We have introduced a 6.5 Mb human mini-chromosome with a complex centromere structure into DT40 cells and have used sequence targeting and telomere-directed chromosome breakage to dissect the sequence requirements for centromere function. These experiments proved that a vertebrate centromere with two blocks of functional alphoid DNA separated by 2.5 Mb can exist as a stable structure in some but not all vertebrate cells. Further experiments indicated that recovery of chromosomes with less than ∼100 kb of alphoid DNA is very inefficient, suggesting that a functional centromere requires a minimum of ∼100 kb of alphoid DNA. Mini-chromosomes with minimal centromeres segregate accurately in some but not all vertebrate cells and should be useful for the detection of sequence-specific factors required for vertebrate centromere maintenance.

INTRODUCTION

The minimal chromosome size and sequence composition consistent with accurate chromosome segregation in vertebrate cells are important both for the development of chromosome-based vectors and of screens for genes important for chromosome function. Three basic approaches have been developed to define these parameters. The first has been to study the structural diversity occurring naturally in human chromosomes. This has shown that alphoid DNA (1) and a variety of other sequences (2,3) will function as centromeres. Alphoid DNA is found at the centromeres of all normal human chromosomes and since stable marker chromosomes lacking alphoid DNA occur only in rare clinical cases it has generally been assumed, although not proven, that centromeres form most readily over alphoid DNA. The second approach to understanding the sequence requirements for vertebrate chromosome function has been to try to build artificial chromosomes from defined sequence components. This approach is potentially ideal but so far has been restricted by the fact that the chromosomes assembled in vitro bear no simple structural relationship to the input DNA (4,5). The third is to use telomere-directed chromosome breakage and homologous recombination (6) to fragment a single natural chromosome into products of defined structure and sequence composition and to study their behavior. This has become practical with the demonstration that human chromosomes can be moved into chicken DT40 cells (7) where they can be targeted efficiently (8). In principle the ability to engineer mini-chromosomes in DT40 cells should allow both the development of vectors for the mammalian germ line (9) and the dissection of vertebrate mini-chromosomes to a minimal size and sequence composition consistent with maintenance as autonomous molecules.

A central problem in understanding metazoan chromosome function is how centromeres work. Centromeres are complex and their study resolves into a variety of different problems. One issue which is fundamental to the others is how centromeres form at a single specific site on a chromosome. This question has attracted considerable interest recently as a result of the demonstration, originally in humans (2) but subsequently in Drosophila (10,11), that centromeres can form over DNA sequences that do not normally function as such. This work has led to widespread speculation about the mechanism of centromere formation and maintenance in eukaryotes. An influential set of ideas have been put forward by Karpen and Allshire (12) who have proposed that, once formed, centromeres function as self-propagating structures independently of underlying DNA sequence. According to this proposal the information that determines whether a centromere is present at a particular position in the genome is contained within the centromeric protein complex and not within the underlying DNA sequence. A well studied and conceptually analogous mechanism of such epigenetic inheritance is provided by the maintenance of specific patterns of DNA methylation at cytosine residues in the CpG dinucleotide in many eukaryotes (13). A second feature of the proposal is the possibility that centromeres are relatively dynamic structures with the self-replicating protein complex being able to crawl or jump along chromosomes.

Previously we described a set of mini-chromosomes derived from the human Y chromosome (14). These mini-chromosomes originated from both the long and short arms of the chromosome and were the results of two or more telomere-directed chromosome breakage reactions with the first occurring in the centromeric alphoid DNA. One interesting
aspect of this set of mini-chromosomes was that, whereas the short-arm-derived mini-chromosome had a simple structure consistent with two truncation events, the three long arm chromosomes had rearranged in more complex ways. The causes of these rearrangements were not known but we speculated that we might have damaged the centromere in the first breakage reaction and the rearrangements somehow compensated for this. Here we have defined the structure and sequence composition of one of these long-arm-derived mini-chromosomes and dissected it further in DT40. We have also carried out an extensive series of telomere-directed chromosome breakage experiments to see whether we could use telomere-directed chromosome breakage to detect centromere movement along the chromosome. The results indicate that centromeres are not dynamic structures when considered over the resolution of 100 kb but, rather, are fixed over a single specific region.

Recently we have shown that a specific centromere on a single mini-chromosome with a short (100 kb) tract of alphoid DNA had different levels of activity in different types of cell (M.H. Shen et al., submitted). The mini-chromosome was stable in chicken and human cells where it formed a centromere, but was rapidly lost from mouse LA-9 cells. We have interpreted this observation to indicate that centromeres are maintained by an activity which functions in trans and is sensitive to the amount of alphoid DNA. Similar conclusions are suggested by experiments carried out in Drosophila by Platero et al. (11). It should be possible to use the mini-chromosomes described here as reagents with which to detect the genes encoding the components of this activity.

RESULTS

Structure and manipulation of a mini-chromosome in DT40 cells

Δ128 is a 6 Mb mini-chromosome derived from the long arm of the Y chromosome by two rounds of telomere-directed chromosome breakage in hprt-deficient Chinese hamster ovary (CHO) cells (14). It is marked at one end by an Escherichia coli ecogpt gene and at the other by a gene conferring resistance to G418. Both genes are driven by sv40 early region promoters and flank the telomeres used in the breakage reactions which generated the mini-chromosome. The sequence-tagged site (STS) content of Δ128 indicated that it was rearranged in a complex way and we wanted to understand...
which sequences were functioning as centromeres on this mini-chromosome. We therefore wished to dissect \( \Delta 128 \) in DT40 cells. The \( sv40 \) early region promoter does not work efficiently in DT40 cells and so we needed to generate a derivative of \( \Delta 128 \) marked with a suitable resistance gene. We therefore assembled a random telomere-directed chromosome breakage construct termed pBS\(~\Delta\)CCAGHisDTel (Fig. 1) containing a 1.2 kb stretch of telomeric DNA (15) and a histidinol-resistance gene driven by a promoter (16) including both a chicken \( \beta \)-actin gene and a cytomegalovirus immediate early region enhancer (Fig. 1). We linearized this construct to reveal the telomere and then transfected the \( \Delta 128 \)-containing \( hpr^{-} \) CHO cells with the pBS--CCAGHisDTel and selected for uptake of the construct by histidinol resistance and for loss of

![Figure 2](image-url)

**Figure 2.** Mapping and manipulation of mini-chromosomes in DT40 cells. (A) PFGE analysis of mini-chromosomes \( \Delta 128 \), \( \Delta\Delta\)His1 and \( \Delta\Delta\)His35. Mini-chromosome \( \Delta 128 \) was broken by random telomere-directed chromosome breakage in Chinese hamster chromosomes into mini-chromosomes \( \Delta\Delta\)His1 and \( \Delta\Delta\)His35, which were size fractionated by PFGE and analysed by filter hybridization with an alphoid DNA probe. (B) Alphoid DNA on DNA of mini-chromosomes \( \Delta 128 \), \( \Delta\Delta\)His1 and \( \Delta\Delta\)His35. DNA extracted from cells containing the indicated mini-chromosome was digested with \( Bgl II \), size fractionated by PFGE and probed either with a probe recognizing the histidinol-resistance gene used to tag the chromosome or with an alphoid DNA probe. (C) Mapping \( \Delta\Delta\)His35. DNA from cells containing \( \Delta\Delta\)His35 was digested with the indicated enzyme, size fractionated by PFGE and probed with a probe recognizing the histidinol-resistance gene, the neomycin-resistance gene, an alphoid DNA probe or a probe recognizing the DYZ1 repeat. (D) Restriction site maps of mini-chromosomes \( \Delta\Delta\)His1 and \( \Delta\Delta\)His35. The restriction site map of \( \Delta\Delta\)His35 was deduced from the data indicated in (C). The map of \( \Delta\Delta\)His1 was established using similar methods in both CHO and DT40 cells. The only difference between the respective maps was the absence of an \( Sst II \) site on the mini-chromosome in the DT40 cells. This \( Sst II \) site lies \(~\)800 kb to the right of the 100 kb alphoid block. (E) Stability of \( \Delta\Delta\)His1 in DT40 cells. DT40 cells containing \( \Delta\Delta\)His1 were cultured for the indicated number of generations and the structure of \( \Delta\Delta\)His1 analysed by PFGE and the retention of the mini-chromosome by cells in the culture analysed by FISH using an alphoid probe. The histogram indicates the percentage of cells with the specified number of alphoid signals of \( \Delta\Delta\)His1. The two signals of alphoid DNA were very close together and thus we assume that each signal pair corresponds to a single chromosome.
the gpt gene on Δ128 by 6-thioguanine resistance. This led to two cell lines containing mini-chromosomes in which the pBS–CCAGHisDTel had seeded a new telomere and the Δ128 had lost the svgrp gene. These were referred to ΔΔHis1 and ΔΔHis35. Pulsed field gel electrophoresis (PFGE) established that ΔΔHis1 was slightly bigger than Δ128 and that ΔΔHis35 was 2.5 Mb in size (Fig. 2A). It might be thought surprising that ΔΔHis1 was slightly bigger than Δ128 but it has previously been noted by others (17) that telomere-directed truncation events are sometimes accompanied by duplications around the site of truncation and we suppose that this is the cause of the fact that ΔΔHis1 is bigger than Δ128.

The small size of ΔΔHis35 made it an attractive candidate for restriction site mapping. We started the mapping by comparing the alphoid DNA on ΔΔHis35, Δ128 and ΔΔHis1. The alphoid DNA in ΔΔHis1 and Δ128 was present on BglII fragments of 11, 90 and 100 kb in size (Fig. 2B). In ΔΔHis35 the 100 kb array was truncated to a 70 kb fragment which included sequences recognized by a probe specific for the pBS–CCAGHisDTel breakage construct. We extracted DNA from ΔΔHis35 and then cut it with SfiI, BsiWI, BssHIII, EagI, SsrI, MluI or NruI, and analysed the digests by gel electrophoresis and filter hybridization with probes specific for the two telomeric markers: Neo and His, for alphoid DNA, and for another repetitive sequence DYZ1 also present on the mini-chromosome (Fig. 2C). These results could be interpreted in a restriction site map (Fig. 2D). The map was at too low a resolution to show the position of the 11 kb array of alphoid DNA but cloning placed it immediately adjacent to the neo gene at the left end of the mini-chromosome (Fig. 3B). Restriction analysis also showed that the DYZ1 sequence was present on a 1400 kb fragment that was uncut by many restriction enzymes which otherwise cut frequently in the bulk of euchromatic DNA, including Apal, HindIII (Fig. 4D), BclI, BglII and BstEII. Also present on these fragments were sequences recognized by a probe specific for the DYZ2 repeat which like the DYZ1 sequence is a major component of the heterochromatin of the long arm of the Y chromosome (data not shown). It is of interest to consider how similar the three different arrays of alphoid DNA on ΔΔHis1 are from one another. In previous work (18) it was shown that two independent 5.7 kb units of Y chromosome alphoid DNA differed by only an average of 1% sequence divergence. We therefore conclude that, since the three arrays of alphoid DNA on ΔΔHis1 originated from a single starting array, they are very similar in structure.

We used a similar approach to map ΔΔHis1 and this revealed that as expected ΔΔHis1 and ΔΔHis35 differed only by the presence of an extra 4.0 Mb of DNA (Fig. 2D). STS analysis (Table 1) revealed that this stretch of DNA included sequences from interval 5 of the long arm of the human Y chromosome. We wished to move either ΔΔHis35 or ΔΔHis1 into DT40 cells. ΔΔHis35 was present in CHO cells at an average copy number of ~4 as indicated by the greater hybridization intensity shown by alphoid cognate fragments from the ΔΔHis35 samples compared with the ΔΔHis1 samples in Figure 2A and B. In light of this evidence for instability we used microcell transfer to move ΔΔHis1 into DT40. This led to two histidinol resistant clones of which one DTHis1 contained a mini-chromosome the same size as ΔΔHis1. We mapped this mini-chromosome (data not shown) and showed that, with the exception of one methylation-sensitive SsrI site, the maps of ΔΔHis1 and the mini-chromosomes in DTHis1 were identical. The structural similarity of the mini-chromosome in the CHO and chicken DT40 cells suggested that we had moved ΔΔHis1 intact from CHO cells to DT40 cells and that it had not incorporated chicken DNA. ΔΔHis1 was stably retained in DT40 cells at an average of one copy per cell for 100 generations in the absence of applied selection indicating that it contained a functional centromere (Fig. 2E). Two signals of alphoid DNA hybridization were seen on the fluorescence in situ hybridization (FISH) analysis of ΔΔHis1; these were very close together in the spread and arose presumably because the mini-chromosome contains two large arrays of alphoid DNA.

**ΔΔHis1 is a stable functionally dicentric chromosome**

The central question posed by the structure of ΔΔHis1 was which sequences were functioning as a centromere. There seemed to be two simple alternatives. The chromosome may be a stable dicentric with centromeric activity situated on the two alphoid arrays. Alternatively the centromere may be located between the alphoid DNA, possibly on the DYZ1, 2 array. In order to resolve between these possibilities we obtained a cosmid (a kind gift of Pauline Yen, University of California) containing the STS sequences sY142 and sY143 (19) which we had mapped (Table 1) to between the two blocks of alphoid DNA and used this to construct a pair of targeted breakage

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**Table 1. Size and sequence content of mini-chromosomes Δ128, ΔΔHis35, ΔΔHis1 and its products 49B(B)G7, 49B(A)A9**

<table>
<thead>
<tr>
<th>Mini-chromosome</th>
<th>Locus</th>
<th>Size (Mb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ128</td>
<td>neo</td>
<td>1.0</td>
</tr>
<tr>
<td>ΔΔHis1</td>
<td>α11kb</td>
<td>1.4</td>
</tr>
<tr>
<td>ΔΔHis35</td>
<td>α90kb</td>
<td>1.3</td>
</tr>
<tr>
<td>49B(A)A9</td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>49B(B)G7</td>
<td></td>
<td>4.7</td>
</tr>
</tbody>
</table>

The respective sequences were analysed by PCR for the STS sequences and markers or by PFGE for the alphoid DNA and DYZ1 and DYZ2 sequences. +, sequence is present; –, sequence is absent; T, array is truncated.
Table 2. Co-localization of alphoid DNA and CENP-C in interphase nuclei containing mini-chromosomes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Mini-chromosome</th>
<th>49B(B)G7</th>
<th>49B(A)A9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔΔHis1</td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>13</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>Aggregate</td>
<td>13</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>3, 4</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Aggregate</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Interphase nuclei containing the indicated mini-chromosome were analysed immunocytochemically for CENP-C and then alphoid DNA was located by FISH. The columns show the cells containing the indicated number of alphoid signals which co-localize with CENP-C.

constructs in which an arbitrary 8 kb EcoRI targeting fragment (termed fragment 49B) was orientated in opposite orientations with respect to the telomere in plasmids termed pBS–PurTel49B(A) and pBS–PurTel49B(B) (Fig. 1). The orientation of the cosmid with respect to ΔΔHis1 was unknown and so we screened by PCR for clones which had been stably transfected with the 49B targeting constructs and had lost either the left or right ends of the mini-chromosome. The markers that we used for the left and right ends of the chromosome were a URA3 gene from Saccharomyces cerevisiae, which immediately flanks the telomere of the left end of the chromosome (14), and the histidinol resistance gene in the pBS–CCAGHisDTel breakage construct. We screened 288 ΔΔHis1 clones stably transfected with the telomere-directed chromosome breakage construct, pBS–PurTel 49B(A), and isolated two clones that were URA+His+. We sized these by PFGE and showed that they were ∼1.8 Mb in size. Screening of 301 ΔΔHis1 clones that had been stably transfected with pBS–PurTel49B(B) identified three that were URA+His+ and that they were ∼4.6 Mb in size. We studied one of each type of chromosome: PurTel49B(A)–A9 and PurTel49B(B)–G7. Restriction analysis, filter hybridization (Fig. 4) and STS analysis (Table 1) of the two types of chromosome indicated that they had reciprocal structures. The bulk of PurTel49B(B)–A9 was comprised of the 90 kb array of alphoid DNA and the 1.4 Mb array of DYZ1 and DYZ2 sequences, whereas PurTel49B(B)–G7 included the 100 kb array of alphoid DNA and the euchromatic sequences from the long arm of the chromosome. We cultured cells containing the 49B(A)–A9 mini-chromosome and or the 49B(B)–G7 mini-chromosome for 34 days in the absence of selection. Gel electrophoresis (Fig. 5A) and cytogenetic observation (Fig. 5B) showed that the mini-chromosomes were retained intact. We thus conclude that both chromosomes have a functional centromere.

Two interpretations of these observations were possible: either the separate arrays of alphoid DNA were functioning as independent centromeres or the starting chromosomes was monocentric but the functional centromere extended across sequences homologous to the targeting construct. In order to address this question we used a combination of immunocytochemistry for the essential centromere protein CENP-C with FISH for alphoid DNA and asked whether the centromere as judged by CENP-C staining co-localized with alphoid DNA (Fig. 6). In ΔΔHis1 we could clearly resolve two sites of alphoid DNA in 71% of the nuclei (Table 2). This observation was consistent with the fact that 80% of the cells contain a single copy of the mini-chromosome ΔΔHis1 and that two loci separated by as little as 100 kb can be resolved from one another (20). Although it is possible that two loci of alphoid hybridization might correspond to single array of alphoid DNA present in a G2 nucleus, the fact that we see few such figures in the cells with the mini-chromosomes 49B(A)–A9 and 49B(B)–G7 suggest that the contribution of such cells to the study is small and that two loci of alphoid hybridization do correspond to two distinct arrays of alphoid DNA. In 40% of the ΔΔHis1-containing cells with two resolvable arrays of alphoid DNA (30/74) both loci of alphoid DNA were associated with CENP-C. In 36% (28/74) only one of the two loci were associated with alphoid DNA. In the rest of the sample the two alphoid loci were either not resolved from one another (13%) or appeared as more than two loci, possibly because of replication. In 18% of nuclei where two loci of alphoid DNA could be detected the CENP-C was aggregated and it was not possible to be confident whether only one or both of the arrays were associated with CENP-C. In 75% of the nuclei containing the 49B(A)–A9 mini-chromosome and in 61% of the nuclei containing the 49B(B)–G7 mini-chromosome the alphoid DNA was associated with CENP-C. These results suggest that the alphoid DNA is the functional centromeric DNA on the 49B(A)–A9 and 49B(B)–G7 mini-chromosomes and that in about half of the cells containing the ΔΔHis1 minichromosome both arrays of alphoid DNA are functional and that the mini-chromosome is functionally dicentric in these cells. Furthermore, the fact that a pair of mini-chromosomes as structurally different as 49B(A)–A9 and 49B(B)–G7 both contain functional centromeres is evidence for the minor role played by non-alphoid DNA in vertebrate centromere function.
Table 3. Targeted breakage of mini-chromosomes in DT40 cells

<table>
<thead>
<tr>
<th>Targeting construct</th>
<th>Target chromosome</th>
<th>Size of broken chromosome (Mb)</th>
<th>No. of colonies screened by PCR</th>
<th>Targeted breakagea</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBSβsdTel1mer(A)</td>
<td>∆His1</td>
<td>2.5</td>
<td>363</td>
<td>6</td>
</tr>
<tr>
<td>pBSβsdTel2mer(A)</td>
<td>49B(A)A9</td>
<td>1.7</td>
<td>586</td>
<td>1</td>
</tr>
<tr>
<td>pBSβsdTel2mer(B)</td>
<td>49B(B)G7</td>
<td>3.9b</td>
<td>720</td>
<td>0</td>
</tr>
<tr>
<td>pBSβsdTel2mer(A)</td>
<td>49B(B)G7</td>
<td>0.7a</td>
<td>507</td>
<td>0</td>
</tr>
<tr>
<td>pBSpurTel49B(A)</td>
<td>∆His1</td>
<td>1.8</td>
<td>288</td>
<td>2</td>
</tr>
<tr>
<td>pBSpurTel49B(B)</td>
<td>∆His1</td>
<td>4.6</td>
<td>301</td>
<td>3</td>
</tr>
</tbody>
</table>

DT40 cells containing the indicated mini-chromosome were targeted with the indicated construct.

aNumber of clones containing mini-chromosomes generated by targeted breakage.

bSize of the anticipated mini-chromosome.

Recovery of mini-chromosomes with <90 kb of alphoid DNA is very inefficient

The small size of the 49B(A)–A9 and 49B(B)–G7 mini-chromosomes made them favorable as substrates for further rounds of telomere-directed chromosome breakage. In particular we wanted to know whether we could make further deletions of the alphoid DNA in these mini-chromosomes and, if we could, whether there was any evidence that this was accompanied by a reduction in centromere activity or in movement of the centromere. We therefore constructed a set of telomere-directed targeted breakage constructs containing either one or two units of alphoid DNA in each of the two possible orientations (Fig. 1). We transfected the appropriately linearized construct into cells containing either ∆His1 or the mini-chromosomes 49B(A)–A9 or 49B(B)–G7. Although we obtained efficient targeted breakage of the alphoid DNA in ∆His1, targeted breakage was very inefficient in 49B(A)–A9 and undetectable in 49B(B)–G7 (Table 3). We describe the successful breakage of 49B(A)–A9 in more detail because this targeting event supported the general conclusion that we were at or close to the limit of what could be achieved by telomere-directed chromosome breakage. The targeting construct, pBS-βSsdTel α2mer(A), contained two alphoid units orientated head to tail in an orientation that we arbitrarily call ‘A’. We screened 586 stably transfected clones for retention of the mini-chromosome by PCR using the STS sY160 and for loss of the URA marker at the left end of the mini-chromosome. One clone, A9-2D6 (Fig. 2), was isolated which had lost the sequences at the left end of the mini-chromosome but had an alphoid array that was similar in size to that on the starting array in 49B(A)–A9 (Fig. 2C). Detailed analysis of the presumed event was difficult because probes for the blasticidin resistance gene hybridized to chicken DNA. Nevertheless we did show that the chromosome had lost all sequences telomeric of the 90 kb alphoid array in 49B(A)–A9 and was accurately retained after 34 days in culture in the absence of selection and thus that it contained a centromere (Fig. 5B).

The conclusion from these experiments was that targeted breakage could not be used to reduce the size of the alphoid DNA to much below 100 kb in length. This suggested that the centromere was relatively fixed on the alphoid DNA. We were interested in identifying chromosomes where sequences other than the centromere had acquired centromeric activity. One obvious idea is that centromeres jump in response to DNA

Figure 3. Reducing the size of an alphoid DNA array by sequence targeting and breakage induced repair. (A) A map of mini-chromosome 49B(A)A9 indicating the region shown in more detail in (B). (B) Restriction site map of the left end of mini-chromosome 49B(A)A9 and breakage-induced repair triggered by sequence targeting. DNA flanking the 11 and 90 kb arrays was cloned in cosmids and phage by screening libraries of 49B(A)A9 DNA with alphoid DNA. Only one end of the 90 kb array was recovered and it was unclear as to whether this was the right or left ends with respect to the orientation of the map in (A). However, the alphoid targeting experiment which generated mini-chromosome A9-2D6 suggested the orientation shown here and this was confirmed in the experiment indicated below. The diagram also indicates the sequence of breakage and homologous recombination reactions giving rise to truncation of the alphoid DNA. We designed a targeting construct pBS–CCAGHisDTel5 using a segment of DNA flanking the left hand of the 90 kb array of alphoid DNA. Targeted breakage with this construct should generate an acentric HisD-tagged fragment and a centric fragment with a broken end. Chromosomes lacking a telomere are unstable but the presence of shared alphoid sequences between the two fragments orientated in opposite directions allowed repair of the broken end and recovery of the histidinol selectable marker on a centromere containing fragment. If degradation of the broken end occurred between telomere-directed chromosome breakage and homology-driven repair then the products should include truncated alphoid DNA arrays. (C) The products of the breakage and homologous recombination reactions have incorporated the HisD gene. DNA extracted from cells containing the indicated mini-chromosome was size fractionated by PFGE and probed with a probe recognizing the histidinol-resistance gene. (D) DNA flanking the 11 kb array now lies adjacent to a truncated 90 kb alphoid DNA array in the products of the breakage and homologous recombination reactions. DNA extracted from cells containing the indicated mini-chromosome was digested with ScaI, size fractionated by PFGE and probed with a probe recognizing sequences immediately adjacent to the 11 kb array (J13) probe or an alphoid probe. (E) The products of the breakage and homologous recombination reactions have lost the left end of the mini-chromosome. DNA extracted from cells containing the indicated mini-chromosome was analysed by PCR for the indicated sequence marking either the left (URA) ends of the molecule or the marker used in targeting (His). (F) The products of the breakage and homologous recombination reactions are structurally stable on culture in the presence and absence of selection. DNA extracted from cells containing the indicated mini-chromosome grown in the presence and absence of selection for 34 days was size fractionated by PFGE and filter hybridized with an alphoid DNA probe. (G) The products of the breakage and homologous recombination reactions are numerically stable on culture in the presence and absence of selection.
damage and so we designed an experimental strategy which placed a double strand break within the centromeric DNA and then asked how easy was it now to derive a mini-chromosome with much less than 90 kb of alphoid DNA. In order to apply this strategy we cloned the DNA flanking the neo marker at the left end of the mini-chromosome A9 and flanking the 90 kb array of alphoid DNA in cosmids and phage (Fig. 3B). Mapping this DNA showed that the 11 kb BglII fragment containing alphoid DNA immediately flanked the neo marker. Although we could not fill the gap between the 11 and 90 kb alphoid arrays the results of the pBS–BSdTel α2mer(A) targeting experiment, described above, suggested that the alphoid DNA in the 90 kb array and in the 11 kb fragment had opposite orientations (Fig. 3B). We therefore built a targeted breakage construct pBS–CCAGHisDTel5 containing a 7 kb sequence flanking the 90 kb array. If the orientation of the array was as suggested by the pBS–BSdTel α2mer(A) targeting experiment then targeting and telomere-directed chromosome breakage with this construct should generate a small acentric fragment from the left end of the 49B(A)–A9 mini-chromosome and a centric fragment with a broken end from the rest of the mini-chromosome (Fig. 3B). Sequence homology should allow the 11 kb array of alphoid DNA on the small acentric fragment to recombine with the broken 90 kb array and thereby generate a His‘Neo’ fragment. If degradation of the broken end of the 90 kb alphoid DNA array occurred prior to the homology-driven repair event then products of the repair should include some with truncated arrays. If the double strand break had triggered centromere movement then it should be possible to isolate a range of mini-chromosomes with alphoid DNA arrays of varying size including some much less than 90 kb in size. We transfected the 49B(A)–A9 cells with pBS–CCAGHisDTel5 and screened for Y160*neo* stably transfected clones. We isolated 23 such clones of which 5 contained a mini-chromosomes that included the His marker and in which sequences that had previously flanked the 11 kb block of alphoid DNA now lay adjacent to a truncated 90 kb array as judged by hybridization with a probe, λ13, that lay within the penultimate SstI fragment of the 49B(A)–A9 mini-chromosome. The size of the alphoid DNA in five of the clones with the inverse rearrangement was approximately the same as in the starting mini-chromosome but one had an array that was reduced to 65 kb in size. We studied the structure (Fig. 3C and D) and stability (Fig. 3F and G) of two clones in detail: Nt-2, in which the array was reduced in size to ∼65 kb, and Nt-5, in which the alphoid DNA was the same size as the starting chromosome. Both mini-chromosomes were stably retained in the absence of selection indicating that they both had functional centromeres (Fig. 3F and G). We interpret these results to indicate that both chromosomes had functional centromeres and that our failure to recover any mini-chromosomes with <65 kb of alphoid DNA suggests that centromeres form very infrequently at sites other than alphoid DNA.

**DISCUSSION**

Here we have used telomere-directed chromosome breakage to define the minimal size and sequence requirements consistent with vertebrate chromosome function. Our major conclusion is that we cannot readily generate a mini-chromosome with <65 kb of Y-chromosome-derived centromeric alphoid DNA. It is now clear that chromosomes which lack alphoid DNA can be completely stable in human cells (3) and thus our failure to generate a chromosome with <65 kb of Y chromosome alphoid DNA is unlikely to reflect a specific requirement for this sequence. Rather it may reflect a requirement for any centromeric sequence and the low frequency with which centromeres can be induced to move. Thus, we would expect to find other sequences capable of functioning as centromeres in DT40 cells but, given our experience with alphoid DNA in mini-chromosomes 49B(A)–A9 or 49B(B)–G7, we would expect that these sequences would extend over at least 100 kb and that they would be hard to remove. Can we use our data to estimate the frequency with which centromeres locate on non-alphoid DNA in DT-40 cells? Two comparisons allow a rough estimate of an upper limit. The frequency of targeted breakage of a construct containing a single alphoid unit to the 100 kb array on ΔΔHis1 is ~2% (Table 3). Targeted breakage constructs with two alphoid units target but fail to truncate the 90 kb alphoid array in mini-chromosome 49B(A)–A9 and fail to target and truncate the 100 kb array in mini-chromosome 49B(B)–G7. If the alphoid DNA in both chromosomes was as easy to target as the 100 kb array in ΔΔHis1 then we would expect ~20 targeting and truncation events. The fact that we see none suggests that in <10% of cells the alphoid DNA is not functioning as a centromere. This estimate makes no allowance for the fact that the 100 kb alphoid array in ΔΔHis1 appears to function as a centromere in at least 30% of cells as judged by CENP-C staining. Our conclusion as to the minimal length of functional centromeric DNA strikingly complement those of Lo et al. (21) who analysed a large set of human karyotypes in order to define the minimal length of alphoid DNA compatible with accurate segregation and centromere function. They observed that the minimal length of alphoid DNA present on any human chromosome was to be found on human chromosome 21, where the shortest α� array was estimated to be between 51 and 184 kb. The mean minimal estimate was 78 kb which is consistent with what we have observed.

One question that motivated this study was the location of the centromere on mini-chromosome ΔΔHis1. ΔΔHis1 was derived from a mini-chromosome called A128 that arose following the second round of telomere-directed chromosome breakage of a long arm acrocentric chromosome generated by targeting a telomere to the centromeric array of alphoid DNA (14). We wondered whether the telomere-directed chromosome breakage at the centromere of the starting Y chromosome had damaged the centromere, had caused the centromere to jump onto sequences on the long arm heterochromatin and this in turn had led to the appearance of rearranged mini-chromosomes obtained in the second round of telomere-directed chromosome breakage. Although this idea was speculative it was consistent with the suggestions of others about the possible mechanism of formation of neo-centromeres (12). The work described here, however, disproves the idea that the centromere jumped onto the long arm heterochromatin. However, we have confirmed the demonstration that functionally dicentric chromosomes can be stable in vertebrate cells (22). One important point on which our work may cast light is the mechanism by which functionally dicentric chromosomes are stabilized. Previously we showed that when a mini-chromosome with the centromere described here was moved from hamster cells into mouse cells it was unstable and broke with...
the characteristics of a dicentric chromosome (23). It is now clear why this might be so. The fact that stability of a dicentric chromosome is cell-type dependent suggests that stability reflects the presence of a factor present in either the host or donor cells which actively coordinates the activity of the dispersed centromeric DNA. Coordination of function over relatively long stretches of DNA is poorly understood mechanistically and it will be of interest to develop this area of study further.

The demonstration of a minimal size of alphoid DNA consistent with vertebrate centromere function could not, despite the limitations expressed above, readily have been arrived at in any way other than by the chromosome engineering techniques described here. Other work from our group has suggested the existence of a cell-type-specific centromere assembly activity (M.H. Shen et al., submitted). The demonstration of cell-type-specific centromere function may be important if it offers a basis for identifying genes involved in centromere function and assembly. An understanding of this pathway is necessary if we are to understand how centromeres form at different sequences and determine whether, as one might expect, this pathway is subject to attack in genetic disease. The extensive set of mini-chromosomes with different sequence compositions described here offer a set of reagents with which to try to isolate these genes.

**MATERIALS AND METHODS**

**Plasmids and molecular biology**

Plasmids were constructed from standard components using conventional recombinant DNA techniques. The cosmid 5F49 used to isolate the 49B targeting constructs was a kind gift of Pauline Yen. It was constructed in the cosmid vector pWE15 from DNA extracted from the Y chromosome YAC yOX5 (24). The telomeric DNA used in the breakage constructs was...
as described before (15). Cloning of the DNA around the 11 and 90 kb arrays of alphoid DNA was done primarily by constructing a cosmid library from a mini-chromosome 49B(A)–A9 and screening for cosmids containing alphoid DNA. Two groups of junction fragment were isolated. Subcloning and filter hybridization established that one group derived from the edge of the 11 kb alphoid array and included the neo gene adjacent to one telomere. A second group derived from one of the two edges of the 90 kb array. The results of the pBS–BsdTel α2mer(A) targeting experiment indicated that the edge that we had cloned was the edge nearest the neo-marked telomere or the left edge in the map in Figure 1.

Cell culture, transfection and microcell fusion

The chicken B cell line DT-40 (7) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 1% chicken serum, 2 mM L-glutamine, 10 μM β-mercaptoethanol, penicillin and streptomycin. Mouse LA9 and human fibroblast HT1080 were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics. For electroporation DT-40 hybrids with human mini-chromosomes were grown to mid-log phase (3–6 × 10^5/ml), centrifuged and washed twice with phosphate-buffered saline (PBS) at room temperature. The cells were suspended in 0.8 ml of PBS and mixed with 10 μl of 2 μg/μl linear targeting construct. Cells were electroporated using a BioRad Gene Pulser II (BioRad, Hercules, CA) with a voltage of 560 V and a capacitance of 25 μF. The cells were allowed to recover for 5 min on ice and then re-suspended in 150–300 ml of medium, distributed into 15–30 96-well plates and allowed to recover overnight under standard culture conditions. Selective antibiotics were added the next day and colonies picked 8–10 days later. Microcell fusion was carried out according to the method of Sanford et al. (25). Log phase donor cells were cultured with colcemid (0.5 μg/ml) overnight. Cells were collected the next day and re-suspended in 40 ml of Percoll/DMEM (1:1) at 37°C supplemented with 20 μg/ml Cytochalasin B. The cell suspension (~0.7–1.5 × 10^6 in 40 ml) was then centrifuged 34°C in a 30 ml Nalgene tube at 28 000 g for 75 min in a Beckman JA-20 fixed-angle rotor maintained at 34°C. Two bands formed after centrifugation. The bands was collected and washed with DMEM three times. The recipient cells were also grown to log phase (see above), harvested by standard procedures and washed three times in DMEM. After washing, recipient and microcells were mixed in 10 ml of DMEM, centrifuged and re-suspended in 1 ml of DMEM. The suspension was kept at 37°C for 7 min and re-centrifuged. Fusion was carried out by adding 1 ml of PEG 1500 (Roche, Lewes, UK) to the pellet. After 90 s of incubation, 10 ml of DMEM was added drop-wise. The cell suspension was then incubated at room temperature for 30 min. The cells were then washed twice in DMEM, re-suspended in serum containing DMEM and cultured overnight. Selection was applied the next day.

Screening of targeted clones by PCR and PFGE

Transfected clones in which the construct had targeted and broken the mini-chromosome were identified by PCR. Cells from 150 μl of cell culture were centrifuged in a round-bottom microtitre plate, the supernatant removed and the pellet lysed, in 5 μl of 2× lysis buffer [1× lysis buffer is: 32 mM (NH_4)_2SO_4, 134 mM Tris–HCl pH 8.3, 0.9% Tween-20, 0.9% NP-40] containing 100 μg/ml pronase (Roche), for 60 min at 55°C. A further 20 μl of 1× lysis buffer were added into each well and mixed. Lysate (5 μl) was used as template in each reaction which was typically for 35 cycles. PFGE was as described in previous publications (13). The resolution of different size chromosomes or fragments was according to the following conditions: for mini-chromosomes of 3.0–6.0 Mb, 28 V, 120 min pulse time, 3.4°C, 0.6% agarose run for 8 days; for mini-chromosomes of 1.0–3.0 Mb, 65 V, 8 min pulse, 3.4°C, 0.75% agarose, run for 87 h; for fragments of 0.3–1.0 Mb, 150 V, 60 s pulse, 17.5°C, 1.5% agarose, run for 19 h effective run time; for fragments of 0.02–0.3 Mb, 170 V, 4 s pulse, 17.5°C, 1.5% agarose run for 20 h effective run time. Filter transfer was to Genescreen (NE Life Sciences, Boston, MA). Hybridization was as described previously (13).

FISH analysis

FISH was carried out according to standard methods. Cells were cultured as above, cultured to mid-log phase and...
colcemid added to 0.1 μg/ml. Cells were cultured for a further 2–3 h and then harvested, swollen in hypotonic solution (40 mM KCl, 0.5 mM Na₂EDTA, 20 mM HEPES, pH 7.4) for 10 min at 37°C, pelleted and fixed in methanol/acetic acid at –20°C. The nuclei were dropped onto microscope slides, dehydrated in ethanol, and denatured in 70% formamide, 2× SSC for 5 min at 70°C. Probes for hybridization were nick-translated with biotin-16-dUTP (Roche) and hybridized in 50% formamide, 10% dextran sulphate, 2× SSC, 40 mM sodium phosphate pH 7.0, 1× Denhardt’s solution, 0.5 mM Na₂EDTA, 120 µg/ml sonicated salmon sperm DNA at 42°C overnight. Biotin-labelled probe was detected with Cy3-conjugated avidin (Amersham Pharmacia Biotech, Little Chalfont, UK) and the signal was amplified with biotin-conjugated goat anti-avidin (Vector Laboratories, Peterborough, UK) and a second round of Cy3-conjugated avidin. Chromosomes and nuclei were counterstained with DAPI at 0.5 µg/ml.

Simultaneous immunofluorescence and FISH analysis

The production of rabbit anti-chicken CENP-C antibody was described previously (25). The simultaneous mapping of mini-chromosome with anti-CENP-C and human α-DNA was based on the method described of Page et al. (26) with slight modifications. Freshly grown DT40 cells containing a mini-chromosome were cultured in colcemid (0.1 µg/ml) for 2–3 h. Cells were fixed in methanol/acetic acid (3:1) after hypotonic treatment and nuclei spread as described above. The slide was rinsed with PBS and incubated in potassium chromosome medium (KCM: 120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, 0.5 mM Na₂EDTA, 0.1% v/v Triton X-100) for 15–30 min at room temperature. Immunofluorescence was performed with rabbit anti-chicken CENP-C antibody, diluted 1:500 with 1× TEEN [1 mM triethanolamine–HCl pH 8.5, 0.2 mM Na₂EDTA, 25 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin (BSA)], for 30 min at 37°C. The unbound

Figure 6. Alphoid DNA and CENP-C co-localize in interphase nuclei. Cells containing the indicated mini-chromosome were immunocytochemically stained for CENP-C and then alphoid DNA was localized using FISH. The arrows indicate loci of CENP-C which co-localize with alphoid DNA. Scale bar, 10 μm.
antibody was washed away with 1× KB (10 mM Tris–HCl pH 7.7, 0.15 M NaCl, 0.1% BSA), three times for 4 min each. The bound antibody was then detected with Cy3-conjugated mouse anti-rabbit IgG (Amersham Pharmacia Biotech), diluted 1:200 in 1× KB, by incubation for 30 min at 37°C. The slide was washed once with 1× KB, fixed with 4% formaldehyde for 10 min at room temperature, and washed again twice in PBS. Finally, the slide was fixed with methanol/acetic acid (3:1) for 15 min at room temperature and air dried with protection from the light. FISH was then carried out as described in the preceding section except that the biotin-labelled DNA was detected using FITC-conjugated avidin.

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