</td>
</tr>
<tr>
<td>7213-hybrid</td>
<td>Xcen</td>
<td>Mitosis</td>
<td>1.1 ± 0.2</td>
<td>10</td>
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<td>GM7213</td>
<td>Xpet</td>
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<td>1.1 ± 0.4</td>
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<td>13</td>
</tr>
</tbody>
</table>

n, Number of cells analyzed.

*Mean ± SD.

Interphase Position of Telomeres in Normal X Chromosomes. Because either Xp or Xq telomere of isodicentrics was capable of telomere association, we analyzed hybrids having normal X chromosomes, using a mixture of Xpet and Xqtel probes. The mean distance between these signals in hybrids with the wild-type active X chromosomes was 10.4 ± 5.3 μm, with a wide range from 3 to 22 μm (DB hybrid in Table 2). In striking contrast, the two signals were close (1.5 ± 0.3 μm) in hybrids with only the normal inactive X chromosome, showing proximity of long- and short-arm telomeres (Fig. 6A and Table 2, G1 hybrid).

DISCUSSION

Evidence That the Inactive X Chromosome Forms a Loop Structure with Telomeres Associated. These studies show that the inactive X chromosome, whether isodicentric or structurally normal, is not a linear structure. The studies with single probes show that the two ends of these chromosomes are too close together for linear structures. Because we only measured distance between two discernible signals and our probes were subtelomeric, the distance between telomeres may be even less than measured. In any event, based on interphase studies of Lawrence et al. (27, 28), Trask et al.
Significance of Telomere Association. Although both telomeres of the inactive X chromosome are close to the nuclear membrane, we have no evidence of membrane attachment. There is some electron microscopic evidence for a network of filaments emanating from the membrane toward the Barr body (3), and in meiosis telomeres are frequently reversibly associated with the nuclear envelope (18, 32). Yet, the proximity of telomeres in mitotic cells (in absence of nuclear membrane) suggests that the nuclear envelope is not required to maintain telomere association. Hinton (33) showed that terminal adhesions between nonhomologous ends of polytene chromosomes were inherent in the nature of the telomere. The common DNA sequence (telomere) at the ends of all human chromosomes could predispose to this kind of association; however, our observations that the telomeres of the active X chromosome are not close suggest that association of the two ends of the inactive X chromosome is not a general characteristic of interphase chromosomes and may be a unique attribute of inactive chromosomes. How telomere association occurs and what role the looped configuration of the chromosome plays in silencing transcription of the inactive X chromosome are subjects for further study.

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