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Novel generation of human satellite DNA-based artificial chromosomes in mammalian cells

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SUMMARY

An in vivo approach has been developed for generation of artificial chromosomes, based on the induction of intrinsic, large-scale amplification mechanisms of mammalian cells. Here, we describe the successful generation of prototype human satellite DNA-based artificial chromosomes via amplification-dependent de novo chromosome formations induced by integration of exogenous DNA sequences into the centromeric/rDNA regions of human acrocentric chromosomes. Subclones with mitotically stable de novo chromosomes were established, which allowed the initial characterization and purification of these artificial chromosomes. Because of the low complexity of their DNA content, they may serve as a useful tool to study the structure and function of higher eukaryotic chromosomes. Human satellite DNA-based artificial chromosomes containing amplified satellite DNA, rDNA, and exogenous DNA sequences were heterochromatic, however, they provided a suitable chromosomal environment for the expression of the integrated exogenous genetic material. We demonstrate that induced de novo chromosome formation is a reproducible and effective methodology in generating artificial chromosomes from predictable sequences of different mammalian species. Satellite DNA-based artificial chromosomes formed by induced large-scale amplifications on the short arm of human acrocentric chromosomes may become safe or low risk vectors in gene therapy.

Key words: Human satellite DNA-based artificial chromosome, rDNA, Large-scale amplification

INTRODUCTION

Mammalian artificial chromosomes (MACs) could represent a tool for studying the structure and function of chromosomes of higher eukaryotes. Furthermore, successful construction of MACs would have a substantial impact on the further development of vectors and methods for gene delivery in various fields of biotechnology including cellular protein production, transgenics, and gene therapy.

Construction of a mammalian artificial chromosome from cloned structural elements is hindered mainly by the lack of an isolated, functional, mammalian centromere. The best characterized human DNA associated with the centromeric region of the chromosomes is the human α-satellite present at the centromeres of all normal human chromosomes. However, results of experiments aimed at in vitro construction of centromeres or human artificial chromosomes with cloned α-satellite sequences (Harrington et al., 1997; Ikeno et al., 1998; Warburton and Cooke, 1997; Henning et al., 1999) are controversial. There is increasing evidence suggesting that α-satellite is neither absolutely necessary nor sufficient for centromere function (Lewin, 1998; Murphy and Karpen, 1998; Barry et al., 1999), and centromeres have an epigenetic structure that is not determined simply by the sequence (Lewin, 1998). Consequently, if epigenetic mechanisms are responsible for centromere activity (Murphy and Karpen, 1998; Wiens and Sorger, 1998) the formation of a neo-centromere from cloned sequences introduced into cells may be incidental. In addition, undefined endogenous or carrier DNA sequences contributing to the centromere formation, or providing the necessary minimal size for stability of the synthetic artificial chromosomes make their structure elusive.

Development of telomere directed fragmentation of mammalian chromosomes (Farr et al., 1991) led to an elegant in vivo approach for the generation of human minichromosomes (Farr et al., 1992; Barnett et al., 1993) that represents an important step towards constructing a human artificial chromosome-based gene delivery system (Mills et al., 1999).

We have developed an alternative in vivo methodology for construction of mouse satellite DNA-based artificial chromosomes. This approach is based on the induction of large-scale amplifications and formation of de novo centromeres and chromosomes in rodent cells upon the integration of exogenous DNA sequences into mouse chromosomes (Hadlaczky et al., 1991; Praznovszky et al.,

1991). Recently, we demonstrated that integration of exogenous DNA sequences into the pericentric regions of mouse chromosomes led to de novo formation of stable, heterochromatic, mouse major satellite DNA-based artificial chromosomes (Keresö et al., 1996)(mSATACs). Cytological evidence supported the role of replication-directed large-scale amplification as the possible mechanism underlying the formation of such new chromosomes (Holló et al., 1996). Several lines of direct and indirect evidence suggest that in amplification-mediated chromosome formation the specific regions susceptible to large-scale amplification are the rDNA containing chromosomal sites, and the rDNA itself may have significance in the de novo chromosome formation (K. Fodor et al., unpublished).

To our knowledge, so far, SATACs are the only mammalian artificial chromosomes that can be purified in meaningful quantity (DeJong et al., 1999). They can be transferred into cells and embryos of different species (Telenius et al., 1999; Co et al., 2000) preserving their structural integrity and function. Furthermore, generation of transgenic animals with purified mammalian artificial chromosome and germ line transmission of this mammalian artificial chromosome has only been demonstrated with mouse SATAC (Co et al., 2000).

The question of reproducibility and generality of the inductive approach for in vivo construction of mammalian artificial chromosomes was raised (Vos, 1998). Considering the basic similarities between the chromosomes of higher eukaryotes, we hypothesized that the inducible, replication-directed large-scale amplifications and de novo chromosome formations observed in rodent cells (Keresö et al., 1996; Holló et al., 1996) might be general in higher eukaryotes. Reproducing these events in other mammalian species would offer a novel, inductive approach for in vivo construction of mammalian artificial chromosomes from predictable sequences utilising the intrinsic cellular mechanisms of mammalian cells. To test this hypothesis, we attempted to generate a human satellite DNA-based chromosome (hSATAC) in a human-Chinese hamster hybrid cell line (94-3) (Ledbetter et al., 1991) containing rDNA bearing human chromosomes. In this paper, we show the feasibility of the inductive in vivo construction of human artificial chromosomes, and describe the successful generation and basic characterization of these prototype hSATACs.

MATERIALS AND METHODS

Culture of cell lines and transfection of cells

Line 94-3 (NIGMS, GM 10664) was produced by fusion of lymphoblasts of a patient with a t(X;15) (q25 or q25;q26) and HPRT deficient Chinese hamster RJK88 cells (Ledbetter et al., 1991), and obtained from NIGMS Human Genetic Mutant Cell Repository, Coriell Cell Repositories, Coriell Institute for Medical Research, Camden, New Jersey, USA. Cells were cultured in F-12 (Sigma) medium supplemented with 10% foetal calf serum (F12-10FCS). The EJ30 human bladder carcinoma cell line was maintained in F12-10FCS. Cotransfection of cells with plasmid DNAs was carried out by calcium phosphate DNA precipitation (Chen and Okayama, 1987) using 1-2 μg of a mixture of plasmid DNAs of pBabe Puro linearized with *Eco*RI, pCH110 linearized with *Bam*HI, and 70 μg of carrier rDNA (pK161) linearized with *Cla*I restriction endonuclease per 5×10^6 recipient cells. Selection of transformants was started 48 hours after transfection using 10 μg/ml puromycin in F12-10FCS medium.

DNA manipulations and plasmids

All general DNA manipulations were performed by standard procedures (Sambrook et al., 1989); pBabe Puro plasmid (Morgenstern and Land, 1990) was a kind gift from Dr L. Székely (MTC, Karolinska Institutet, Stockholm); pCH110 carrying β-galactosidase was obtained from Pharmacia; pK161 carrying a 9 kb coding sequence of mouse rDNA in pWE15 was cloned in our laboratory. ACR-1, a plasmid clone (ATCC 61650) of a human 4.3 kb unique sequence distal to the rDNA cluster on the short arm of all 5 acrocentric chromosomes (Worton et al., 1988) was obtained from the American Type Culture Collection (ATCC), Rockville, USA.

Chromosome banding

Constitutive heterochromatin was detected by Giemsa/barium hydroxide C-band staining (Sumner, 1972).

PCR amplification of DNA probes

To avoid cross hybridization of vector sequences, DNA probes were generated by PCR. Primers were synthesised on a Pharmacia Gene Assembler.

List of forward and reverse primers:
LacZ-F (lacZ 121-143) 5′-GAAGAGGCCCGCACCGATCGCCC;
LacZ-R (lacZ 772-750) 5′-TTACCCGTAGGTAGTCACGCAAC.
Puromycin: Puro-F primer 5′-ATGACCGAGTACAAGCCCACGGT-GCGC;
Puro-R primer 5′-TCAGGCACCGGGCTTGCGGGTCATGCA.
Human rDNA 5′-external transcribed spacer (Gonzalez and Sylvester, 1995): HETS-F (1-24 of U13369) 5′-GCTGACACGCTGTCCTCTG-GCGAC;
HETS-R (U13369: 1520-1497) 5′-GTCCTCTGCGAGCGGGTCGC-TACG.
rDNA (Gogel et al., 1996) coding sequence pK161-M7 carrying a part of conserved 18S RNA sequence: pK161 M7-F primer 5′-AGGTGC-GTCTGCGGGTTGGGGCTCGTC;
pK161 M7-R primer 5′-AAGGATCCTCGTTAAAGGATTTAAAGT-GGAC.
Chromosome-specific α-satellites: α-satellite primer 1 (pS12, Cserpán, I., unpublished, X60716: 3920-3898) 5′-CCTGAAAGCGC-TCCAAATGTCCA;
α-satellite primer 2 (pS12, X60716: 3582-3605) 5′-CCTAAGGTGA-AAAAGGAAATATCT.
Centromeric *Not*I repeat DNA (Thoraval et al., 1996): Notrep-F primer (U53226: 50-75) 5′-GGGTTTAAATAGCCTCGGGCGCAG-C;
Notrep-R primer (U53226:571-545) 5′-TAGTAGATTGGATTATCT-GGAGCCACA.
β-satellite (distal) hybridizing to the p13 region of acrocentric chromosomes (Greig and Willard, 1992): β-satdist F primer (M81228: 1-26) 5′-ATAAGCTTAGGCAAGAGTTGCATCACCT;
β-satdist R primer (M81228: 955-930), 5′-TGAAGCTTTGCCTAC-AGGGGATTGTGAC.
β-satellite (proximal) hybridizing to the p11 and p13 regions of acrocentric chromosomes (Greig and Willard, 1992): β-satprox F primer (M81226: 38-65) 5′-CAAAGCTTAGACAAGAGTTACATC-ACCT;
β-satprox R primer (M81227: 200-176) 5′-TGAAGCTTTCCTAGA-GGCACATTGGGAC.
Satellite III DNA (Vissel et al., 1992): Satellite III F primer (M21305: 1-25) 5′-TGGGAATTCAATAGAATGGAATGGTAT, Satellite III R primer (M21305: 1608-1582) 5′-TGCAATAGAATGGAATGGA-ATCAACTC, 147 bp repeat sequence located at the proximal junction of the human rDNA cluster on all acrocentric chromosomes (Sakai et al., 1995): 147rep22F (D31961, 156-177) 5′-CGGGTTGGGA-CCAGTTAGGT;

147rep22 (D31961, 358-379) 5′-TGGAGTCTGTGAGTCTGTAGGT.

All PCR products listed above were isolated by agarose gel electrophoresis, and identity of the fragments was verified by sequencing from both ends with the amplification primers on an ABI Prism automated DNA sequencer.

The PCR generated telomeric probe was produced by the template-free method (Ijdo et al., 1991), and labelled either directly by PCR or nick translation with DIG-11-dUTP (Boehringer) then purified on Nick Spin column (Pharmacia).

Immunostaining of chromosomes and in situ hybridization

Indirect immunofluorescence staining with human LU851 anti-centromere serum was performed as described previously (Keresö et al., 1996; Nicol and Jeppesen, 1994). Immunostaining with fluorescein conjugated anti BrdU monoclonal antibody (Boehringer, Mannheim) was performed according to the manufacturer's suggestions.

For in situ hybridizations biotin or digoxigenin (DIG) labelled human genomic painting probe (total genomic DNA from EJ30 cell line), a 1 kb synthetic telomere (I. Cserpán et al., unpublished), PCR generated telomere probe (Ijdo et al., 1991), 147 bp human rDNA flanking sequence probe, human 9 kb repeat, ACR-1, LacZ PCR, Puro PCR, β-satellite, and satellite III probes were prepared by using a nick translation kit (Gibco BRL) according to the manufacturer's suggestions. Biotin and DIG labelled human 15, 22, and X chromosome specific painting probes, D15Z, D22Z1 and DXZ1 α-satellite centromeric probes were obtained and used according to the suggestions of the supplier (Oncor). Standard in situ hybridizations were performed as described earlier (Hadlaczky et al., 1991). To detect human chr #15 centromere a biotin or DIG labelled monomeric α-satellite variant was also used that was isolated in our laboratory (I. Cserpán, unpublished). The specificity of all human probes used was verified by in situ hybridization on human lymphocyte preparations made from the peripheral blood of a healthy individual. Microscopy was carried out using an Olympus AH-2 photomicroscope equipped with Quips XL Genetics Workstation system including a Photometrics KAF1400-G2 CCD camera (Vysis).

Flow cytometry

Metaphase chromosomes were isolated from colchicine-blocked cells and stained with Hoechst 33258 and Chromomycin A3 using standard procedures (DeJong et al., 1999).

Flow analysis and purification of the hSATACs were performed on a FACS Vantage (BDIS, San Jose, CA) equipped with a turbosort option and two Inova 305 lasers (Coherent, Palo Alto, CA). Condensing agents were added to the sheath buffer to maintain condensed chromosomes after sorting. The final buffer contains 15 mM Tris-HCl, 0.1 mM EDTA, 20 mM NaCl, 1% hexylene glycol, 100 mM glycine, 20 μM spermine and 50 μM spermidine, pH 7.6. The sorted SATACs were collected in 1.5 ml screw-capped Eppendorf tubes at 4°C, at a sort concentration of approximately 1×10^6 chromosomes/ml, which were then stored at 4°C until needed. For FISH analysis of sorted fractions, aliquots of approximately 10,000 chromosomes from each sorted region were placed on glass slides after addition of 0.2% formaldehyde for 5 minutes. Slides were then left to dry and dehydrated before being hybridized to labelled probe (pBabe Puro plasmid and PCR-generated telomere) using standard conditions. Following FISH detection, at least 20 chromosomes were scored for each sorted region.

RESULTS

Generation of de novo chromosomes

Initial characterization of the 94-3 cell line revealed that in 100% of the cells, human t(X;15) chromosome (referred herein as t(X;15q25)) and human chromosome #22 were retained. In addition, 15% of the cells had human/hamster translocated chromosomes, and 20% of the cells carried human chromosome fragments (NIGMS Human Genetic Mutant Cell Repository).

First we verified the presence of human chromosomes in 94-3 cells by in situ hybridizations with biotin labelled human genomic DNA probes, and also with biotin labelled human α-satellite DNA sequences, and confirmed that 96.8% of the cells carried human chromosomal material. Two hundred and ninety-six metaphases of 94-3 cells were analyzed in detail by in situ hybridization using a human genomic painting probe (EJ30), human chromosome-specific painting probes (chr #15, #22, and X), and human chromosome-specific α-satellite centromeric probes (D15Z, D22Z1, and DXZ1). Details of the FISH analysis of the 94-3 cell line is presented in Fig. 1 and Table 1.

Our aim was to integrate marker gene(s) into the ribosomal DNA region (rDNA, NOR = nucleolus organising region) of a human chromosome via site-specific integration, and induce large-scale amplification and de novo chromosome formation. Large amounts of target sequences used as a carrier in cotransfection, can promote site-specific integration of the exogenous DNA sequences (Keresö et al., 1996; Raimondi et al., 1996). The ribosomal RNA coding sequences are highly conserved in eukaryotes (from yeast to human), therefore mouse rDNA sequences are suitable for targeting the rDNA region of human (or hamster) chromosomes. In the case of human acrocentric chromosomes, besides the rDNA a number of neighbouring endogenous sequences have been identified in the NOR regions. These sequences are: the proximal 147 bp repeat (Sakai et al., 1995) proximal and distal β-satellite (Waye and Willard, 1989), ACR-1, a unique flanking sequence distal to the rDNA cluster (Worton et al., 1988), a methylated human 9-kb repetitive sequence (Thoraval et al., 1996) (*Not*I repeat), telomeric sequences on the distal ends, D15Z1 satellite III (Vissel et al.,1992), and D15Z α-satellite DNA sequences towards the centromere. Except for ACR-1 sequences, all these sequence elements were detectable on the human chr t(X;15q25) in the 94-3 cell line by in situ hybridization with the corresponding probes.

The result of a large-scale amplification of the NOR region(s) would be the formation of a new heterochromatic

Table 1. Analysis of 94-3 cell line by FISH

Human chromosome (intact or fragment)	FISH probe	Percentage in 296 metaphases analyzed
Human chromosome material	Genomic painting	96.8
Chromosome t(X;15q25)	15 painting, D15Z	56.0
Chromosome X	X painting	56.0
Chromosome # 22	22 painting, D22Z1	15.5
Chr #15 centric fragment	D15Z	3.4
Chr # 22 centric fragment	D 22Z1	34.1
Chr X centromere	DXZ1	0.0
Translocated human/hamster chromosome*	Genomic painting, D15Z and D22Z1	24.7

*These metaphases contained an acrocentric hamster chromosome with a centromere and short arm composed of human chromosomal material. The majority (89%) of these translocated chromosomes showed centromeric hybridization with D22Z1, 7% of the centromeres were labelled with D15Z (Fig. 1C), and in 4% of the centromeres no human alphoid sequences were detected.

chromosome arm ('sausage' chromosome) (Keresö et al., 1996) with amplified satellite and rDNA sequences as well as amplification of the integrated exogenous DNA sequences. Further, duplication of the centromere would produce a dicentric chromosome; subsequent breakage of such a dicentric chromosome would result in the formation of a de novo heterochromatic SATAC (for details see Keresö et al., 1996; Holló et al., 1996). To test whether large-scale amplification can be induced on human chromosomes in a host cell of another species, a semiconfluent culture of 94-3 cells was cotransfected with a mixture of plasmid DNAs of pBabe Puro linearized with *Eco*RI, pCH110 linearized with *Bam*HI, and carrier rDNA (pK161) linearized with *Cla*I restriction endonuclease as described in Materials and Methods. After 10-16 days of puromycin selection, 68 individual puromycin resistant colonies were rescued, and propagated for an additional 18-25 days. Initial cytological analyses of the clones were performed on days 28-42 after transfection, and at the same time the primary screening for amplification was carried out by Southern blot hybridization with a plasmid probe on *Eco*RI digested DNAs purified from the individual clones.

The generation time of the primary clones was estimated by BrdU incorporation (Holló et al., 1996), and was found to be 14-16 hours. Therefore, these primary clones represented cultures of 45th-70th generations after transfection.

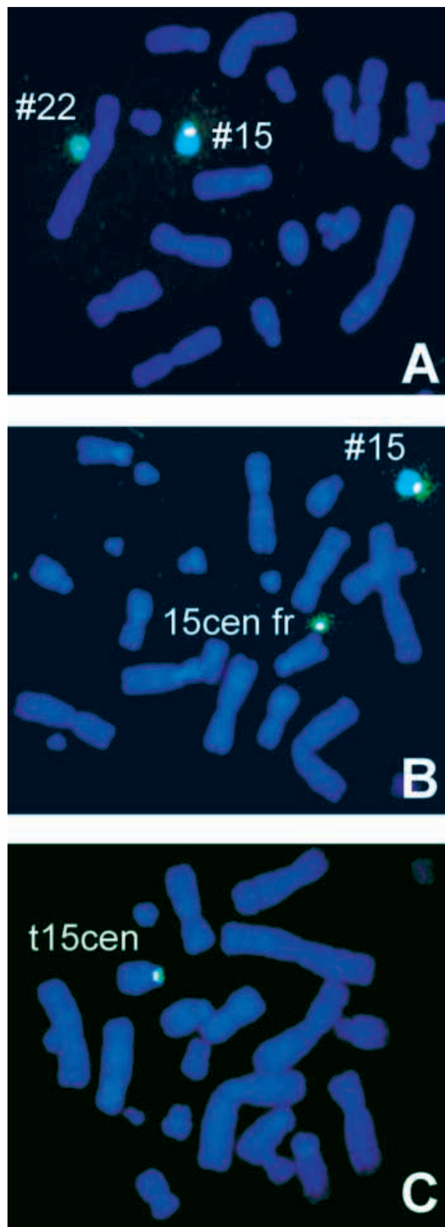

Fig. 1. Two color FISH on metaphases of 94-3 cells with biotin-labelled human genomic painting probe (green signal), and digoxigenin (DIG)-labelled D15Z probe (pink-white signals). (A) human chromosomes t(X;15q25) and #22. (B) Human chr t(X;15q25) and a small human chromosome fragment with chr #15 centromere. (C) Acrocentric hamster chromosome with translocated human chr #15 centromere and short arm region.

Analysis of primary clones

Over 50% of the 68 rescued clones showed 'high copy number' hybridization signals with plasmid probe indicating either the integration of multiple copies of the bacterial constructs or the amplification of the integrated exogenous DNA sequences. Representative examples of the hybridizations on the primary clones are shown in Fig. 2. Twenty-one primary clones showed β-galactosidase expression detected with the standard LacZ staining technique.

The first cytological analysis was made by C-banding. Because hamster chromosomes contain no large constitutive heterochromatic regions, newly formed chromosome segments and SATACs of human origin would easily be detectable in Chinese hamster cells, by C-banding (Sumner, 1972).

Two clones (c23 and c48) were found to carry large de novo

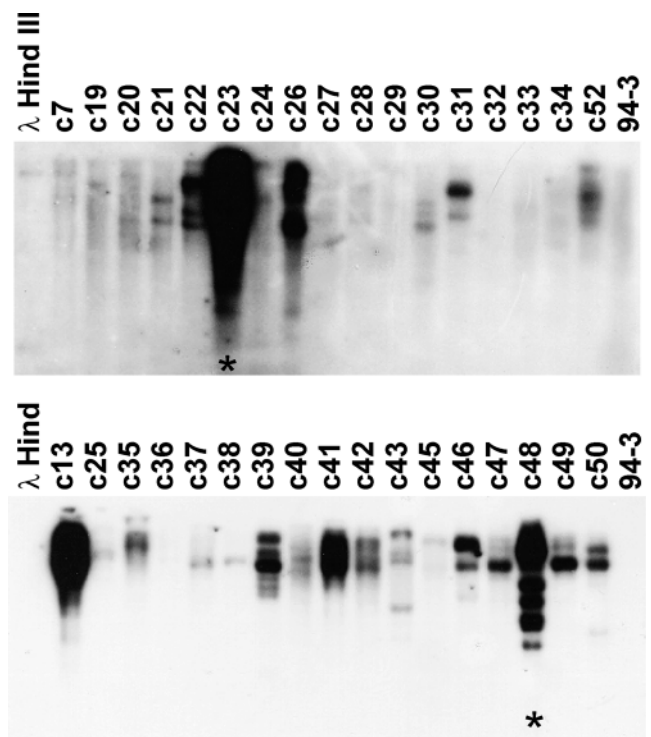

Fig. 2. Representative example of Southern blot hybridization on approx. 10 μg *Eco*RI digested DNA of 34 primary clones with pBabe Puro plasmid probe. Lanes representing clones c23 and c48 are marked with asterisks.

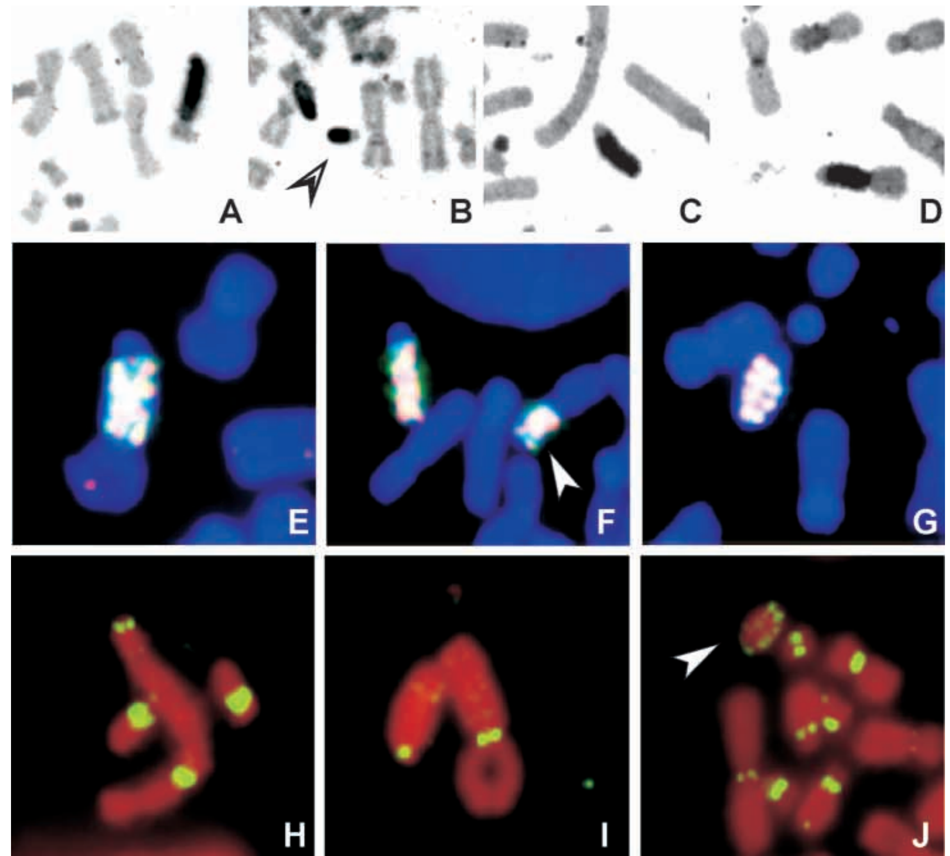

Fig. 3. Heterochromatic staining on c23 and c48 primary clones. (A) 'Sausage' chromosome in c23. (B) Independent heterochromatic chromosome in c23 (arrowhead). (C) Independent heterochromatic chromosome in c48 metaphase. (D) Heterochromatic 'sausage' chromosome in c48. Subsequent steps of de novo chromosome formation in primary clones. Two color FISH with DIG-labelled D15Z (red), and biotin-labelled pBabe Puro probes (green). Colocalized signals appear pink/white. Chromosomes are counterstained with DAPI (blue). (E) The 'sausage' chromosome. (F) Breakage of the 'sausage' chromosome. Arrowhead points to the formerly sausage chromosome with signals on the broken arm. (G) Independent de novo chromosome. Immunostaining of similar structures with LU851 human anti-centromere serum and FITC-conjugated second antibody (yellow) on propidium iodide (red) stained chromosomes. (H) Dicentric 'sausage' chromosome. (I) Breakage of the dicentric chromosome. (J) Independent hSATAC (arrowhead).

formed heterochromatic segments. In clone c23, 71% of the cells contained a typical 'sausage' chromosome with a heterochromatic arm of 30-150 MB (Fig. 3A). The sizes of the de novo structures were estimated by comparing them to the 250-300 Mb sized hamster chromosome 1. 15% of the c48 metaphases also contained heterochromatic 'sausage' chromosomes (Fig. 3D). In addition to the amplified sausage chromosomes, free de novo heterochromatic chromosomes were observed in 5 out of 347 metaphases of the c23 clone (Fig. 3B), and heterochromatic chromosomes of different size (approx. 50-250 MB) were found in 107 out of 219 metaphases of the c48 clone (Fig. 3C). The heterochromatic segments in both clones were derived from the short arm of an acrocentric chromosome. Fluorescence in situ hybridization revealed one integration site of the pBabe Puro sequence in the short arm of an acrocentric chromosome in clone c23, and there were two integration sites in clone c48: one in the centromeric region of an acrocentric chromosome, and the other in a tiny metacentric chromosome. Initiation of the amplification detected by hybridization with the D15Z probe correlated with these integration sites, and enlarged human chr #15 α-satellite (D15Z) regions were detected on the acrocentrics, and on a centric fragment of human chr #15 (not shown).

Concurrent with the C-banding, results of in situ hybridizations (FISH) with human genomic, human chr #15 and chr #22 painting, and centromere specific probes showed that the large amplified regions were of human origin and contained human D15Z sequences. In clone c23, the short arm of human chr t(X;15q25) hosted the new amplified arm, while in clone c48, the 'sausage' chromosome derived from the acrocentric hamster chromosome with translocated human chr #15cen/short arm region (not shown). In situ hybridization on c23 and c48 metaphases with chr #15cen/Puro PCR probes and immunostaining with LU851 human anti-centromere serum (Hadlaczky et al., 1991) confirmed that these de novo formed heterochromatic chromosomes carried amplified exogenous DNA (Fig. 3E-G) and functional centromeres (Fig. 3H-J). Standard immunostaining on hSATACs with anti-centromere serum resulted in a typical centromeric label showing terminally located kinetochores. In addition, faint signals along the amplified chromosomal segments were observed (Fig. 3H-J).

The morphology of the transitional structures found in the primary clone suggests that de novo chromosome formation proceeds through the following subsequent steps: (i) the integration(s) of exogenous DNA into centromeric regions induced large-scale amplifications; (ii) the formation of heterochromatic arms with an active centromere ('sausage' chromosome formation) (Fig. 3A,D,E,H), (iii) breakage of the dicentric 'sausage' chromosome separated the heterochromatic arm (Fig. 3B,F,I), which finally became an independent new chromosome (Fig. 3B,D,C,J).

Isolation of stable subclones

The occurrence of hSATACs in primary clones indicated that they were mitotically stable, and might allow selection of stable subclones. By single-cell subcloning we managed to establish subclones carrying stable hSATACs of different size and morphology from both primary clones. We regarded subclones as stable when at least 98% of cells retained the

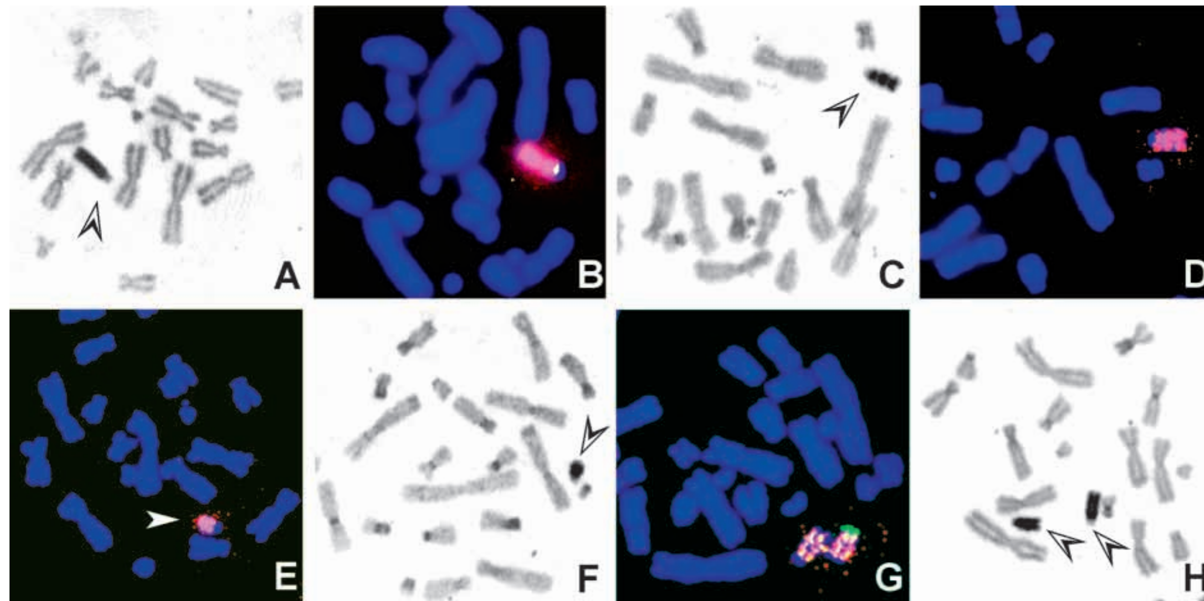

Fig. 4. Chromosomes of stable hSATAC subclones with C-banding and FISH. (A) Heterochromatic staining of clone c23-Z41/15. (B) Two-color FISH with DIG-labelled human chr #15 α-satellite D15Z (red) and biotin labelled (green) human satellite III probes. Intense colocalized signals appear pink/white. (C) Clone c48-6B5 by C-banding. (D) FISH with DIG labelled (red) D15Z α-satellite probe. (E) FISH on clone c48-6D6 with human D15Z α-satellite (red) probe. (F) C-band staining on clone c48-6D6. (G) FISH on clone c48-5B4 with D15Z α-satellite (red), biotin labelled pBabePuro plasmid (pink/white) probes, and with biotin labelled β-satellite probe (green) that localized exclusively on the tip of one hSATAC. (H) C-banding of clone c48-5B4. Arrowheads on pictures point to the hSATACs. Chromosomes on B,D,E, and G were counterstained with DAPI (blue).

hSATAC under selective conditions, and more than 95% of cells retained cytologically unchanged SATAC under non-selective conditions cultured for 33 days (approx. 50 generations). In the two most extensively used subclones (c23-Z41/15 and c48-5B4-D2), under normal selective growth hSATACs remained cytologically unchanged after 274 and 731generations, respectively.

Expression of the non-selected β-galactosidase marker gene have been persisted over 300 generations, in c23 clones. Analysis of the long-term stability of hSATACs and the persistence of gene expression in the host cells, and in cells of different mammalian species, are in progress.

Sequence composition of hSATACs

To identify DNA sequences present on the hSATACs other than the alpha-satellite (D15Z) and exogeneous sequences, in situ hybridizations were also performed with a number of different probes of known human DNA sequences specific to the short arm regions of acrocentric chromosomes (Table 2). Analysis of sequence composition of hSATAC of clone c23-Z41/15 by FISH is demonstrated on Fig. 5. All known sequences specific to the short arm of human chr #15 from the centromere (α-satellite, and satellite III, Fig. 4B and Fig. 5E) to the telomere (ACR-1, telomeric repeats, Fig. 5K,L) were detected on the hSATAC. Results of in situ hybridizations revealed that the most abundant sequences on the hSATACs were: chr #15 α-satellite (D15Z), human rDNA, satellite III (D15Z1), β-satellite (both distal and proximal), and exogenous DNA (Fig. 5). These observations suggest that the heterochromatic nature of the newly formed chromosomes can be attributed to the presence of these amplified DNA sequences. The presence of interspersed telomeric sequences on hSATACs (Fig. 6B, R1, 2) indicates that these sequences also coamplified with the other sequences specific to the short arm of human acrocentric chromosomes. Following the chromosome breakages that occur during the SATAC formation, these interspersed telomeric repeats may contribute to the stability of the de novo chromosomes.

On hSATACs of c23 clones no hamster specific sequences were detected by FISH, while on SATACs of the c48 clone that derived from the acrocentric hamster chromosome with a translocated human chr #15cen/short arm region hamster centromeric sequence was found. This sequence was cloned, sequenced and identified as hamster chr #5 centromeric repeat (unpublished).

Table 2. Sequence analysis of hSATACs by FISH

	hSATAC clones			
Probes	c23-Z41/15	c48-6D6	c48-5B4*	c48-6B5
22cen D22Z1	–	–	– –	–
15cen D15Z	+	+	++	+
Puro PCR	+	+	+	++
LacZ PCR	+	–	– –	–
rDNA(M7)	+	+	++	+
rDNA HETS	+	–	– +	–
Telomere	+	+	++	+
147bp	+	+	++	+
9kb *Not*I	+	+	– +	–
ACR	+	–	– –	–
β-satellite	+	–	– +	–
Satellite-III	+	–	– +	–

*c48-5B4 cell line contains two hSATACs with similar morphology but different sequence composition (for an example see Fig. 4G).

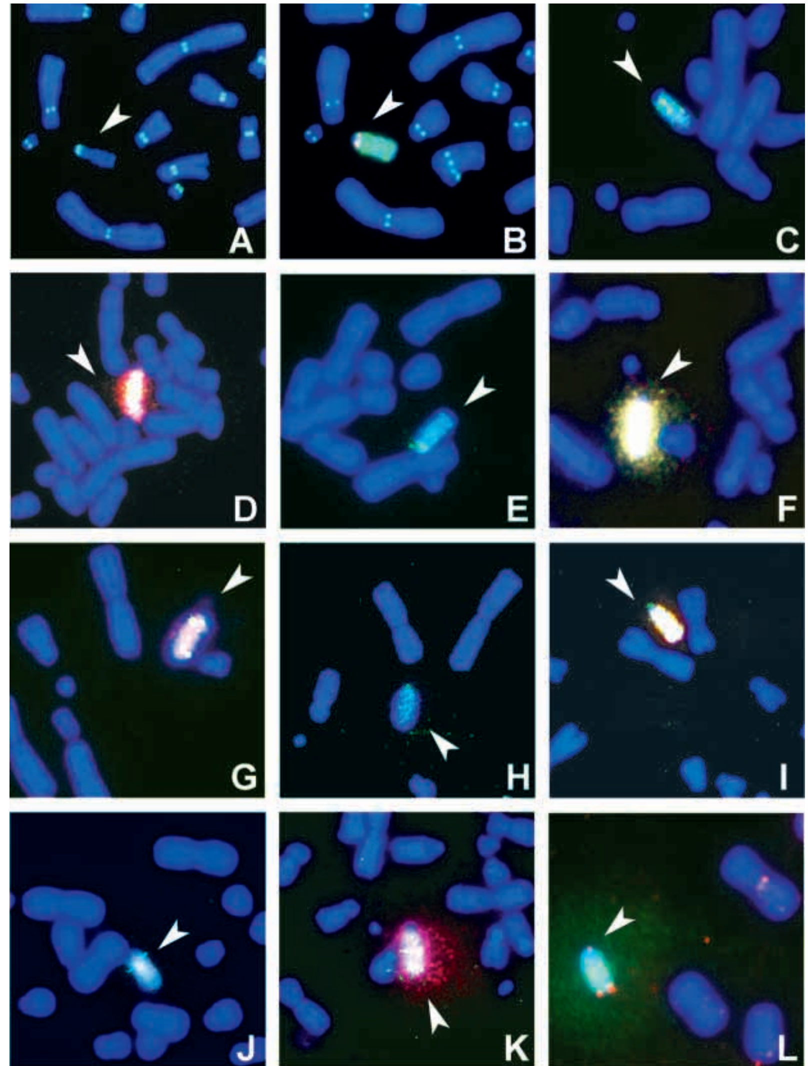

Fig. 5. Sequence analysis of hSATAC (indicated with arrowheads) of clone c23-Z41/15 by FISH. Chromosomes are counterstained with DAPI (blue). (A) Indirect immunofluorescence staining of hSATAC with LU-851 anti-centromere serum showing terminally located centromere. (B) FISH on the same metaphase with human chr #15 monomeric α-satellite probe (red) localized exclusively in the centromere and with plasmide probe (pBabe Puro) (green) showing interspersed signal on the hSATAC. (C) Interspersed LacZ (green) signal on the hSATAc. (D) Colocalized (pink-white) Puro PCR (green) and D15Z (red) signals. (E) Centromeric and interspersed localization of satellite III (D15Z1) sequences (green). (F) Colocalized M7 rDNA (green) and D15Z (red) signals. (G) Human rDNA externally transcribed spacer (HETS) (green) and D15Z (red) signals. (H) Interspersed signal (green) with NotI centromeric repeat probe. (I) Colocalized DIG labelled β-satellite (red) and biotin labelled D15Z (green) signals. (J) Biotin labelled (green) 147bp repeat probe. (K) Two color FISH with D15Z (red) and ACR-1 (green) probes. (L) Two color FISH with biotin labelled D15Z (green) and DIG labelled PCR generated telomere (red) probes on hSATAC, the presence of centromeric telomere sequences are characteristic to some hamster chromosomes.

Expression of the marker gene from heterochromatic hSATACs

Twenty-one out of 68 primary clones show β-galactosidase expression by conventional lacZ staining. Clone c23 is one of the 21 β-galactosidase positive primary clones. Expression of the non-selected marker gene in hSATAC carrying subclones (c23-Z41/15) of c23 primary clone was also confirmed by immunoblotting using monoclonal β-galactosidase antibody (not shown).

Expression of the selectable marker gene (puromycin phosphotransferase) was detected by resistance to 10 μg/ml puromycin, which is twofold higher than the lethal concentration established for the host cell line (94-3). Z41/15 cells were also tested at high antibiotic concentrations, and at up to 150 μg/ml puromycin no killing effect or stunted growth was observed. In addition, northern hybridizations were also used to detect the mRNA of the selectable marker gene in subclones of both c48 and c23 primary clones (not shown).

Separation of hSATACs

The cell line containing two hSATACs, C48-5B4, was used to demonstrate the separation of hSATACs by flow cytometry. Metaphase chromosomes were isolated from mitotic cells, stained with two fluorescent dyes, and passed through two laser beams: by plotting the fluorescence of each dye and chromosome, a ‘flow karyotype’ was obtained from both the parental cell line (94-3) (Fig. 6A) and the hSATAC line (Fig. 6B). The two novel clusters (R1 and R2) in the hSATAC line were assumed to represent the two hSATACs. Chromosomes from these two clusters were physically purified by the cytometer and placed on slides for FISH analysis. Two other regions (R3 and R4) were also isolated as controls. The FISH experiment using either plasmid probes or PCR generated telomere probes showed conclusively that the chromosomes isolated in regions 1 and 2 contained interspersed plasmid/telomere DNA consistent with hSATACs, while the chromosomes in regions 3 and 4 contained no plasmid DNA, and the telomere DNA signal was consistent with hamster chromosomes (Fig. 6B, R3-4). From R1 and R2, 62 chromosomes were analysed by in situ hybridization and 60 chromosomes proved to be hSATACs. Based on these results, the purity of separated hSATACs was >96%.

Purified SATACs can be used in quality control by FISH as templates to generate hybridization probes with degenerate oligonucleotide primed PCR (DOP PCR) (Telenius et al.,

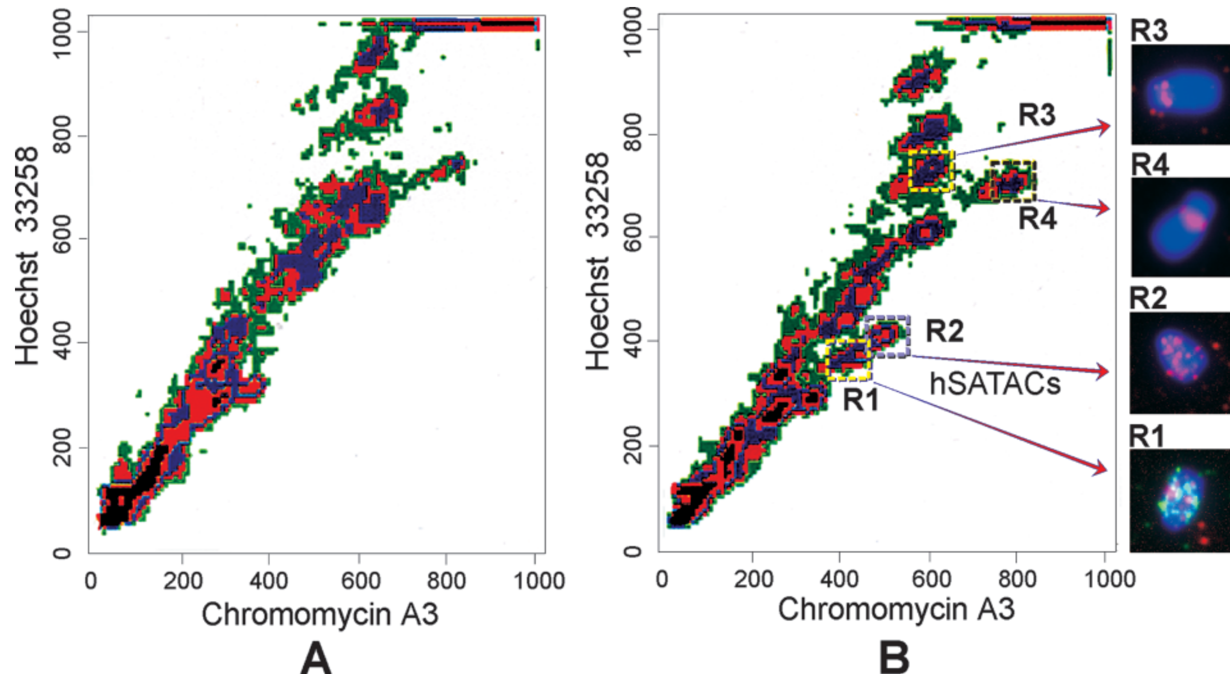

Fig. 6. Separation of hSATACs with fluorescence activated cell sorter (FACS). (A) FACS karyotype of the host 94-3 cell line. (B) FACS karyotype of the c48/5B4 clone containing two hSATACS. R1-R4 represent the separated chromosome fractions, on the right side of the panel the verification of the separated chromosomes are shown by in situ hybridization with biotin-labelled pBabe Puro probe (green) (R1), and DIG labelled PCR generated telomere probe (red) (R1-R4) on DAPI counterstained chromosomes (blue).

1992). Fluorescence in situ hybridization with these probes to the chromosomes of host cell and SATACs can serve in monitoring the purity of separated SATACs, and to analyze the sequence composition of SATACs at the level of FISH detection. Details of DOP PCR FISH analysis will be published elsewhere.

DISCUSSION

We described the successful generation of the human counterpart of mouse satellite DNA-based artificial chromosomes (Keresö et al., 1996). Here we demonstrate that human satellite DNA-based artificial chromosomes can now be generated via induced large-scale amplification on the centromeric/short arm region of the human acrocentric chromosomes. These results confirm that the methodology we initially used in mouse cells to create mouse satellite DNA-based artificial chromosomes is reproducible, and can be successfully applied to other species, and also in other host cells using different foreign DNA sequences. The ability to target amplifiable sites, as we did here using rDNA sequences, greatly accelerates the generation of SATACs. These mammalian artificial chromosomes are formed in vivo and acquire all the structural and functional elements necessary for chromosome formation from endogenous sequences. Consequently, the successful generation of de novo chromosomes, at the same time, is the ultimate test of the functionality of these components together with the integrated exogenous genetic material.

Human-hamster hybrid cells used in these experiments proved to be an efficient platform for de novo chromosome formation, and for establishing stable cell lines with hSATACs. The short arms of the human acrocentric chromosomes are specific chromosomal regions (NOR) with well characterized DNA sequences. Amplification of these endogenous sequences of the short arms offers several advantages, which make these sites ideal for the construction of human artificial chromosomes in vivo: (i) coamplified telomeric sequences can provide functioning telomeres for the de novo formed chromosome. (ii) The non-coding centromeric satellite DNAs lack transcription units for undesired and unknown genes and hence are safe ‘filling sequences’. (iii) Despite the heterochromatic nature of the hSATACs, coamplified rDNA sequences may provide a suitable chromatin environment (Lucchini and Sogo, 1992; Karpen et al., 1988) for the expression of the integrated ‘foreign’ gene(s). (iv) Non-coding repeated sequences specific to the short arm of the human acrocentric chromosomes can serve as useful markers to characterize newly formed chromosomes. (v) With respect to the possible use of hSATACs as vectors in human gene therapy, it is important that apart from the rRNA genes, no unsuspected or unwanted coding sequences are localized in these chromosomal regions, and it would seem unlikely that the amplification of rDNA or the known sequences identified at these chromosomal regions would cause phenotypic effects. In contrast, at least in tissue culture, amplification of rDNA presented selective advantage (Roberts et al., 1987). The spontaneous amplification of the short arm of the human acrocentric chromosomes is the most frequent structural polymorphism without phenotypic effect (Conte et al., 1997). Follow up studies on an unselected group of children with

small supernumerary chromosomes revealed that in all 12 cases out of 14, where the inherited or de novo extra chromosomes were derived from the short arm of a NOR chromosome, the affected children developed normally (Gravholt and Friedrich, 1995). Additionally there is a report of 8 healthy members of a three generation family carrying a small supernumerary chromosome derived from the short arm of acrocentric chromosome (Fu et al., 1992). These examples indicate the existence of intrinsic cellular mechanisms that give rise to stable de novo chromosomes under natural circumstances without any dire consequences. The in vivo generation of SATACs is based upon the induction of these intrinsic mechanisms, and it is conceivable that satellite DNA-based artificial chromosomes formed by induced large-scale amplifications on the short arm of human chromosomes may become a safe or low risk vector in gene therapy.

The cell population of primary clones represents 45-70 generations of transfected cells, and the presence of hSATACs in these clones indicated that the de novo chromosomes appeared early after the transfection. We showed that stable subclones could be established with different hSATACs from the primary clones, and that these de novo chromosomes were retained in the majority of the cells. These results indicate that hSATACs are mitotically stable, and demonstrate the feasibility and relative ease of establishing stable cell lines with hSATACs by prolonged culturing and repeated subclonings.

We have shown that mouse SATACs can be efficiently transferred by microcell mediated chromosome transfer to mammalian cells derived from different species (Telenius et al., 1999) preserving their structural integrity, stability, and function. Further engineering of hSATACs can be performed with targeted integration of the required exogenous genetic material either in the host cells or in a homologous recombination system such as chicken DT-40 cells (Dieken et al., 1996). Here we demonstrated that hSATACs could be purified efficiently from the host cell chromosomes by flow cytometry. Purified hSATACs may allow their extensive molecular characterization and may be used for direct chromosome transfer. Recent results of direct transfer experiments with mouse SATACs indicated that the FACS procedure did not abolish the biological activity of purified artificial chromosomes. Furthermore, by pronuclear microinjection of FACS purified artificial chromosomes, successful generation of transgenic mice and germ line transmission of mouse SATAC was demonstrated (Co et al., 2000).

The generation of prototype hSATACs presented here demonstrates that the inductive approach represents an effective method for construction of human artificial chromosomes in vivo. In one transfection experiment, and by rounds of single-cell subcloning, different stable clones could be produced with different hSATACs. We showed that DNA sequences specific to the centromeric/short arm regions of human chromosomes are appropriate tags, and useful markers in characterizing the hSATACs generated in the NOR region of human chromosomes. For possible human gene therapy applications, construction of hSATACs on a human NOR chromosome of choice in stable monochromosomal human-rodent hybrids (Cuthbert et al., 1995), may serve as an appropriate platform.

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