Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7125–7130, July 1996
Genetics

Mini-chromosomes derived from the human Y chromosome by telomere directed chromosome breakage

(centromere/artificial chromosome)

R. HELLER, K. E. BROWN*, C. BURGTORF†, AND W. R. A. BROWN‡

Cancer Research Campaign Chromosome Molecular Biology Group, Biochemistry Department, Oxford University, South Parks Road, Oxford OX1 3QU, England

Communicated by Maynard V. Olson, University of Washington, Seattle, WA, January 30, 1996 (received for review September 20, 1995)

ABSTRACT **We have used telomeric DNA to break two acrocentric derivatives of the human Y chromosome into mini-chromosomes that are small enough to be size-fractionated by pulsed-field gel electrophoresis. One of the mini-chromosomes is about 7 Mb in size and sequence-tagged site analysis of this molecule suggests that it corresponds to a simple truncation of the short arm of the Y chromosome. Five of the mini-chromosomes are derived from the long arm, are all rearranged by more than a simple truncation, and range in size from 4.0 Mb to 9 Mb. We have studied the mitotic stabilities of these mini-chromosomes and shown that they are stably maintained by cells proliferating in culture for about 100 cell divisions.**

We would like to define the sequence requirements for human chromosome function, to develop chromosome-based vector systems and to understand chromosome behavior during cell division. Telomeres are the one cis-acting functional component of human chromosomes that have been cloned and shown to function upon re-introduction into human cells (1, 2). This created the possibility of using telomeric DNA to systematically winnow down a functional human chromosome into mini-chromosomes, which would help define the minimal size and sequence composition consistent with accurate mitotic segregation and regular replication (3).

The Y chromosome is among the best characterized in the human karyotype (4). We have therefore used telomeric DNA to engineer mini-chromosome derivatives from the human Y chromosome. This has involved three rounds of telomere-directed chromosome breakage (summarized in Fig. 1). In the first (5), we targeted telomeric DNA to the centromeric array of alphoid DNA in each of the two possible orientations. This generated a pair of acrocentric derivatives. One of these, ΔYq74, was composed of Yp, 140 kb of alphoid DNA, and the breakage construct; the other, ΔYp134, was composed of Yq, 550 kb of alphoid DNA, and the breakage construct. Both segregated accurately at mitotic anaphase, although ΔYp134 showed occasional aberrant anaphase movement. These results suggested that alphoid DNA is sufficient for accurate chromosome segregation but that other sequences may be required for full centromere function. Here we describe the second and third round of telomere-directed chromosome breakage.

MATERIALS AND METHODS

Pulsed-field gels were run in 0.5 TAE as described in ref. 6. Gels to size-fractionate mini-chromosomes were of 0.5% agarose (Sigma; low electro endo osmosis) and were run with a voltage gradient of 0.75 V/cm^{-1} across the gel at 5°C for 14 days with a pulse time of 2 h. Cells were embedded in agarose plugs at a concentration of 10^7/ml. Blotting was to Genescreen (DuPont) membranes in 10 × SSC overnight. Membranes were baked for 1 h at 80°C *in vacuo* and then irradiated with pre-calibrated UV light at 250 nm to fix the DNA onto the nylon. Hybridization and washing were as described (5). Typically we used probes labeled to 2×10^5 dpm/ng DNA at a concentration of 10 ng/ml in the hybridization solution. Hybridization was for between 14 and 18 h at 68°C and the washed filters were then exposed to Fuji-RX x-ray film for up to 14 days at −70°C. The probes were all as described in ref. 5 with the exception of the 0.7 kb *Hin*dIII–*Eco*RV fragment of pSVgpt (7) which we used as the *gpt* probe.

The *svgpt* gene is described in ref. 7, the hygromycin phosphotransferase thymidine kinase fusion gene in ref. 8, and the *S. cerevisiae* ADE2 gene in ref. 9. We used hygromycin B at 200 μg/ml, hypoxanthine/aminopterin/thymidine (HAT) at 100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine and 6-thioguanine (6-TG) at 5 μg/ml. Cells were cloned by two (ΔΔ2) or three rounds of single colony isolation prior to stability analysis. All other materials and methods are described in ref. 5.

RESULTS

Strategy and Screens. Targeted breakage during the first stage of the experiment was inefficient (5). We have therefore used random telomere-directed chromosome breakage for the second and third rounds of truncation. We have introduced a plasmid, gptADE2TEL (Fig. 1*B*), into Chinese Hamster Ovary cells containing either ΔYq74 or ΔYp134 and selected clones that were either $G418^r$, HAT^r, $DXYS20^-$ or $G418^r$, HAT^r, $DYZ1^-$, respectively, using filter hybridization to colony lifts of CHO hybrid cells as described (5). This generated four mini-chromosomes: Δ1, Δ7, Δ128, and Δ196 (Fig. 1*A*).

The *gpt* gene can be counter-selected using 6-TG and so we carried out a third round of breakage using HyTkADE2TEL (Fig. 1*B*) selecting for cells with 6-TG, G418, and hygromycin. This produced two further mini-chromosomes; ΔΔ2 and ΔΔ8 (Fig. 1*A*).

A Mini-Chromosome Derived from the Short Arm of the Y Chromosome. We screened approximately 18,000 ΔYq74-containing cells that had been stably transfected with linearized gptADE2TEL plasmid. We isolated 30 $DXYS20^-$ clones and then screened these for mini-chromosomes by *in situ* hybridization of Y chromosome centromeric alphoid DNA to metaphase chromosomes. This led to the isolation of 16 clones containing autonomous Yp derived mini-chromosomes. The remaining clones contained translocations between ΔYq74 and one or another hamster chromosome. We analyzed the Y

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PFGE, pulsed-field gel electrophoresis; 6-TG, 6-thioguanine; STS, sequence tagged site.

*Present address: Medical Research Council Clinical Research Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London, England.
†Present address: Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, Berlin, Dahlem, Germany.
‡To whom reprint requests should be addressed.

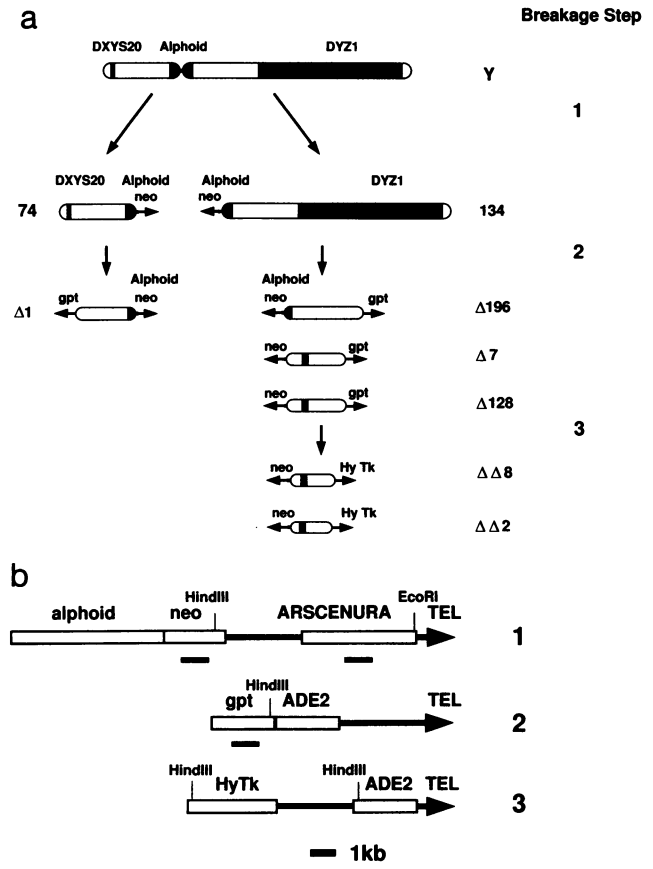

FIG. 1. Dissecting the Y chromosome into mini-chromosomes. (*A*) Sequence of breakage steps used to generate the mini-chromosomes described in this paper. Solid blocks indicate the repeated sequences studied in the course of this work. The DXYS20 and DYZ1 sequences were used for selecting broken chromosomes and the alphoid DNA was the target of the first round of breakage described in ref. 5. The mini-chromosomes Δ7, Δ128, and the Δ128 third round breakage derivatives, ΔΔ2 and ΔΔ8, have deleted most of their alphoid DNA and extensively rearranged their euchromatic sequences. The block in the icons representing these mini-chromosomes indicates the 1.5 Mb array of DYZ1 and DYZ2 (4) sequences remaining on these chromosomes. (*B*) Plasmids used to break the Y chromosome into mini-chromosomes. Each of the linearized plasmids used in the three rounds of telomere directed breakage is indicated schematically together with the positions of the probes and restriction sites used in the experiments illustrated in Fig. 2*C*. There are no *Eco*RI sites in the second round breakage construct.

chromosome sequence content of the clones containing autonomous chromosomes using filter hybridization. They fell into a simple nested set (results not shown). In nine clones, however, the repeated sequences DYZ5 (4) or DYZ3 (alphoid) were rearranged and these clones were excluded from subsequent study. We analyzed the remaining seven clones by pulsed-field gel electrophoresis (PFGE) and filter hybridization (Fig. 2*A*) to a probe specific for the Y alphoid DNA, the first round breakage construct (neo) and for the second round breakage construct (gpt). Clones Δ1 and 17c contained alphoid and neo cognate mini-chromosomes and were size-fractionated by gel electrophoresis. The size of the 17c mini-chromosome was approximately the same as *S. pombe* chromosome 1, which is 5.7 Mb in size (10). The Δ1 chromosome cannot be sized accurately because it is bigger than the largest *S. pombe* chromosome. However, the size fractionation characteristics of the gels (6) in the region just below the mobility limit suggest that the size of Δ1 is about 7 Mb. The other five mini-chromosomes were larger and were not examined further. Chromosome Δ1 contains gpt cognate sequences and is therefore a candidate for a mini-chromosome that has been truncated by the second round breakage construct. Chromosome 17c does not and was not studied further. *In situ* hybridization of total human DNA to metaphase chromosomes demonstrated that Δ1 contained just a single human derived mini-chromosome (Fig. 2*B*).

We wanted to confirm that Δ1 had been truncated by telomere directed chromosome breakage with the second round breakage construct and that the first round construct was intact. We therefore digested DNA extracted from ΔYq74 cells and from Δ1 cells with either *Eco*RI or *Hin*dIII. Digests were analyzed by gel electrophoresis and filter hybridization to a probe either specific for the first (the *S. cerevisiae* cen4 sequence) or the second round breakage construct (the gpt probe). As expected from the maps of the construct (Fig. 1*B*), the cen4 probe recognizes a fuzzy band in the *Hin*dIII digests of both ΔYq74 and Δ1 and a sharp band in the corresponding *Eco*RI digests (Fig. 2*C*). This is consistent with the first round construct being adjacent to a telomere. The gpt probe shows some cross hybridization to the neo sequences in the first round breakage construct and correspondingly recognizes weak signals in the *Hin*dIII and *Eco*RI digests at 7 and 21 kb, respectively. (The gpt probe does not recognize a fuzzy band in the *Hin*dIII digest of ΔYq74 because there are *Hin*dIII sites between the gpt cognate fragments and the telomere.) The gpt probe recognizes two strongly hybridizing, discretely sized fragments at 5.9 and 7.0 kb in the *Hin*dIII digests of Δ1: the larger of these comigrates with the cross-hybridizing neo fragment. This observation suggests that there are two sites of integration of the second round breakage construct in the Δ1 line. There are two gpt cognate *Eco*RI fuzzy bands in the Δ1 line at 11 and 13.2 kb indicating that both of these sites are telomeric. This was confirmed by digestion with the exonuclease BAL31 (not shown). *In situ* hybridization confirmed that the second round breakage construct had integrated at two sites; one signal was on a mini-chromosome and one signal was at the tip of a hamster chromosome (not shown). These results, considered with the results of the gel electrophoresis and filter hybridization, suggested that the second round breakage construct had truncated ΔYq74 to produce Δ1, but that there was a second site of integration on one of the hamster chromosomes. To confirm this we transferred Δ1 to the human fibroblast line HT1080 by micro-cell transfer using the G418 resistance gene to select for transfer. This led to the isolation of 11 G418-resistant lines. PFGE established that the Δ1 chromosome was intact in 8 of these 11 lines. Conventional gel electrophoresis showed that the 5.9 kb gpt cognate *Hin*dIII fragment was also present in these eight lines but not in the other three. Analysis of one of these lines, HTΔ1, is also illustrated in Fig. 2*C*. Comparison of the sequence content of the original Δ1 line with that of ΔYq74 suggests that Δ1 was generated by a simple truncation; sY34 to sY78 (11) were present and the rest of the short arm STS markers that we analyzed were deleted (Fig. 4*A*). Although the length of the short arm of the Y chromosome is unknown it is likely to be between 10 and 12 Mb (4). The size and sequence composition of Δ1 are therefore consistent. In conclusion, these results show that we have generated a Yp derived mini-chromosome of approximately 7 Mb in size by two rounds of telomere directed chromosome breakage.

Mini-Chromosomes Derived from the Long Arm of the Y Chromosome by Two Rounds of Telomere-Directed Breakage. We screened approximately 62,000 ΔYp134 containing hprt$^-$ Chinese Hamster Ovary hybrid cells that had been stably transfected with linearized svgptADE2TEL by colony hybridization with DYZ1 and selected 26 DYZ1$^-$, HAT^r, G418^r clones. These were then analyzed by *in situ* hybridization of a Y alphoid probe to metaphase chromosomes and 13 clones containing autonomous mini-chromosomes were isolated. PFGE and filter hybridization with an alphoid probe showed

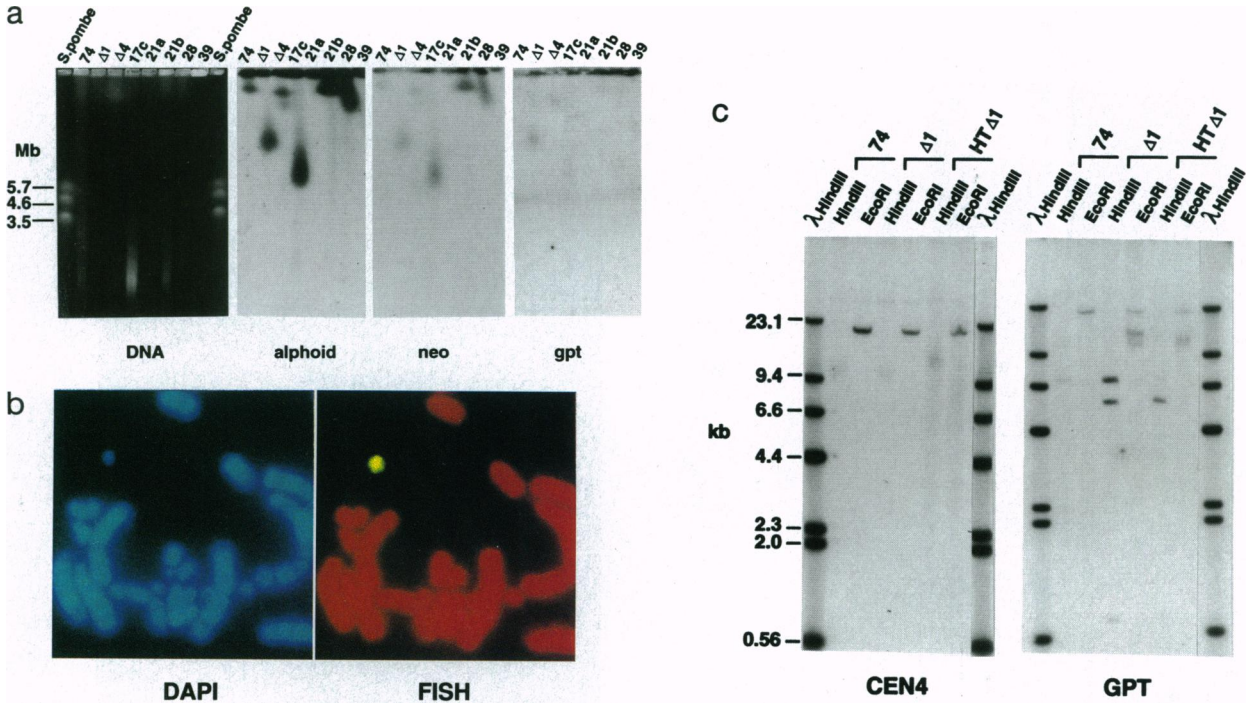

FIG. 2. Δ1: A mini-chromosome derived from the short arm of the Y chromosome. (*A*) PFGE of Y chromosome short arm derived mini-chromosomes. DNA extracted from each of the indicated cell lines was size-fractionated by PFGE as described in *Materials and Methods*. The gel was stained with ethidium bromide, photographed, and analyzed by filter hybridization with the indicated probes. The gel consisted of two identical panels of which one was analyzed first with the neo probe and then the alphoid probe, the other panel was hybridized with the gpt probe. (*B*) Analysis of Δ1 using fluorescence *in situ* hybridization. Metaphase chromosomes from the original Δ1 cell line were hybridized *in situ* to biotin-labeled total human male DNA. Hybridized DNA was detected using fluorescein-labeled avidin. The chromosomes were counterstained with DAPI and propidium iodide and photographed through an epifluorescent microscope. The Δ1 mini-chromosome is the small 4′,6-diamidino-2-phenylindole (DAPI)-stained object in the top left of the left panel and the yellow object in the same position of the right panel. No other human DNA was detected in this line (×2000). (*C*) Conventional gel analysis of the telomeric location of the breakage plasmids used to construct Δ1. DNA from the cell lines ΔYq74, Δ1, and HTΔ1 was digested with either *Hin*dIII or *Eco*RI and then analyzed after gel electrophoresis by filter hybridization with the cen4 or gpt probes indicated in Fig. 1*B*. Positions of the *Hin*dIII and *Eco*RI sites in the first and second round breakage constructs are also indicated in Fig. 1*B*.

that 9 of the 13 mini-chromosomes were identical (not shown). We therefore analyzed one (Δ113) and the four other chromosomes (Δ7, Δ128, Δ194, and Δ196) by PFGE and filter hybridization with the alphoid, neo, and gpt probes (Fig. 3*A*). All of the chromosomes were size fractionated and, as expected, hybridized to the first round breakage construct probe (neo) and the alphoid probe. Chromosomes Δ7, Δ128, and Δ196 also hybridized to the gpt probe. These chromosomes were therefore candidates for ΔYp134 derived chromosomes that had been truncated a second time by cloned telomeric DNA. Δ7 and Δ128 were close to the size of chromosome 1 of *S. pombe* and were only 6 Mb in size. Δ196 was too large to be accurately sized but seems likely to be around 9 Mb given the separation characteristics of the gel. Analysis of conventional digests using the approach described above showed that there is a single telomeric copy of the second round breakage construct in each of these mini-chromosomes and that the first round construct is still telomeric (not shown). The results of Fig. 3*A*, however, suggested that the structure of the alphoid DNA in these chromosomes had been rearranged because DNA from equal numbers of cells was loaded in each of the four tracks, but the amount of hybridization to the alphoid probe is variable. To examine the nature of rearrangements we first compared the structure of the alphoid DNA in these lines with that in the starting line ΔYp134. We digested DNA extracted from each of the lines with *Bam*HI; an enzyme that does not cut within the alphoid array on the Y chromosome, size fractionated the digests by PFGE, filter transferred, and hybridized the filter with a neo probe specific for the first round breakage construct and with an alphoid probe (Fig. 3*B*). The results confirmed that the structure of the alphoid DNA array has rearranged in Δ7, Δ113, Δ128, and Δ196 with respect to the starting line ΔYp134. The size of the alphoid DNA array in Δ7, Δ113, and Δ128 was reduced from 550 kb to 110 kb in size and had become separated by at least one *Bam*HI site from the first round breakage construct that was present on a *Bam*HI fragment less than 50 kb in size. In Δ196, the alphoid DNA appears to have been partially duplicated with only one of the two blocks in this line remaining associated with the first round breakage construct detected by the neo probe. Further evidence for rearrangement in these lines was provided by mapping the sequence content of these chromosomes (Fig. 4*B*). Most of the proximal long arm extending from sY79 to sY129 was deleted from Δ7 and Δ128, whereas Δ196 retained from sY78 to sY102. These results show that Δ7, Δ128, and Δ196 are derived from the Y chromosome by more than a simple pair of truncations. It is difficult however to interpret these data in terms of any simple pattern of rearrangement because several of these STSs are repeated and dispersed along the Y chromosome. In light of this uncertainty, we therefore used a total human genomic DNA probe and fluorescent *in situ* hybridization to show that in the Δ7, Δ128, and Δ196 lines the human DNA was localized on a mini-chromosome (Fig. 3*C*). (There was no hybridization of the probe elsewhere in any of the spreads.) There was one copy of the cognate mini-chromosome in more than 50% of the Δ7 and Δ128 cells, but 90% of the Δ196 cells contained two cognate mini-chromosomes. We have not mapped the Δ196 mini-chromosomes that are identical in size and appearance; both contain the first and second round breakage constructs and similar amounts of alphoid DNA. It therefore seems likely that the presence of two copies of the Δ196 chromosome is the

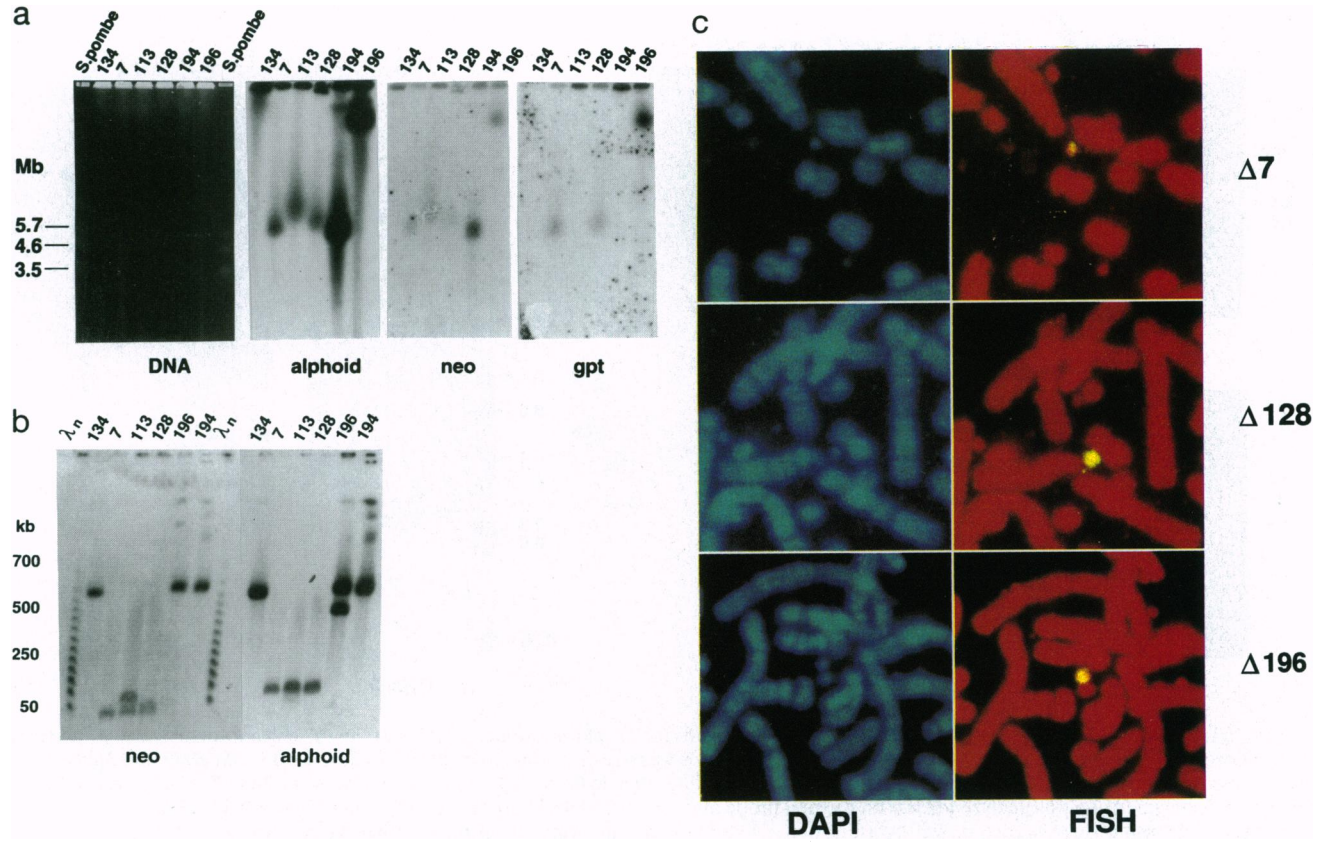

FIG. 3. Mini-chromosomes derived from the long arm of the Y chromosome. (*A*) PFGE of Y chromosome long arm derived mini-chromosomes. DNA extracted from each of the indicated cell lines was size fractionated by PFGE as described. The gel was then stained with ethidium bromide, photographed, and analyzed by filter hybridization with the indicated probes. The gel consisted of two identical panels of which one was analyzed first with the neo probe and then the alphoid probe and the other panel was hybridized with the gpt probe. (*B*) Analysis of alphoid DNA on the Yq derived mini-chromosomes. DNA from each of the indicated lines was restricted with *Bam*HI and then size-fractionated by PFGE together with multimers of phage λ DNA. The gel was filter transferred and hybridized to a neo probe mixed with a small amount of radiolabeled λ DNA (*left*), and then hybridized to an alphoid probe. (*C*) Analysis of long arm derived mini-chromosomes using fluorescence *in situ* hybridization. Metaphase chromosomes from the Δ7, Δ28, or Δ196 cell lines were hybridized *in situ* to biotin-labeled total human male DNA. Hybridized DNA was detected using fluorescein-labeled avidin. The chromosomes were then counterstained with DAPI and propidium iodide and photographed through an epifluorescent microscope. The mini-chromosome is the small DAPI stained object (*left*) corresponding to the yellow object in the same position (*right*). No other human DNA was detected in any of these lines (×1900).

result of an inaccurate segregation event early in the culture of this clone. To check for the possibility that these long arm derived mini-chromosomes had incorporated hamster DNA during the rearrangements that appear to have occurred during their generation we also analyzed them by fluorescent *in situ* hybridization using both total hamster DNA and hamster Cot1 DNA as probes. No hamster DNA was detectable on these mini-chromosomes. We have also recovered DNA sequences flanking the constructs at the neo end of Δ7 and shown that they derive from the human Y chromosome.

Long Arm Derived Mini-Chromosomes Produced by Three Rounds of Telomere-Directed Chromosome Breakage. We used the linearized plasmid HyTkADE2TEL (Fig. 1*B*) for a third round of telomere-directed breakage and isolated 12 Δ128 derived clones of which 2, ΔΔ2 and ΔΔ8, contained chromosomes, which at 4.0 Mb and 4.5 Mb in size (Fig. 5) were smaller than the starting Δ128 chromosome and that were further deleted for sequences present in Δ128 (Fig. 4). Further analysis showed that both the ΔΔ2 and ΔΔ8 mini-chromosomes had been truncated by the third round construct (not shown). Seven of the remaining clones contained mini-chromosomes that were similar to Δ128 in size and that may have been broken very close to the *gpt* gene or have inactivated the *gpt* gene by mutation or by an epigenetic mechanism. The remaining three clones had lost a detectable mini-chromosome. It is surprising that although ΔΔ2 is only about one-half the size of the starting chromosome Δ128, ΔΔ2 is deleted for only 6 of the 31 STSs present on Δ128. This may reflect the fact that many of the STSs in this set are repeated and the possibility that some sequences were duplicated during the rearrangement, which gave rise to the precursor of Δ128.

Stability of Mini-Chromosomes upon Prolonged Mitotic Proliferation. Do mini-chromosomes small enough to be size-fractionated by gel electrophoresis segregate accurately at mitosis and are they retained by cells proliferating in culture?

We first used whole cell *in situ* hybridization (5) to analyze the behavior of Δ1 at mitosis. In the 1066 cells scored, more than 99% of the cells contained mini-chromosomes segregating either 1:1 or 2:2. As measured by this assay the segregation of this mini-chromosome Δ1 was comparable to that of the starting Y chromosome or the precursor chromosome ΔYq74 (5).

Next we examined the stability of Δ1, Δ7, Δ196, and ΔΔ2 on prolonged culture in the presence and absence of selection on the neo^r gene. Both the Δ7 and Δ196 contained significant numbers of cells with two chromosomes suggesting the occasional inaccurate segregation event; however, the results of gel electrophoretic analysis (Fig. 6) and cytogenetic studies (Table 1) indicate that the chromosomes are maintained stably under either regime. We could easily have detected a 25% loss of any of the mini-chromosomes during the 3 months of culture. The doubling time of our cells is about 20 h so the rate of loss is unlikely to exceed 1 event in every 400 doublings.

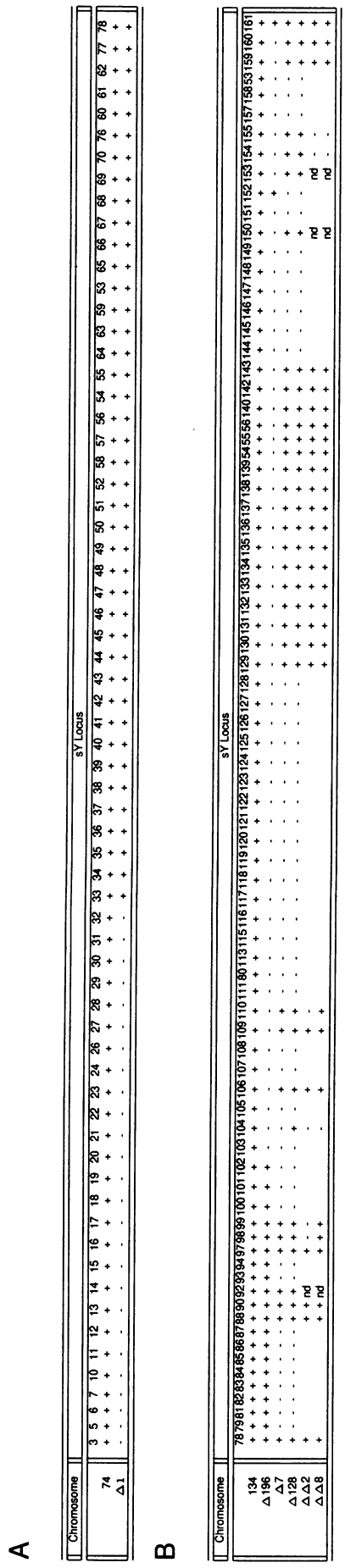

FIG. 4. STS content of the Y derived mini-chromosomes. (*A*) STS content of the short arm derived mini-chromosome Δ1 and of ΔYq74. (*B*) STS content of the long arm derived mini-chromosome and of ΔYp134. The STS contents of the indicated mini-chromosomes were determined using PCR and the primer sequences defining the respective STSs as described in ref. 11. Empty spaces indicate STSs that were absent from Δ128 and were not assayed in the derived mini-chromosomes, ΔΔ2 and ΔΔ8. nd corresponds to STSs that were present in the Δ128 and were not assayed in the derived mini-chromosomes, ΔΔ2 and ΔΔ8. The STSs in each panel are oriented in the conventional way, running from the telomere of the short arm on the left to the centromere (*A*) and from the centromere to the most telomeric of the long arm sequences on the right (*B*). STS sY78 is an STS that identifies the alphoid DNA sequence and is held in common by all of the chromosomes.

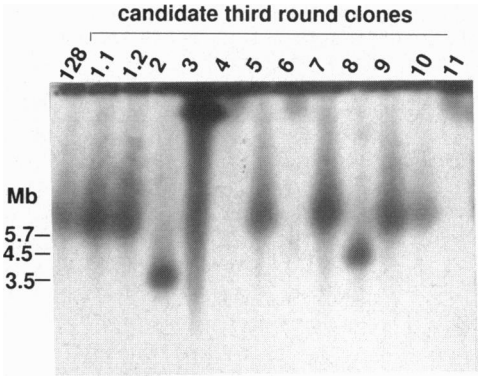

FIG. 5. Construction of mini-chromosomes by a third round of telomere directed chromosome breakage. PFGE of candidate third round breakage derived clones. DNA extracted from the Δ128 line and from Δ128 derived clones that had been transfected with HyTkADE2TEL and were G418, 6-TG, and hygromycin resistant was size-fractionated by PFGE and analyzed by filter hybridization with a Y alphoid probe.

DISCUSSION

This report describes the construction and characterization of a set of mini-chromosome derivatives of the human Y chromosome. PFGE indicates that they range from about 3.5 Mb to an

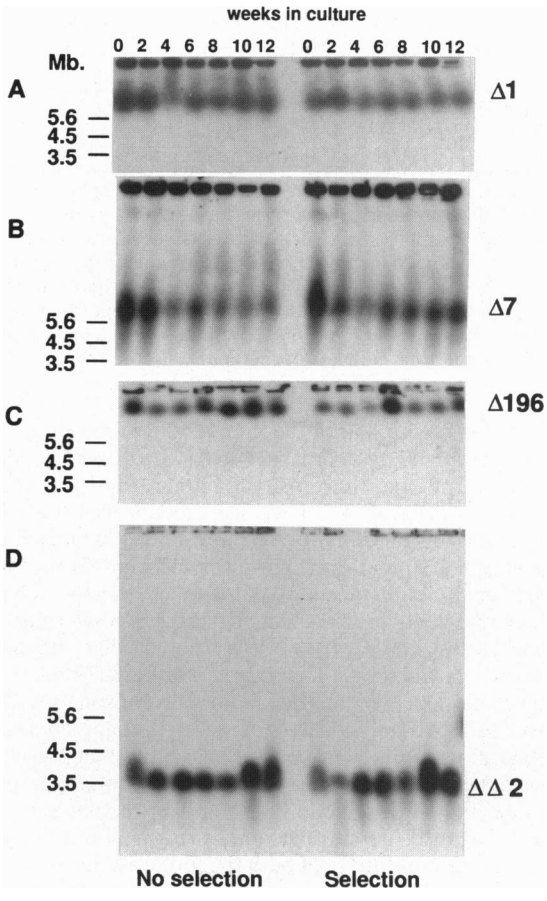

FIG. 6. Stability of the mini-chromosomes Δ1, Δ7, Δ196, and ΔΔ2 analyzed by PFGE. DNA extracted from cells of (*A*) Δ1, (*B*) Δ7, (*C*) Δ196, or (*D*) ΔΔ2, grown either in the absence of G418 (No selection) or in the presence of 100 μg/ml G418 (Selection) for the indicated time, was size-fractionated by PFGE and then analyzed by filter hybridization with an alphoid DNA or DYZ1 (ΔΔ2) probe to detect the mini-chromosome, the position of which is indicated on the right hand side of the respective panel.

Table 1. Stability of the Y derived mini-chromosomes assessed by fluorescent *in situ* hybridization to metaphase spreads

Weeks in culture	Selection	No. of chromosomes per metaphase					
			Δ1				
		0	1	2	3	4	>4
0		4 (3)	118 (96)	1 (1)	0	0	0
6	+	2 (2)	83 (95)	2 (2)	0	0	0
12	+	3 (4)	80 (97)	2 (2)	0	0	0
6	−	4 (7)	80 (94)	1 (1)	1 (1)	0	0
12	−	10 (9)	100 (89)	2 (2)	0	0	0
			Δ7				
0		3 (2)	63 (45)	55 (39)	10 (7)	9 (6)	0
6	+	2 (2)	30 (23)	70 (52)	20 (15)	7 (4)	4 (3)
8	+	2 (2)	28 (26)	55 (52)	14 (13)	6 (6)	1 (1)
12	+	3 (2)	20 (12)	87 (52)	39 (24)	13 (8)	4 (2)
4	−	2 (2)	56 (43)	58 (45)	12 (9)	1 (1)	0
8	−	10 (7)	65 (44)	58 (40)	14 (10)	0	0
12	−	2 (1)	55 (38)	83 (57)	5 (3)	0	1 (1)
			Δ196				
0		2 (1)	18 (11)	135 (85)	4 (3)	0	0
4	+	2 (2)	17 (16)	82 (77)	5 (5)	1 (1)	0
8	+	0	34 (21)	108 (67)	19 (12)	0	0
12	+	3 (2)	39 (25)	94 (61)	18 (12)	1 (1)	0
4	−	3 (2)	26 (17)	117 (75)	10 (6)	0	0
12	−	5 (3)	29 (19)	115 (73)	7 (5)	1 (1)	0
			ΔΔ2				
0		14 (7)	183 (90)	5 (2)	2 (1)	0	0
2	+	14 (11)	107 (87)	2 (2)	0	0	0
6	+	6 (5)	118 (92)	4 (3)	0	0	0
12	+	18 (10)	150 (88)	3 (2)	0	0	0
2	−	13 (9)	120 (87)	5 (4)	2 (1)	0	0
6	−	7 (5)	142 (95)	0	0	0	0
12	−	21 (14)	125 (82)	5 (3)	0	1 (1)	0

Cell lines containing either Δ1, Δ7, Δ196, or ΔΔ2 were cultured for the indicated time in the absence or presence of 100 μg/ml of G418. Metaphase chromosomes were analyzed by fluorescence *in situ* hybridization using total human genomic DNA as a probe. Approximately 100 spreads at each point were analyzed. The figures in these columns represent the number of spreads of each type seen at the indicated time point. In the brackets these numbers are expressed as percentages of the total number of metaphases scored at each time point.

estimated 9 Mb in size. All of the mini-chromosomes have two ends defined by the two breakage constructs used in their construction and we therefore conclude that the mini-chromosomes are linear. We have size fractionated the mini-chromosomes using pulsed-field gels run at both 120-min (shown above) and either 90-min (not shown) or 150-min (Chris Tyler-Smith, personal communication) pulse times and the relative mobilities of the mini-chromosomes and of the *S. pombe* markers vary consistently. These observations suggest that the mini-chromosomes have a simple topology and are unknotted.

We have analyzed the stability of mini-chromosomes Δ1, Δ7, Δ196, and ΔΔ2. All are as stable as we can reliably measure. Our estimate of stability ignores the possibility that the chromosomes are being retained as a result of complementation. We consider that such selection is an unlikely explanation for our results because the chromosomes are small and because they include representatives of both the long and short arms of the Y chromosome. In earlier experiments (5), we transferred the long or short arm acrocentric chromosomes from the first round of breakage to human fibroblasts and demonstrated that they were as stable in human cells as in the hybrids. These results suggest also that selection is not responsible for the stability of the mini-chromosomes in the Chinese hamster ovary cells. The estimates of stability that we provide are qualitative. However, we want accurate measures of the rate of inaccurate chromosome segregation in mammalian cells as a function of size and sequence organization. The HyTk gene like the *gpt* gene is counterselectable and so it should be possible to engineer still smaller derivatives of the Y chromosome and then use fluctuation analysis to approach this problem. Fluctuation analysis will be worthwhile once we have more mini-chromosomes and have entered the domain in which these chromosomes are obviously unstable. All of the work that we have carried out hitherto has involved transformed cells that have been through many rounds of intense selection. Mini-chromosome stability in these cells may therefore reflect mutations in checkpoint control genes. Because of this limitation, it would also be valuable to introduce our mini-chromosomes into mice and measure their stabilities on genetically defined backgrounds as well as during meiosis.

Although the sample size is small it is striking that all of the mini-chromosomes that are derived from ΔYp134 have rearranged their alphoid DNA sequences. Δ7 and Δ128 were derived from distinct second round truncation events yet appear to be rearranged in similar ways. The simplest explanation for this is that there existed a rearranged precursor of both Δ7 and Δ128 in the ΔYp134 cells prior to the second round breakage. We noted earlier (5) that ΔYp134 showed evidence of aberrant centromere function as it sometimes lagged with respect to the other chromosome during mitotic anaphase. The rearrangements in the ΔYp134 derivative chromosomes may reflect this aberrant behavior.

Our observations may be of interest to those trying to assemble human artificial chromosomes from their constituents by recombination in *S. cerevisiae*. ΔΔ2 is about the size of the largest yeast artificial chromosome to have been described (Zoia Larin, personal communication) and slightly larger than the largest natural chromosome of *S. cerevisiae* that is variable in size but that can be as big as 2.5 Mb. It is as yet unclear whether it will be possible to use yeast to construct human artificial chromosomes, but our results suggest that size will not limit this approach.

An alternative to constructing artificial mammalian chromosomes from their constituents in yeast is to engineer mini-chromosomes directly in mammalian cells and then to shuttle them into a suitable host for further manipulation and analysis. The mini-chromosomes described here allow us to pursue this strategy and then return them to mammalian cells for functional study.

We thank Chris Tyler-Smith, Aarti Chand, and Ming Hong Shen for their comments on the manuscript. We also thank Chris Tyler-Smith for pulsed-field gel conditions. The work was supported by the United Kingdom Cancer Research Campaign and the Medical Research Council.

1. Farr, C., Fantes, J., Goodfellow, P. N. & Cooke, H. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7006–7010.
2. Barnett, M. A., Buckle, V. J., Evans, E. P., Porter, A. C. G., Rout, D., Smith, A. G. & Brown, W. R. A. (1993) *Nucleic Acids Res.* **21,** 27–36.
3. Brown, W. R. A. (1992) *Curr. Opin. Gen. Dev.* **2,** 479–486.
4. Affara, N. A., Bishop, C., Brown, W., Cooke, H., Davey, P., Ellis, N., Graves, J. M., Jones, M., Mitchell, M., Rappold, G., Tyler-Smith, C. Yen, P. & Lou, Y. F. C. (1994) *Cytogenet. Cell Genet.* **73,** 360–399.
5. Brown, K. E., Barnett, M. A., Burgtorf, C., Shaw, P., Buckle, V. J. & Brown, W. R. A. (1994) *Hum. Mol. Genet.* **3,** 1227–1237.
6. Southern, E. M., Anand, R., Brown, W. R. A. & Fletcher, D. S. (1987) *Nucleic Acids Res.* **15,** 5925–5943.
7. Mulligan, R. C. & Berg, P. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2072–2076.
8. Lupton, S. D., Brunton, L. L., Kalberg, V. A. & Overell, R. W. (1991) *Mol. Cell. Biol.* **11,** 3373–3378.
9. Stotz, A. & Linder, P. (1990) *Gene* **95,** 91–98.
10. Fan, J.-B., Chikashige, Y., Smith, C. L., Niwa, O., Yanagida, M. & Cantor, C. (1988) *Nucleic Acids Res.* **17,** 2801–2819.
11. Vollrath, D., Foote, S., Hilton, A., Brown, L. G., Beer-Romero, P., Bogan, J. S. & Page, D. C. (1992) *Science* **258,** 52–59.