Mammalian artificial chromosome formation from circular alphoid input DNA does not require telomere repeats

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Received 2 March 2000; Revised and Accepted 2 May 2000

Mammalian artificial chromosomes (MACs) form in HT1080 cells after transfecting linear yeast artificial chromosome constructs minimally containing competent alphoid arrays, a selectable marker and terminal human telomere repeats. Restrictions on the structure of input DNA in MAC formation were investigated by transfecting circular or linear alphoid constructs with or without human telomere arrays and by varying the position and orientation of the telomere arrays on input linear constructs. Circular input DNA efficiently produced MACs. Absence of telomere arrays from circular input molecules did not significantly alter MAC formation rates. Linear constructs capped with telomere arrays generated MACs effectively, but a severe reduction in MAC formation was observed from linear constructs lacking telomere arrays. Human telomere arrays positioned 1–5 kb from linear construct ends and in either orientation were able to promote MAC formation with similar efficiencies. Both circular and linear input constructs generated artificial chromosomes that efficiently segregated in the absence of selection. Telomeres were not detected on the MACs, regardless of the inclusion of telomere arrays on input DNA, suggesting that circular chromosomes were formed. We found no evidence for acquisition of host cell DNA, which is consistent with de novo chromosome assembly.

INTRODUCTION

Chromosomes in eukaryotes have evolved as vehicles for nuclear genes and have developed specialized nucleoprotein structures for this purpose. Centromeres, telomeres and origins of replication are the best studied of these structures and in yeast are sufficient for artificial chromosome formation (1–4).

In human chromosomes the centromere is defined cytologically as the primary constriction observed in metaphase spreads and is the site of kinetochore formation and of spindle attachment. Alphoid arrays are found at all human centromeres (5,6) and consist of a 171 bp monomer organized in higher order repeats encompassing 0.5–5 Mb. They have long been considered the best candidate for the specific DNA requirement for centromere function, however, in rare instances neocentromeres form on rearranged human chromosomes in regions lacking alphoid repeats (7,8), which suggests an epigenetic component to kinetochore assembly.

De novo mammalian artificial chromosome (MAC) formation by transfection of constructs containing alphoid DNA with human telomere repeats into HT1080 cells supports the view that alphoid DNA alone is sufficient for centromere function in human cells. A transfection containing an unlinked mixture of several hundred thousand base pairs of alphoid arrays multimerized from a 2.7 kb higher order repeat from chromosome 17, human telomere sequence (T<sub>2</sub>AG<sub>3</sub>)<sub>α</sub> amplified by PCR and total human genomic DNA produced a cell line with a MAC in 100% of cells (9).

MAC formation was observed following transfection of a 100 kb yeast artificial chromosome (YAC) containing alphoid sequence derived from chromosome 21 (array α<sub>21-I</sub>) (10,11) with uniform higher order repeats and frequent CENP-B boxes, a conserved motif binding the CENP-B protein (12–14). The YAC had been retrofitted with terminal human telomere sequence, but no other human DNA was included. This YAC construct generated cell lines containing MACs at frequencies ranging from 10 to 100% of cells. A second YAC construct containing diverged alphoid DNA was separately transfected but was not able to form MACs, indicating that some alphoid repeats are poor centromere templates.

An ~1 Mb partly characterized YAC has also been used to generate MACs (15). This YAC contained alphoid sequence but also non-alphoid DNA apparently derived from several chromosomes. The YAC was retrofitted with terminal human telomere sequences, but no other human DNA was included. This YAC construct generated cell lines containing MACs at frequencies ranging from 10 to 100% of cells. A second YAC construct containing diverged alphoid DNA was separately transfected but was not able to form MACs, indicating that some alphoid repeats are poor centromere templates.

An ~1 Mb partly characterized YAC has also been used to generate MACs (15). This YAC contained alphoid sequence but also non-alphoid DNA apparently derived from several chromosomes. The YAC was retrofitted with terminal human telomere arrays and a candidate human replication origin from the β-globin locus. PCR pre-screening enriched for clones containing both ends of the YAC and 25% of these contained a MAC in all cells.

Although the input constructs differed in these experiments, the MACs generated were in the same size range, varying between 1 and 10 Mb, and though tiny in comparison with native chromosomes, were substantially larger than the input DNA. Efficient segregation of the MACs in some of the cell

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lines was demonstrated by long-term culture in the absence of selection. These results give some insight into the MAC formation process and are a useful reference point for future MAC vector design. Cloned alphoid arrays of ~100–200 kb originating from the centromeres of different chromosomes were effective as templates for centromere formation, with the exception noted above. Only alphoid and telomere arrays needed to be present on input linear DNA for MAC formation in HT1080 cells. Inclusion of non-alphoid genomic DNA or replication origins was not essential, presumably because replication was initiated adequately from alphoid or vector sequence. The input DNA was subject to amplification, which may have been necessary for MAC formation under these conditions, and resulted in concatemers, minimally of 1–2 Mb. Linear MACs formed which seeded functional telomeres and migrated into pulsed field gels (PFGs) (9). However, a second type, generated from YAC constructs, were not detected on PFGs. The reasons for this difference are not clear and may have to do with variable size of the MACs from a given clone, the upper limit of resolution of PFGs or, perhaps, the formation of circular MACs which failed to seed telomeres and as megabase size circles would not migrate into a PFG.

Input DNA has been linear in all reported experiments. Capping the ends of linear input DNA by human telomere repeats (T2AG3) is thought to promote MAC formation by inhibiting DNA end processing activity and by seeding extending telomeres at the ends of a newly formed linear chromosome. YAC constructs which are retrofitted with human telomere arrays remained capped with several hundred base pairs of yeast telomere repeats (TG1–3). Though yeast telomeres alone are not expected to be proficient at seeding human repeats in human cell lines, addition of human repeats from human telomere arrays with subterminal positions on plasmids or YACs integrated at the ends of fragmented chromosomes has been shown (16,17). With current methods MACs are formed at variable efficiency. Mitotically stable MACs can be produced, but others show a significant loss rate. While many factors may have an influence on the efficiency of mitotically stable MAC formation, here we considered some variable configurations of input DNA in P1 artificial chromosome (PAC) constructs based around a previously characterized alphoid fragment from the α21-I locus (10). We compared MAC formation rates among alphoid DNA input as circular constructs either with or without human telomere arrays and input as linear constructs either with two terminal, subterminal or inversely oriented human telomere arrays, with a single array present or with no arrays. Our results show that telomere arrays are essential to obtain efficient MAC formation from input linear constructs but that they are not required for MAC formation in HT1080 cells when the input DNA is circular. Telomeres were not detected on any examined MACs, suggesting that both circular and linear input constructs may generate circular artificial chromosomes.

RESULTS

PAC constructs based on the α21-I repeat fragment 7c5

YAC construct α7c5hTEL, containing an ~70 kb insert derived from the α21-I alphoid array and retrofitted human telomere arrays, has been shown previously to be competent at de novo MAC formation in HT1080 cells (10,12). The 70 kb alphoid fragment from α7c5hTEL was transferred to a PAC vector giving high yields of DNA which would be amenable to in vitro manipulation. After transfer to the PAC vector, microgram quantities of DNA with the 7c5 alphoid insert were purified for visualization on a gel. Following EcoRI digestion, fragments predicted for vector and α21-I repeats (11) were observed on a gel after ethidium bromide staining, indicating that the 7c5 alphoid fragment is composed mainly of the 1.9 kb, 11mer higher order repeat (data not shown).

Several PAC constructs were built with the 7c5 alphoid fragment (Fig. 1) and were individually lipofected into HT1080 cells. In addition to pPAC_7c5 (construct I, Fig. 1), which does not have human telomere arrays, additional circular PAC constructs were created having either two 800 bp human telomere arrays flanking the alphoid insert in a configuration reflecting normal chromosomes (pTAT_7c5, III) or with an inverted configuration (pT-inv, V). These three circular constructs were enzymically cut to yield linear forms either capped by two telomere arrays (IVa), with two internal arrays (IVb), two internal and inverted arrays (VIa), two internal arrays flanking the alphoid to one side (IVc), a single internal inverted array (VIb) or with no telomere arrays present (II).

Figure 1. Circular PAC constructs and linear derivatives (not to scale). Thick line, 70 kb of α21-I alphoid repeats; thin line, vector; arrowheads, telomere arrays (800 bp). BS, blasticidin S-methylase selectable marker.
Circular input DNA and artificial chromosome formation

In previous reports of MAC formation input DNA has been linear and included telomere arrays, since a linear artificial chromosome requiring telomeric ends for stability was the expected outcome. To see if MACs would assemble from circular input DNA and if telomere arrays had an influence on the outcome, PAC constructs containing the 7c5 alaphoid fragment either with or without telomere arrays present were separately lipofected into HT1080 cells.

MACs formed efficiently when input DNA was circular and lacking telomere arrays (Fig. 1, construct I). A high frequency MAC (observed in ≥50% of spreads) was seen in 10 of 21 randomly picked clones and three of these had a MAC frequency >90% (Table 1). MAC copy numbers of one or two per nucleus and MAC size range, as judged from cytogenetic observation, appeared similar to that reported for YAC-derived MACs. A fluorescence in situ hybridization (FISH) probe, which only detects α21-I and related arrays on chromosome 13, localized to the MACs, most often with an intensity level equivalent to or greater than the signal from endogenous arrays seen in the same chromosome spread (Fig. 2A, inset). This is consistent with amplification and concatemerization of the transfected DNA. A probe for the PAC vector occasionally hybridized to the MACs but was variable in signal intensity arising from a combination of reasons. For example, FISH detection is not 100% efficient, either because of a low signal or loss of small chromosomes during the spreading process. In addition, the variability in PAC signal intensity due to loss of construct sequences could give rise to plastidicin S-methylase (BS)-resistant lines in which the 2.6 kb BS resistance marker is not detectable.

When telomere arrays were included on the input circular DNA (construct III) efficient MAC formation was also observed. A MAC was found at high frequency in 16 of 25 randomly picked clones. Three of these contained a MAC in >90% of cells. As before, alphoid signal on these MACs was often more intense than signal from the endogenous arrays. Size and copy number of these MACs were in the normal range. The telomere probe was used to determine if newly seeded telomeres had formed from this type of circular input construct. Of three analysed cell lines, tugp7 and t3.3c, examined at early passage, and t14c, examined at 90 days culture, none showed any signal on scored MACs, only on host chromosome ends (tugp7, Fig. 2B and Table 2).

As a control for effectiveness of the telomere probe in detecting artificially seeded telomeres on small minichromosomes, we performed FISH analysis on a cell line containing 2.4 Mb human X-derived minichromosomes in HT1080 cells produced by telomere-associated chromosome fragmentation (19). The minichromosomes on these minichromosomes are in the same size range as endogenous telomeres on HT1080 chromosomes (20) and were clearly detected with the telomere probe (Fig. 2C).

In total, input circular constructs produced 26 cell lines with a high frequency of MAC from 46 randomly picked clones. Sixteen cell lines had a MAC in at least 75% of cells and six had a MAC in >90% of cells, demonstrating the proficiency of the circular PAC constructs. The presence of the telomere arrays seemed to have no significant influence on the rate of MAC formation. Detectable background integration events were very rare and, when observed, were usually PAC vector

<table>
<thead>
<tr>
<th>Construct (L)</th>
<th>High frequency MAC formation</th>
<th>MAC frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (3)</td>
<td>10/21</td>
<td>100, 97, 96, 87, 80, 80, 80, 60, 50, 50</td>
</tr>
<tr>
<td>II (4)</td>
<td>1/45</td>
<td>100</td>
</tr>
<tr>
<td>III (3)</td>
<td>16/25</td>
<td>100, 100, 92, 87, 87, 87, 84, 77, 76, 72, 65, 63, 63, 60, 60, 50</td>
</tr>
<tr>
<td>IV (a)</td>
<td>(3) 6/26</td>
<td>90, 83, 80, 63, 57, 55</td>
</tr>
<tr>
<td></td>
<td>(b) 2/42</td>
<td>96, 93, 92, 85, 84, 83, 83, 80, 77, 76, 73, 68, 64</td>
</tr>
<tr>
<td></td>
<td>(c) 6/10</td>
<td>96, 92, 85, 75, 73, 65</td>
</tr>
<tr>
<td>VI (a)</td>
<td>(1) 4/10</td>
<td>96, 84, 80, 68</td>
</tr>
<tr>
<td></td>
<td>(b) 2/11</td>
<td>100, 50</td>
</tr>
<tr>
<td>pac F15</td>
<td>(1) 0/10</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. MAC formation in HT1080 cells with the different PAC constructs shown in Figure 1 (L), number of lipofectons carried out for each construct, Construct V was not used. High frequency MAC formation is the number of cell lines in which >50% of the cells contained a MAC (visible by FISH) out of the total number of cell lines analysed. MAC frequency (%) indicates the percentage of cells containing a MAC in each of the positive cell lines. A χ² test of the significance of the results gave a value of 22.8 (P = 0.002). Omitting four experiments where numbers were low reduced this χ² value to 14.4 (P = 0.006), still a highly significant value.
fragments. As a negative control for MAC formation from circular constructs with large inserts, a random PAC clone with a 190 kb mouse insert (F15) and a BS selectable marker was transfected as a circle and assayed for MAC formation. In 10 cell lines scored after FISH with a F15 PAC probe, no MAC or MAC-like structures that hybridized to the probe were seen (Table 1). The successful transfer of the F15 PAC could be seen as chromosomal integration in five cases. This shows that alphoid arrays are the significant feature of MAC-forming circular input molecules, not size or circular structure.

MAC formation from linear input DNA with terminal or internal telomere arrays

Since candidate de novo artificial chromosomes formed from 7c5-based circular input constructs without requiring telomere arrays, we also compared linear input constructs with or without terminal telomere arrays for MAC formation. A linear construct with terminal telomere arrays was derived from TAAT7c5 by I-SceI digestion and gel purification (construct IVa) and lipofected into HT1080 cells. In 26 randomly picked clones we obtained six with a MAC present in >50% of spreads. The MACs in cell lines SCE3-8, SCE-6 and SCE3-7 were examined for telomere formation by FISH, but, surprisingly, no evidence for telomere seeding was found (Table 2).

When no telomere array is present, linear input DNA was ineffective at MAC formation. We lipofected gel-purified pPAC_7c5 after linearization with either SmaI+AscI, KpnI, XhoI or AscI, which gave an increasing vector ‘tail’ 3′ of the BS selectable marker of 22 bp or 2.5, 5.5 or 10.6 kb, respectively (Fig. 1, II). All four constructs produced a similar

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### Table 2. Tabulation of MAC validation and mitotic segregation data from characterized cell lines derived from constructs I–III, IVa and IVb

<table>
<thead>
<tr>
<th>Input construct</th>
<th>Cell line</th>
<th>Probes</th>
<th>CENP_E</th>
<th>CENP_C</th>
<th>Tel</th>
<th>Pan alpha</th>
<th>Alu paint</th>
<th>Yα</th>
<th>rDNA</th>
<th>60 days culture off selection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I circle, no tel.</td>
<td>C13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>C16</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>2_3c</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>80</td>
</tr>
<tr>
<td>II linear, no tel.</td>
<td>A3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>III circle with 2 tel.</td>
<td>Tugg7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>13.3C</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>t14c</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>tugp2</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>77</td>
</tr>
<tr>
<td>IVa linear with 2 tel.</td>
<td>SCE3_8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>SCE_6</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>SCE3_7</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>90</td>
</tr>
<tr>
<td>IVb linear, 2 internal tel.</td>
<td>Tx2</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Tx7</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Tx12</td>
<td>+</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>83</td>
</tr>
</tbody>
</table>

+, presence of signal; –, no signal; ND or blank, not determined.

* Extrapolated from day 40 count. The time point of telomere FISH is early passage if not given in parentheses as d (=days). t14c was found in 30% of cells at 90 days.

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**Figure 2.** Alphoid and telomere content of MACs generated from circular alphoid input DNA either without telomeres (construct I, cell line c13) or with telomeres (construct III, cell line tugp7). DAPI stained chromosomes hybridized with an α21-I probe (red) or a PNA telomere probe (green). A single MAC with no telomere signal (arrowhead) is shown in cell line c13 (A) and cell line tugp7 (B). (Insets) The same spreads re-probed with the α21-I repeat. Note the relative host chromosome 21 alphoid staining intensity in the inset to (A). (C) Minichromosomes of ~2.4 Mb (arrowheads), derived from a telomere-directed truncation of a human X chromosome, are clearly stained with the telomere probe. This chromosome has been passaged through chicken cell line DT40 which adds longer arrays of telomeric repeats than are found in human cell lines, resulting in a more intense signal.
number of BS-resistant colonies compared with input circular DNA, suggesting that loss of BS marker function by exonuclease activity was not significant. Of 45 cell lines scored, only one case of a high level MAC was found, in cell line A3, generated from AscI-linearized DNA (Table 2). The remainder had either no MAC or a very low level of putative MAC formation (<10%). We observed a significantly higher rate of detectable integration events in cell line A3 than was found in MAC-containing cell lines produced with circular or linear telomere capped constructs. Thus, while telomere arrays were required for efficient MAC formation from linear constructs, a rare event, perhaps circularization soon after transfection, can occasionally overcome the inhibition imposed on linear telomere-minus molecules.

The human telomere arrays in the I-SceI-linearized pTAT_7c5 input molecule are capped by ~20 bp, mainly derived from the I-SceI site, whereas in YAC constructs they are more internal, since a few hundred base pairs of yeast telomere repeats remain at the YAC ends. Nevertheless, the efficiency of MAC formation from I-SceI-cut pTAT_7c5 was consistently poorer than circular input DNA over several trials and so we investigated MAC formation from a linear PAC construct with more internally positioned telomere arrays by lipofecting XhoI-cut and gel-purified pTAT_7c5 DNA (construct IVb). Digestion with XhoI leaves a 0.75 and a 3.1 kb cap of PAC vector sequence distal to the human telomere arrays of the transfected molecule. These vector DNA caps are 2–10 times larger than yeast telomere arrays present on the ends of YAC constructs. Of 24 randomly picked clones, 14 had a high frequency MAC and three of these had a MAC in >90% of cells. There was no indication of inhibition of MAC formation, although one end had over 3000 bp of non-telomeric sequence before the T2AG3 repeat began.
The PNA telomere FISH probe did not detect telomeres on the MACs in three tested cell lines, Tx2, Tx7 and Tx12 (Table 2). We have therefore been unable to detect telomere formation by FISH analysis on any examined MACs, including those generated from linear constructs with terminally positioned telomere arrays.

Inverted telomere arrays promote MAC formation from linear DNA

We examined other potential constraints on MAC formation by testing whether linear constructs with bilateral inverted and internal human telomere arrays or linear constructs with one end lacking a telomere would be capable of promoting MAC assembly. pTAT_7c5 or pT-inv DNA was cleaved with the relevant restriction enzymes to yield linear constructs with one or two inverted and internal telomere arrays.

Linear construct VIa, in which bilateral arrays were inverted and internal (1.8 and 5.0 kb), was competent at MAC formation. Of 10 cell lines examined, four had a MAC present at frequencies between 68 and 100% of nuclei. There was no significant inhibition of MAC formation with this construct.

Linear constructs in which only one end contained an inverted array were able to form MACs but appeared prone to elevated background integrations. Construct IVc, which had one end containing one inverted array internalized 1.8 kb and a second array internalized 12 kb, formed MACs in six of 10 cell lines at frequencies of 65–100% of nuclei. This formation rate is similar to results using circular input DNA. Pericentric input alphoid and PAC vector integrations were observed in most spreads in six of the 10 cell lines, four of which also had a MAC. Linear construct VIb had a single inverted and internal array 6 kb from one end. When transfected, a modest MAC formation rate was found. Two of 11 cell lines had a MAC present in 50 or 100% of nuclei. Input alphoid and PAC vector integrations were also detected at an elevated frequency from this construct.

MAC validation

A de novo artificial chromosome should assemble a new centromere and form without capturing host DNA. High frequency MACs formed from input constructs I–III and IVa were tested for association with centromere proteins CENP-C and CENP-E, indicative of a functioning centromere (21), and for the presence of extraneous host DNA. Since telomere repeats were not detected on the MACs in the 13 cell lines examined, capture of these as a common event can be ruled out.

Immunocytochemical analysis showed that both CENP-E and CENP-C were present on the MACs in cell lines c13 and tugp7 (Fig. 3A–D and Table 2) which were formed from circular input constructs I and III, respectively. Two additional MACs were tested, generated from constructs II and IVa, and were also found to be associated with both centromere proteins (Table 2). An Alu paint probe did not detect host chromosomal arm fragment acquisition by the MAC in cell line c13 (Fig. 4A–C) produced from circular input DNA (I) nor on the MACs in four other tested cell lines generated from constructs I–III or IVa (Table 2). Capture of non-α21-I repeats was tested by a pan-alphoid FISH probe competed with excess unlabelled α21-I DNA. No evidence of host alphoid capture was observed on the MAC in cell line tugp7 (Fig. 4D–E) derived from circular input DNA (III) nor on the MACs in four other tested cell lines generated from constructs I–III or IVa (Table 2).

Within the limits of sensitivity of our FISH probes, all tested MACs seem to have formed without capturing large fragments of host DNA from either chromosome arms or centromeres and clearly have CENP-E and CENP-C signal and these observations are consistent with the assembly of de novo chromosomes. We have failed to resolve any MAC by PFG. γ-Irradiation can linearize circular chromosomes and permit mobility into PFGs, but this has not yet resulted in detection of a MAC and might be a consequence of MAC size heterogeneity.

Segregation of MACs in long-term culture

Cell lines with high frequency MACs produced from constructs I, III, IVa and IVb were assessed for MAC segregation efficiency during culture off selection for 60 days. MACs maintained at initial levels were produced from each of the input constructs assayed (Fig. 5 and Table 2). The MAC in one of three tested cell lines, formed from circular input DNA lacking telomere arrays (I), was stable and in two of three cell lines in which the input circular construct contained telomere arrays (III) the MACs were maintained for 60 days. One of the stable MACs produced from construct III was tested for an additional 30 days and was maintained without significant loss. The MACs in one of three cell lines generated from linear telomere capped input DNA (IVa) was mitotically stable. When telomere arrays are internalized on a linear input construct (IVb), the resulting MACs can also segregate efficiently since two of three cell lines were found to retain the MAC at initial levels. Differences in mitotic stability may reflect differences in internal organization or size between individual MACs as a consequence of the amplification and concatemerization process.

DISCUSSION

We have shown that MACs form at high frequency in HT1080 cells using both linear and circular input DNA. Altogether 59 randomly picked cell lines with MAC frequencies of at least 50% have been generated and, of these, 15 had MACs present in at least 90% of cells. Circular alphoid constructs were very effective at MAC formation whether or not human telomere arrays were included. Linear alphoid constructs with two
observation to be in the expected size range of 1–10 Mb. The mosomes. These can develop nance of circular DNA in mammalian cells, such as circular is evidence for mechanisms for the replication and mainte-

telomere arrays are perhaps configured as circles. Though we in independent structures. Mitotically stable MACs that lack apotosis (22), and are unlikely to be stable in cell culture as exposed ends would be predicted to stimulate double-strand long-term culture off selection shows that some of them can FISH analysis indicates that the resulting MACs have not mere assembly from telomere seeding in MAC formation. [48]

The generation of MACs from input circular DNA with telomere repeats separates centro-

mere assembly from telomere seeding in MAC formation. FISH analysis indicates that the resulting MACs have not acquired detectable telomere arrays from the host genome and long-term culture off selection shows that some of them can segregate efficiently. Linear MACs with non-telomeric exposed ends would be predicted to stimulate double-strand break repair and allied mechanisms, such as cell cycle arrest or apoptosis (22), and are unlikely to be stable in cell culture as independent structures. Mitotically stable MACs that lack telomere arrays are perhaps configured as circles. Though we provide no direct proof here for a circular MAC structure, there is evidence for mechanisms for the replication and mainte-
nance of circular DNA in mammalian cells, such as circular double minutes, smaller circular episomes and large ring chromosomes. These can develop in vivo and in vitro in human cells and are associated with amplification of genes that confer a selective advantage to cells maintaining them or with disease states (23–25).

In contrast to circular alploid constructs, linear alploid constructs require human telomere arrays for efficient MAC formation. Telomeres function to protect the ends of linear chromosomes from activities of DNA processing proteins, such as double-strand break repair proteins, and to maintain chromosome ends by an end extension activity mediated by telomerase. A possible mechanism for the observed positive effect of the arrays on transfected linear constructs could be telomere array interaction with telomere-binding proteins and assembly of a complex which inhibits DNA end processing activity (26). Terminal positions of the arrays or a specific orientation was not essential, perhaps because some telomere proteins, like TRF1 and TRF2, do not need to be near a terminus to bind TTAGGG repeats effectively (27,28) and these protective proteins might spread to adjacent non-telomeric DNA at construct ends.

We examined by FISH nine cell lines with MACs generated from six linear and three circular telomere repeat-containing constructs for evidence of telomere formation. The failure to detect telomeres may be because they are consistently too small to detect by FISH, but this view is at odds with telomeres seeded on the ends of truncated chromosomes. Such newly formed telomeres are variable in size (16,17,29–31) and can be readily detected by FISH, as we showed with the 2.4 Mb human X-derived minichromosome. Circular MACs not requiring telomeres or linear MACs with telomeric ends might be formed from linear alploid constructs containing human telomere arrays with frequencies dependent on the relative activities of DNA end processing proteins and telomere-binding proteins, two groups of proteins which, interestingly, have some members in common (32,33). A high incidence of circular chromosome formation would raise questions about the relative ability of alploid DNA to form new centromeres compared with the seeding of telomeres from small telomere repeat cassettes in this system. It is conceivable that interfer-

ence between the closely linked alploid and telomere arrays occurs or that the two processes of initiation of telomere exten-
sion and concatamer formation are in conflict.

A baseline of MAC formation efficiency using some simple variations of input DNA has been established here as a starting point for addressing the complex questions about mechanisms of formation or how to control MAC structure. In our best experiment, eight of 10 randomly picked cell lines produced MACs with an average frequency of 90%, demonstrating the feasibility of future more directed experiments. These data can also serve as a reference point from which to compare the consequences, if any, of including elements that might contribute to MAC formation and stability, such as enlarged alploid arrays, candidate human origins and other types of repeat elements found at human centromeres, or of incorpo-
rating gene-containing fragments of various sizes.

MATERIALS AND METHODS

Construction of input DNA

The α21-1 alploid insert from YAC α7c5hTEL was ligated into the NolI site of pPAC4, which contains the BS selectable gene (http://bacpac.med.buffalo.edu/index.html ) and was electroporated into competent DH10B cells. After plating and colony blotting, eight colonies positive for the α21-1 alploid probe, p11-4 (10), were picked, cultured and clone DNA was analysed by BssHII digestion and PFG. Five of eight pPAC4_7c5 clones had an insert size of ~70 kb. Two of the five full-length clones when tested were equally competent in MAC formation. Removal of OriP from pPAC4_7c5 did not alter MAC formation rates.

Construction of pTAT_7c5. Details of construction of the ditelomeric PAC vector pTAT will be described elsewhere. It contains two oppositely oriented 0.8 kb telomere arrays (27) flanked by I-SceI and NorI sites. pTAT was modified by inclu-
sion of the BS gene by cloning a 2.4 kb PvuII–Smal fragment containing the BS gene from pPAC4 into a blunted SalI site between the telomere arrays. The 70 kb alploid fragment was isolated from pPAC4_7c5 clone 8 by BssHII digestion and gel electrophoresis and cloned into a BssHII site in pTAT_BS.
Construction of T-Inv. An 8 kb NotI fragment from pTAT BS containing both telomere arrays was ligated into Bsp120I-cut pPAC4.7c5 DNA. The resulting construct contains two telomere arrays in reverse orientation with respect to the alphoid insert and two BS genes.

Cell culture and transfection

Cell lines with MACs were generated by lipofecting DNA purified on Qiagen columns or precipitated from alkaline preparations. Best results were obtained from linear or supercoiled DNA separated from contaminants by PFG in autoclaved running buffer. The excised gel slice treated withagarase and either phenol/chloroform extracted/alcloholic precipitated or dialysed prior to lipofection. Typically, DNA (0.5–5 µg) was mixed with lipofectamine/OptiMem ( Gibco BRL, Paisley, UK) according to the manufacturer’s instructions and incubated overnight with 70% confluent HT1080 cells, a human male fibrosarcoma cell line, in a 25 cm² flask. The next day the OptiMem/DNA/liposome mix was replaced with medium (DMEM [ Gibco BRL]) and 10% bovine calf serum (HyClone, Basingstoke, UK) and selection applied after 60–70 h using a final concentration of 4 µg/ml blasticidin (ICN Biochemicals, Logan, UT). Colonies were picked to 24-well plates, expanded and subjected to FISH or immunocytochemical analysis. MAC frequencies were counted from metaphase spreads following FISH to detect the input αz1-1 array fragment, 7c5 and PAC vector sequence on DAPI stained minichromosomes. Normally 25–50 spreads were scored.

FISH and immunocytochemistry

In situ hybridization to chromosomes was performed using standard protocols (34). Chromosomes from BS-resistant HT1080 clones were analysed using the 13/21 alphoid probe p11-4 and pPAC4. The pan-alphoid probe was generated by PCR with consensus alphoid primers (10) and HT1080 DNA as template. The 13/21 alpha satellite was competed out from the digoxigenin-labelled pan-alphoid probe by annealing 10 µg of unlabelled p11-4 prior to hybridization. Confirmation of successful competition was demonstrated by subsequent hybridization with biotin-labelled p11-4. Absence of Y α satellite (which is not represented in the pan-alphoid probe) on the MACs was confirmed independently using a Y alphoid probe (Oncor, Westgrove, PA). Absence of host chromosome arm fragments on MACs was assessed using a biotin-labelled Alu PCR probe (containing both inter- and intra-Alu PCR products) (35) amplified from HT1080 DNA and hybridized to chromosomes in the presence of 10 µg of human Cot1 competitor. Following image capture the slide was hybridized with digoxigenin-labelled p11-4 to confirm MAC identity. Telomere signals were detected with a direct FITC-labelled PNA telomere probe (18). Immunocytochemistry was carried out using published protocols (21) with minor modifications. Antibodies to CENP-C (10) and CENP-E monoclonal antibody mAb177 (36) were diluted in TEEN buffer and washes were done in phosphate-buffered saline (three washes for 2 min each at room temperature). TRITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (Iikhirch, France). After the final phosphate-buffered saline wash chromosomes were fixed for 10 min in 10% formalin (made up in KCM buffer), washed in dH₂O for 10 min, air dried and then stored at −20°C prior to hybridization with p11-4.

ACKNOWLEDGEMENTS

This work was supported by the UK Medical Research Council. We would like to thank our colleagues in the Chromosome Biology Section of the HGU for critical reading of the manuscript and Dr P. Perry for assistance with the imaging systems.

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