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Characterization of neo-centromeres in marker chromosomes lacking detectable alpha-satellite DNA

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Recent studies have implicated α-satellite DNA as an integral part of the centromere, important for the normal segregation of human chromosomes. To explore the relationship between the normal functioning centromere and α-satellite DNA, we have studied eight accessory marker chromosomes in which fluorescence in-situ hybridization could detect neither pancentromeric nor chromosome-specific α-satellite DNA. These accessory marker chromosomes were present in the majority of or all cells analyzed and appeared mitotically stable, thereby indicating the presence of a functional centromere. FISH analysis with both chromosome-specific libraries and single-copy YACs, together with microsatellite DNA studies, allowed unequivocal identification of both the origin and structure of these chromosomes. All but one of the marker chromosomes were linear mirror image duplications, and they were present along with either two additional normal chromosomes or with one normal and one deleted chromosome. Indirect immunofluorescence analysis revealed that the centromere protein CENP-B was not present on these markers; however, both CENP-C and CENP-E were present at a position defining a ‘neo-centromere’. These studies provide insight into a newly defined class of marker chromosomes that lack detectable α-satellite DNA. At least for such marker chromosomes, α-satellite DNA at levels detectable by FISH appears unnecessary for chromosome segregation or for the association of CENP-C and CENP-E at a functional centromere.

INTRODUCTION

The centromere of large, complex chromosomes is defined cytologically by the primary constriction and is required for proper chromosome segregation in both meiosis and mitosis. The kinetochore is located on either side of the centromere, and it is the centromere/kinetochore complex that is the location for the motor components responsible for chromosome movement in mitosis (1–5). This complex can be split into three different regions, an inner pairing domain, a central domain and an outer kinetochore domain (2,6). The central domain is composed primarily of centromeric heterochromatin and is where centromere protein B (CENP-B) is localized (7–10). CENP-B binds to a 17 bp motif (CENP-B boxes) within α-satellite DNA (11), the major class of DNA found at primate centromeres. Other proteins have also been localized to the kinetochore domain and have been implicated in chromosome movement. CENP-C has been localized to the inner plate of the kinetochore domain (12) and has been suggested to have a role in kinetochore assembly (13). Cytogenetic studies have indicated that, while CENP-B is present at both active and inactive centromeres, CENP-C is present only at active centromeres (5,7,14). CENP-E is found at the outer plate of the kinetochore structure and can be detected on either side of the centromere by late prometaphase (15,16). This protein is believed to function as a unique motor protein and is associated with microtubules and chromosomal movement (17,18).

One effective genetic approach to understanding mammalian centromeres and the role of centromeric proteins in the proper segregation of mitotic chromosomes is to examine structurally

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rearranged chromosomes with an abnormal number of centromeres. The most accessible rearrangements are Robertsonian translocations, which are the most common dicentric chromosomes in humans. Sullivan and Schwartz (5) studied 12 dicentric Robertsonian translocations and, using immunocytogenetic studies, demonstrated that while CENP-B was invariably present at both active and inactive centromeres, CENP-C and CENP-E were usually located only at active centromeres. These studies confirmed previous observations of CENP-C at active centromeres (7) and provided the first evidence that CENP-E is also found specifically at active centromeres, thus suggesting that at least two specific centromeric proteins are necessary for human centromeric function.

Small accessory unidentified chromosomes ('marker chromosomes') are estimated to occur in 0.05% of livebirths (19). Utilizing FISH, the majority of these can now be identified. In most instances, such chromosomes have been shown to contain α-satellite DNA and other pericentromeric material (20). However, a recently recognized class of marker chromosomes that lack detectable α-satellite DNA has been identified (21,22). In light of recent evidence that indicates a role for α-satellite DNA in centromere function (22–24), this class of marker chromosomes raises the question of what DNA acts as a functional centromere in the absence of normal amounts of α-satellite DNA. In this study, we have investigated eight different marker chromosomes in which α-satellite DNA could not be detected by FISH with either pancentromeric or chromosome-specific α-satellite DNA probes. We explored the relationship between chromosome segregation, stability and the presence and identity of centromeric proteins in this unique set of marker chromosomes.

**RESULTS**

**Cytogenetic studies**

The eight cases were initially studied with high resolution chromosome analysis revealing the presence of a marker chromosome in all eight patients, and a concomitant deletion in three patients (Table 1). These markers were characterized using both G-banding and C-banding. No C-banded region was seen on any marker; however, a constriction was seen in each marker chromosome using G-banding. FISH with several different repetitive sequences was utilized to characterize each marker. FISH studies with α-satellite DNA, β-satellite DNA and satellite III-DNA probes revealed no detectable signal on any of the markers (Table 1). Studies with chromosomal library probes revealed that three of these markers originated from chromosome 15, two from chromosome 9 (25), and one each from chromosomes 13, 11 and 10. Studies with a telomeric probe showed that seven of these markers had telomeres at each end, while one (Case 8) did not show any detectable hybridization.

Based upon both cytogenetic and FISH studies, the markers were subdivided into three groups (Table 1). Five of the cases (patients 1–5) had 46 normal chromosomes plus an additional supernumerary marker chromosome that appeared linear about a symmetrical point. This resulted in four copies of segments of 15q, 13q and 9p in cases 1–3, 4 and 5, respectively. Two cases (6 and 7) showed a terminal deletion in one chromosome along with a symmetrical mirror image resulting from duplication of the deleted segment. This resulted in three copies of segments of 9p and 11q in cases 7 and 6, respectively. The marker in case 8 resulted from an interstitial deletion of chromosome 10q. In this case, there was no duplication of the deleted chromosomal segment, leading to a net loss of material, because of mosaicism.

**Table 1. Description of markers**

<table>
<thead>
<tr>
<th>Patient #</th>
<th>α-Sat</th>
<th>Telomere</th>
<th>Type of abnormality</th>
<th>Karyotype</th>
<th>Stability</th>
<th>lymphocyte</th>
<th>lymphoblast</th>
<th>fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>++</td>
<td>Mirror image duplication</td>
<td>47,XX,+der(15)(qter→q25.3::q25.3→qter)</td>
<td>82%</td>
<td>33%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>++</td>
<td>Mirror image duplication</td>
<td>47,XY,+der(15)(qter→q25.3::q25.3→qter)</td>
<td>74%</td>
<td>3%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>++</td>
<td>Mirror image duplication</td>
<td>47,XY,+der(15)(qter→q26.1::q26.1→qter)</td>
<td>86%</td>
<td>0%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>++</td>
<td>Mirror image duplication</td>
<td>47,XY,+der(13)(qter→q32::q32→qter)</td>
<td>98%</td>
<td>32%</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>++</td>
<td>Mirror image duplication</td>
<td>47,XY,+der(9)(pter→p21.2::p21.2→qter)</td>
<td>100%</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>++</td>
<td>Terminal deletion; mirror image duplication</td>
<td>47,XY,del(11)(q22),+der(11)(qter→q22::q22→qter)</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>++</td>
<td>Terminal deletion; mirror image duplication</td>
<td>47,XY,del(9)(p12),+der(9)(pter→p12::p12pter)</td>
<td>100%</td>
<td>100%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
<td>Interstitial deletion and marker</td>
<td>47,XX,del(10)(q11q23),+der(10)(q11q23)</td>
<td>62%</td>
<td>NA</td>
<td>80%</td>
<td></td>
</tr>
</tbody>
</table>

*Amniotic fluid cultures.
NA, cells not available for testing.
Structural analysis

The structure of each marker chromosome was examined with a series of cosmid and YAC probes. When possible, each marker was studied by FISH with at least two single-copy probes from the relevant chromosome. FISH with site-specific DNA probes revealed the presence of either two signals or no signal in markers 1–7. The information obtained by FISH, in conjunction with high resolution banding, suggests that markers 1–7 are linear ‘mirror image’ rearrangements, which are symmetrical about a central point (Fig. 1). Only in case 8 was the inferred structure of the marker different. In this case, the marker was not symmetrical, appearing as the segment lost from an interstitial deletion in one chromosome 10 (Fig. 2).

Three of these markers were derived from the distal long arm of chromosome 15. In order to explore the structural heterogeneity of these markers, they were studied using several different cosmid and YAC probes along with several PCR primers. These studies showed that the markers are indeed heterogeneous, each having a different breakpoint. The marker from patient 1 is the largest with a break...
around D15S202. Only one signal was seen in the middle of the marker using YAC 802-b-4, whereas more distal probes showed two signals. A similar finding was also seen in case 2, which had a break around D15S158; only one signal was seen in the middle of this marker with YAC 694-g-12. Case 3 had the smallest of the three markers with a breakpoint proximal to D15S87.

**Mitotic stability**

When possible, mitotic stability of the markers was studied in lymphocyte, lymphoblast and fibroblast cultures (Table 1). Studies of lymphocyte cultures revealed that in three of the seven cases (cases 5–7), the marker chromosomes were seen in every cell examined. The marker was present in at least 74% of the cells from the other patients (cases 1–4). These findings suggest that the markers are stable in short-term lymphocyte cultures. The lymphoblast and fibroblast studies did not reveal the same high frequency of marker chromosomes (Table 1). While this may indicate that the marker is less stable in long-term cell culture, the lower frequencies may also be just a chance occurrence, as these lines are oligoclonal in origin.

**Figure 2.** Delineation of the structure of an interstitial deletion and simultaneous linear marker derived from chromosome 10 (case 8). (A) FISH with a chromosome 10 specific paint demonstrating that the marker is derived from this chromosome; (B) FISH with a pancentromeric probe demonstrating the lack of detectable α-satellite DNA sequences; (C) FISH with a telomeric probe showing the absence of detectable telomeres on the marker chromosome.
Table 2. Centromere proteins

<table>
<thead>
<tr>
<th>Patient #</th>
<th>CENP-B</th>
<th>CENP-C</th>
<th>CENP-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NA, not available for testing.
–, Tested but not present.

Centromere proteins

In order to understand how and why these chromosomes which lack detectable α-satellite DNA can segregate in culture, several antigens to centromere proteins were studied using indirect immunofluorescence in combination with FISH to evaluate each marker available for study. Consistent with the absence of detectable α-satellite DNA, CENP-B could not be detected in any of the five cases studied (Table 2; cases 1, 4, 6, 7 and 8). However, CENP-C was present on 5/5 cases studied, while CENP-E was present on 4/4 cases studied (Table 2; Figs 3, 4 and 5). Neither CENP-C nor CENP-E was located in the center of the mirror image markers; however, only one antibody signal was detected on the chromosome.

Timing and parental origin

To better understand the mechanism of formation of these mirror image markers and to determine their parental origin, at least 12 PCR-formatted polymorphisms on a subset of these markers (cases 1–4) were studied. In case 2, the proband always showed three distinct alleles for each informative marker, two from the father and one from the mother. This not only revealed the parental origin, but also indicated that the marker most likely formed during meiosis, although it could have formed, for example, after non-disjunction leading to trisomy 15 followed by marker formation due to a mitotic error. However, for cases 1, 3 and 4, three distinct alleles were not found. We found that the parents of patients 1 and 3 are heterozygous and informative for multiple loci in the marker, but the proband contains two alleles, not three as in case 2. This suggests that a mitotic, rather than meiotic, origin is most likely in these two cases. Dosage studies of multiple primers suggests that these two markers were paternally derived. In case 4 where parental specimens were not available, three signals were never detected, again suggesting a mitotic origin for this marker as well.

Cases 6 and 7 had both the marker and a concomitant deletion and were most likely formed by a different mechanism to the other markers. Parental DNA was not available in either case. However, PCR analysis for multiple loci on both 11q and 9p revealed only two alleles for each locus, never three alleles as in case 2.

DISCUSSION

Identification and delineation of structure

Marker chromosomes occur in approximately one per 2000 livebirths. The vast majority of these markers can now be identified using FISH with either α-satellite DNA probes or with chromosome painting probes (20). In this study, we discuss a recently recognized type of marker chromosome in which the centromere is not detectable by C-banding, and α-satellite DNA is not detectable by FISH with either a pancentromeric probe or any chromosome-specific α-satellite DNA sequence (22). The identity of the marker in three of our cases (cases 6–8) was easily identified cytogenetically because of a concomitant deletion. However, in our other cases (cases 1–5), neither routine nor high resolution chromosome analysis identified the marker, and FISH with a series of DNA probes was required to identify the markers (Table 1).

Telomere probes confirmed that seven of these marker chromosomes (cases 1–7) were linear, with two telomeric segments. In addition, FISH with a series of single-copy cosmids and YACs indicated that all seven of these markers appeared to have a symmetrical mirror image organization. Because three different markers were found to originate from chromosome 15, single-copy cosmids and YACs were utilized to determine if these markers were identical in structure. This analysis clearly showed that each marker had a different breakpoint and involved different segments of chromosome 15q (Table 1).

In contrast, telomere studies on the chromosome 10 marker identified in case 8, revealed that this marker, in addition to having no detectable C-banding or α-satellite DNA, did not have any demonstrable telomeric regions. Most likely this marker was the result of an interstitial deletion not accompanied by duplication (see below). Based on the banding of both the deleted chromosome 10 and the marker, no material appeared lost from those cells with the marker. However, the patient did contain a cell line with the deletion but without the marker, thus resulting in a net loss of material.

Previous studies

Our cytogenetic analyses and FISH studies, revealed three different types of markers, all with no detectable α-satellite DNA. These included: (i) markers that originate as extra supernumerary chromosomes, such that the karyotype contained 46 normal chromosomes plus an accessory symmetrical mirror-image marker; (ii) markers that result from a terminal deletion of a chromosome with duplications of the deleted segment, leading to a symmetrical mirror-image marker; and (iii) markers that result from an interstitial deletion, leading to a non-duplicated marker. Our findings regarding these subtypes are consistent with previously reported cases. Magnani et al. (26) reported a marker chromosome derived from chromosome 14 with a concomitant deletion on one chromosome 14; this has been the only other case of a symmetrical marker chromosome in addition to the presence of a deletion. One case of an extra marker derived from chromosome 8, and three cases with an extra marker derived from chromosome 15 have been reported (21,27,28). Vouillaire et al. (29) reported an unusual supernumerary marker chromosome resulting from an interstitial deletion. All of these marker chromosomes are similar to the eight cases described here in that none demonstrate C-banding nor contain detectable α-satellite DNA.

Origin and mechanism of formation

As described above, both FISH and DNA polymorphism studies were used to examine the mechanism of formation and the parental origin of marker chromosomes. In case 2, three
individual alleles could be detected by PCR; thus the marker found in case 2 is probably the consequence of a meiotic error. In three of the cases, the proband’s marker chromosome showed only two alleles, and therefore, were most likely the consequence of mitotic errors. These observations are consistent with our findings in routine cytogenetic studies of amniotic fluid cell cultures, where a survey of 400 amniotic fluid cultures revealed that 2.5% of the cases examined had an ‘acentric’ symmetrical mirror image marker (data not shown). We believe that the majority of these markers were produced mitotically, are not stable in culture, and are most likely a tissue culture artifact.

Blennow et al. (21) have hypothesized that acentric markers derived from chromosome 15 are formed as reciprocal by-products of the formation of inv dup(15) chromosomes. While this is feasible, it is probably not the method of formation for the majority of these markers for several reasons. First, the markers reported here are much smaller than markers predicted as the acentric reciprocal event of the inv dup(15). Second, although...
there are several markers without normal centromeres derived from chromosome 15, this number is still very small and the breaks are very heterogeneous. In contrast, we have recently found that there are specific hotspots in proximal 15q involved in the formation of inv dup(15) chromosomes (Wandstrat et al., manuscript in preparation). Thirdly, our molecular data suggests that the majority of these markers most likely arise in mitosis, and therefore must be accounted for by a different mechanism than inv dup(15) markers which are the consequence of maternal meiotic errors (Wandstrat et al., manuscript in preparation).

Therefore, it is unlikely that the marker chromosome studied in this report represents reciprocal events.

**Centromere function**

As demonstrated in our studies and in several previously reported cases by others (21,26–29), this new class of markers has neither detectable α-satellite DNA nor detectable C-banding. Additionally in those cases studied, no CENP-B protein could be detected on these markers. This latter finding is not an unexpected

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**Figure 4.** Localization of CENPs to a deletion and simultaneous mirror image duplication derived from chromosome 9 (case 7). (A) Lack of detectable CENP-B on the marker chromosome. Simultaneous FISH with a chromosome 9 specific library allowed the detection of the marker chromosome with the immunofluorescence studies; (B) presence of CENP-C on the marker chromosome; and (C) detection of CENP-E on the marker chromosome.
result, as CENP-B binds to α-satellite DNA (2,7,9,11). Antibodies to CREST antigens were used in the study of three previous cases, and were shown to bind to these markers (27,29,30). As the antibodies present within each batch of CREST serum will vary and recognize several different centromeric proteins, information obtained using CREST serum is of limited value.

In order to better understand the relationship of centromeric proteins with these markers, we examined each of our markers for the presence or absence of specific centromeric proteins, CENP-C and CENP-E. In all five cases studied with CENP-C and the four studied with CENP-E, both proteins were detected. As described in previous studies of Robertsonian translocations (5), this suggests an important role for these proteins in the functional centromere and confirms earlier antibody microinjection studies that showed CENP-C to be required for the assembly of the kinetochore (13).

Because both CENP-C and CENP-E are present on the marker chromosomes, we can hypothesize that these chromosomes must contain functional kinetochore, thus explaining why the markers...
are stable in vivo. However, it is unclear how and where these proteins bind, as no conventional centromere is present. It is possible that a small undetectable amount of α-satellite DNA is present and provides the basis for a functioning centromere, but that the amount of α-satellite DNA present is below our methods of detection. This would be consistent with several studies over the past few years that have shown that by introducing α-satellite DNA into primate and human cell lines, these sequences will provide the necessary requirements for certain aspects of centromere function (24,31). Additionally, Wevrick et al. (32) and Andersen et al. (33) have shown that a partial deletion of α-satellite DNA, while associated with reduced amounts of CENP-B, is consistent with the presence of a mitotically stable human chromosome. However, it seems unlikely that this is the best model for explaining the markers reported here. Our analyses showed that these markers are symmetrical, do not include a typical centromeric sequence, and originated from chromosomal segments distant from the centromeric region. The inclusion of α-satellite into the markers would require an extensive chromosomal rearrangement, a complex event that appears unlikely. An alternative explanation would be the activation of small amounts of ‘ectopic’ α-satellite DNA at non-centromeric positions in these rearrangements (29). Although this is possible, there is no evidence for such ‘ectopic’ α-satellite DNA from extensive physical mapping studies except on chromosome 2q, which is the site of the ancient primate centromere (34–36).

The most likely explanation for our finding is that marker chromosome formation is accompanied by activation of non-centromeric sequences at non-centromeric sites. Such sequences could be normal euchromatic DNA that, in the absence of α-satellite DNA and/or other centromeric DNA, is capable of being activated to form a ‘neo-centromere’ (37). The activation may be sequence-specific, but the sequence-specificity must be broader than currently recognized. The activation may alternatively be sequence-independent or conformation-dependent, in which case the centromere/kinetochore forms at a particular site because the chromatin achieves a proper conformation at that site which permit CENP-C and CENP-E association to form a kinetochore. Based on our studies and previously reported studies, there is a selection bias in the patient samples. Therefore, while these markers are not frequent, they can be formed and become stable if selected for phenotypically.

MATERIALS AND METHODS

Clinical population

Eight patients with de novo supernumerary marker chromosomes were collected from five different genetics centers. Seven of these patients were ascertained because of mental retardation and/or development delay or because of structural anomalies detected at birth; one (case 8) was ascertained through routine prenatal diagnosis studies.

Cytogenetic studies

Metaphase chromosomes were prepared from phytohem-agglutinin (PHA)-stimulated blood cultures, from fibroblast cultures and/or lymphoblast cultures by using standard procedures. When possible, leukocytes from peripheral blood of the probands were cultured for high resolution chromosome studies (38,39). To attain high resolution chromosomes, PHA-stimulated blood leukocytes were cultured for ~72 h in RPMI 1640 with 17% fetal bovine serum. The cultures were synchronized by addition of thymidine for the last 16.5 h of culture and harvested after the addition of ethidium bromide and colcemid for the last 45 and 25 min of culture, respectively. The cells were treated for 8 min with 0.075 M KCl and fixed in 3:1 methanol:acetic acid prior to staining. Chromosomes were GTG- and C-banded, and at least 20 chromosomal spreads were examined from two cultures.

Centromere protein antibodies

Polyclonal antibodies to CENP-B, CENP-C and CENP-E were generated in the laboratories of two of the co-authors (W.E. and H.F.W.) (24). In addition, monoclonal antibodies to CENP-E were provided by Dr Tim Yen (Philadelphia, PA) (15,16). Both monolayer and suspension cultures of cells were used to obtain unfixed metaphase preparations for immunofluorescence. Modifications of the method of Earnshaw (7) were applied to the preparation of metaphase chromosomes for antibody detection (5). Lymphoblastoid cells were grown on polylysine-coated slides and harvested as monolayer cell cultures. The method of Sullivan and Schwartz (5) was used for the visualization of CENP-C and CENP-E.

FISH studies

FISH was performed utilizing a variety of probes (YACs, cosmids, satellite DNA or chromosomal libraries) on unstained slides according to the technique of Pinkel et al. (40) with minor modifications, depending on the probe (41).

In each case at least 10, and in most cases 20, metaphases were analyzed for the presence of probe utilized on both the normal and the derived chromosome.

Digital images were captured using a confocal microscope and/or Zeiss epifluorescence microscope equipped with a cooled CCD camera (Photometrics CH250) controlled by an Apple Macintosh computer. Gray scale source images were captured separately with DAPI, fluorescein and rhodamine filter sets, merged and pseudocolored using Gene Join software (Yale University).

Molecular studies

Highly polymorphic microsatellite markers within the duplicated region of the derived chromosome found in each patient were analyzed by PCR using standard techniques (42). Primer sequences for microsatellite analysis (Operon Technologies, Inc., Alameda, CA, or Research Genetics, Inc., Huntsville, AL) were obtained from the Genome Database.

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