Centromere formation in mouse cells cotransformed with human DNA and a dominant marker gene

(functional centromere/cotransfection/centromere-linked marker/immunofluorescence/in situ hybridization)

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ABSTRACT A 13,863-base-pair (bp) putative centromeric DNA fragment has been isolated from a human genomic library by using a probe obtained from metaphase chromosomes of human colon carcinoma cells. The abundance of this DNA was estimated to be 16–32 copies per genome. Cotransfection of mouse cells with this sequence and a selectable marker gene (aminoglycoside 3′-phosphotransferase type II, APH-II) resulted in a transformed cell line carrying an additional centromere in a dicentric chromosome. This centromere was capable of binding an anti-centromere antibody. In situ hybridization demonstrated that the human DNA sequence as well as the APH-II gene and vector DNA sequences were located only in the additional centromere of the dicentric chromosome. The extra centromere separated from the dicentric chromosome, forming a stable minichromosome. This functional centromere linked to a dominant selectable marker may be a step toward the construction of an artificial mammalian chromosome.

The centromere is a specialized region of the eukaryotic chromosome that is the site of kinetochore formation, a structure that allows the precise segregation of chromosomes during cell division and may play a role in the higher-order organization of eukaryotic chromosomes (1). The isolation and cloning of centromeres is essential not only to understand their molecular structure and function but also to construct stable artificial chromosomes. By taking advantage of centromere-linked genes, functional centromeres of lower eukaryotes (yeast) were isolated (for reviews, see refs. 2 and 3) and then combined with telomeres, which stabilize the chromosome ends, to permit the construction of yeast artificial chromosomes (4, 5) and initiate studies of chromosome function and genetic manipulation.

Unlike yeast, higher eukaryotic cells contain repetitive DNA sequences that form a boundary at both sides of the centromere. This highly repetitive DNA interacts with certain proteins, especially in animal chromosomes, to create a genetically inactive zone around the centromere. This pericentric heterochromatin keeps any selectable marker gene at a considerable distance, and thus repetitive DNA prevents the isolation of centromeric sequences by chromosome walking.

In this report we describe the isolation of a 14-kilobase (kb) DNA fragment obtained from a human genomic library. The probe used for library screening was isolated from mechanically fragmented human metaphase chromosomes by the immunoprecipitation with anti-centromere antibodies. By cotransfection of the 14-kb fragment of putative centromeric DNA and a selectable marker [aminoglycoside 3′-phosphotransferase gene (APH-II gene)], a mouse cell line was produced that carries a functioning additional centromere either in a dicentric chromosome or in a minichromosome. The newly formed centromere was capable of binding mouse centromere proteins, as shown by anti-centromere antibody binding. Furthermore, the human DNA, the APH-II gene, and vector DNA sequences were colocalized to the additional centromere. This transformed cell line, carrying a centromere-linked selectable marker, provides a tool to isolate components of a functional centromere and to build an artificial mammalian chromosome.

MATERIALS AND METHODS

Cell Lines. The human colon carcinoma cell line COLO 320 was grown in suspension in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. Mouse fibroblast cells (LMTK-) were maintained as a monolayer in F12 medium supplemented with 10% fetal calf serum.

Chromosome Isolation and Immunoprecipitation of Chromosome Fragments. Metaphase chromosomes of COLO 320 cells were isolated by our standard method (6). Isolated metaphase chromosomes were resuspended in 1 ml of buffer (150 mM NaCl/50 mM Tris-HCl, pH 7.5/10 mM MgCl2/5 mM 2-mercaptoethanol) at a DNA concentration of 1 mg/ml and digested with 500 units of EcoRI restriction endonuclease for 1 h. The suspension was diluted with 4 ml of IPP buffer (500 mM NaCl/10 mM Tris-HCl, pH 8.0/0.1% Nonidet P-40), sonicated for five 30-s bursts with an MSE 5-70 sonicator, and then centrifuged at 1500 × g for 10 min to remove unbroken chromosomes and large chromosome fragments. The supernatant contained only small (<1 μm) chromosome fragments as judged by light microscopy.

Protein A-Sepharose CL-4B (Pharmacia; 250 mg) was swollen in IPP buffer and incubated with 500 μl of human anti-centromere serum LU851 (7) diluted 1:20 with IPP buffer. A suspension of sonicated chromosome fragments (5 ml) was mixed with anti-centromere-Sepharose (1 ml) and incubated at room temperature for 2 h with gentle rolling. After three subsequent 25-ml washes with IPP buffer, the Sepharose was centrifuged at 200 × g for 10 min.

Abbreviation: APH-II, aminoglycoside 3′-phosphotransferase type II.

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tions were done according to ref. 8. DNA probes were labeled for library screening and for Southern blot analysis by random oligonucleotide priming (9). DNA sequencing was performed by the dideoxyribonucleotide method (10, 11).

Isolation of \( \lambda \) Clones. A \( \lambda \) Charon 4A human genomic library (12) was screened with \( ^{32}P \)-labeled (at 2 \( \times 10^5 \) cpm/\( \mu l \), specific activity) high molecular weight DNA isolated from the immunoprecipitated fragments. Eight strongly hybridizing clones were selected from 4 \( \times 10^5 \) plaques.

Transfection of Mouse Cells. The calcium phosphate method (13), with 40 \( \mu g \) of \( \lambda \)CM8 and 40 \( \mu g \) of AgtWESneo DNA per Petri dish (80 mm) and a 2-min glycerol shock, was used for transfection. Transformed cells were selected on growth medium containing G418 (Geneticin, Sigma; 400 \( \mu g/ml \)).

In Situ Hybridization. In situ DNA DNA hybridization to metaphase chromosomes was done with \( ^{3}H \)thymidine-labeled probes as described (14) or with biotin-labeled probes as described (15).

Indirect Immunofluorescence. Indirect immunofluorescence of mouse metaphase cells was done as described (7). When indirect immunofluorescence and in situ hybridization were performed on the same metaphases, mitotic cells were resuspended in a glycine/hexylene glycol buffer (7), swollen at 37°C for 10 min, cytocentrifuged, and fixed with methanol at -20°C. After the standard immunostaining (7), metaphases were photographed, then coverslips were removed with phosphate-buffered saline, and slides were fixed in ice-cold methanol/acetic acid [3:1 (vol/vol)], air-dried, and used for in situ hybridization.

Microscopy. In the in situ hybridization experiments with biotin-labeled probes, chromosomes were counterstained with propidium iodide (14), whereas in indirect immunofluorescence the DNA binding dye Hoechst 33258 was used.

RESULTS

Isolation of Putative Centromeric DNA. Metaphase chromosomes isolated from a human colon carcinoma cell line were mechanically fragmented by sonication after a limited endonuclease digestion. The suspension containing small chromosome fragments was incubated with human ant centromere serum coupled to protein A-Sepharose CL-4B. DNA was isolated from the chromosome fragments bound to anti-centromere-Sepharose. The centromeric DNA is in the structurally most-stable region of mammalian chromosomes (16). Therefore, this DNA is the most resistant to enzymatic digestion and mechanical shearing. Results of electrophoresis of immunoprecipitated and supernatant DNA support these observations (Fig. 1). The bulk of DNA from chromosome fragments that did not bind to the anti-centromere-Sepharose (supernatant) ranged from several hundred base pairs to 5 kb (Fig. 1, lanes A and B) whereas chromosome fragments bound to the anti-centromere-Sepharose contained an additional population of high molecular weight (9-20 kb) DNA fragments (Fig. 1, lanes C and D). This high molecular weight DNA was isolated from the agarose gel and used as a probe to screen a \( \lambda \) Charon 4A human genomic library (12), and 8 of the most strongly hybridizing clones were selected from 4 \( \times 10^5 \) plaques. Restriction enzyme analysis of the selected clones proved that these clones were identical, probably due to amplification of the genomic library. One of these clones (CM8) containing a 14-kb human DNA insert was used for further experiments. The restriction map of the 14-kb human DNA insert for some relevant restriction endonucleases is shown in Fig. 2. Southern blots of human lymphocyte DNA digested with various restriction endonucleases and probed with subfragments of the CM8 insert suggested that the 14-kb insert represents an uninterrupted part of the human genome (T.P., J.K., and G.H., unpublished results). Thus, extreme left and right EcoRI subfragments of the CM8 insert form parts of 10-kb and 13-kb genomic EcoRI fragments, respectively, and the inner subfragments of the insert are the same size as corresponding fragments of genomic DNA (data not shown).

In Southern blot experiments by simultaneously hybridizing a single-copy DNA probe (XV2c) (17) and the central XhoI-EcoRI fragment of the CM8 insert (Fig. 2) with serially diluted human peripheral lymphocyte DNA, the copy number of CM8 was estimated to be 16-52 per genome (data not shown).

In situ hybridization of \( ^{3}H \)thymidine-labeled CM8 DNA to human (COLO 320) metaphase chromosomes resulted in the preferential localization of silver grains to the centromeres (Fig. 3). We found a centromeric signal on representatives of all chromosome groups, and no preferential hybridization to any individual human chromosome was detected.

In situ hybridization using biotin-labeled subfragments or the whole CM8 insert was not detected by the standard method (15). Furthermore, if a hybridization method that is suitable for single-copy-gene detection with a biotin-labeled probe was used (18), apart from the typical R-band-like Alu hybridization pattern (19), no specific hybridization signal was detected on any of the chromosomes with the whole 14-kb CM8 insert. A likely explanation for this negative result is that centromeric sequences are not accessible to the hybridization probe due to their compact packing.

The sequence of the 13,863-base-pair human CM8 clone showed no homology to any known sequence when compared with a complete nucleic acid data bank [MicroGenie].

![Fig. 1. Agarose gel electrophoresis of DNA fragments obtained by immunoprecipitation. Lanes: A and B, DNA isolated from chromosome fragments remaining unbound to anti-centromere-Sepharose; C and D, DNA isolated from chromosome fragments bound to anti-centromere-Sepharose (note the presence of a population of high molecular weight DNA fragments); B and D, samples treated with RNase A (100 \( \mu g/ml \)) prior to electrophoresis; M, HindIII-digested \( \lambda \) markers.](image)
sequence software (MG-1M-5.0), Beckman). However, a 300-base-pair Alu repeat, deficient in the flanking direct repeat sequences, was found in the 2.5-kb EcoRI-Xho I fragment (Fig. 2), which explains the Alu-type in situ hybridization pattern obtained with the CM8 insert.

**Human DNA and the Dominant Selectable Marker Form a Centromere.** To detect any in vivo centromere function of the human putative centromeric DNA, the CM8 DNA and the selectable APH-II gene were introduced into mouse LMTK- cells by the calcium phosphate coprecipitation method (13). The pAG60 plasmid (20) containing the APH-II gene was cloned into a AgtWES (21) bacteriophage vector. We decided to introduce the whole λCM8 and AgtWES neo constructions into mouse cells for two reasons. (i) By separating the marker gene from the putative centromeric sequences, we hoped that it would be possible to avoid inactivating the APH-II gene, a process that may occur during centromere formation. (ii) λ DNA is capable of forming long tandem arrays of DNA molecules by concatamerization, and in Schizosaccharomyces pombe 4–15 copies of conserved sequence motifs form centromeres (22). Thus a multiplication of the putative centromeric DNA by concatamerization might increase the chance of centromere formation.

Transformed cells were selected on growth medium containing G418 (400 μg/ml). Individual G418-resistant clones were analyzed as follows. The presence of human sequences in the transformed clones was monitored using Southern blots probed with subfragments of the CM8 insert. Excess centromeres were screened for by indirect immunofluorescence using human anti-centromere serum LU851 (7). The chromosomal localization of “foreign” DNA sequences was determined by in situ hybridization with biotin-labeled probes.

Eight transformed clones have been analyzed. All of the clones contained human DNA sequences integrated into mouse chromosomes. However, only two clones (EC5/6 and EC3/7) showed the regular presence of dicentric chromosomes. Individual cells of clone EC5/6 carrying di-, tri-, and multicentromeric chromosomes exhibited extreme instability. In more than 60% of the cells of this cell line, the chromosomal localization of the integrated DNA sequences varied from cell to cell. Due to this instability, clone EC5/6 proved to be unsuitable for the present study. However, cells of clone EC3/7 were stable, carrying either a dicentric (85%) or a minichromosome (10%). Centromeres of dicentric chromosomes and minichromosomes were indistinguishable from the mouse centromeres by immunostaining with anticientromere antibodies (Fig. 4A and B). To determine whether newly introduced DNA was contributing to centromere formation, in situ hybridization with biotin-labeled CM8 and A phage DNA was carried out. Without exception these probes hybridized to the same spots: either the distal centromere of the dicentric chromosome (Fig. 4C) or the centromere of the minichromosome (Fig. 4D). In less than 5% of the EC3/7 cells was an alternative localization of the hybridization signal found. These included more than one

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Fig. 3. **In situ** hybridization of [3H]thymidine-labeled CM8 DNA to human metaphase chromosomes. (A) Preferential localization of silver grains to the centromeres of human chromosomes (arrowheads). (B) Distribution of silver grains (●) on 131 metacentric chromosomes. Numbers indicate the frequency of silver grain localization to certain regions of the chromosomes.

Fig. 4. Detection of dicentric and minichromosome of the EC3/7 cells by indirect immunofluorescence with anti-centromere antibodies (A and B) and by in situ hybridization with biotin-labeled CM8 probes (C) or with λ (vector) DNA sequences (D). A and C are the same field and B and D are the same field. Counterstaining of chromosomes was with propidium iodide. Arrowheads point to dicentric and minichromosomes.
integration site, the lack of a detectable signal, or hybridization to chromosomes other than the dicentric chromosome.

To demonstrate the integration of the human sequence and the APH-II gene in the centromeric region, immunostaining of centromeres with anti-centromere antibodies followed by in situ hybridization with CM8 and APH-II sequences was carried out on the same metaphase plates of EC3/7 cells. The in situ hybridization signals with both biotin-labeled probes colocalized with the immunostained centromeric region of the chromosomes carrying additional centromeres (Fig. 5).

Extra Centromeres Are Functioning. Extra centromeres of dicentric or minichromosomes bound the anti-centromere antibodies of the LU851 serum. By using this anti-centromere serum on a multicentromeric chromosome of a mouse cell line, only one centromere was labeled by the antibodies (data not shown). In hydroxyurea/caffeine-treated cells, where the chromosomes were fragmented in vivo (23), our anti-centromere serum recognized only the active centromeres (Károly Fátyol, J.C., T.P., V.T., F.K., and G.H., unpublished results).

The regular breakage of the dicentric chromosome and the formation of the minichromosome strongly suggested that the extra centromeres were capable of binding mitotic spindles. When the mouse centromere and the extra centromere are pulled in opposite directions, the extra centromere could break-off.

By subcloning EC3/7 cells, we have established two single-cell-derived stable cell lines (EC3/7C5 and EC3/7C6) that carry the extra centromere on minichromosomes. Indirect immunofluorescence with anti-centromere antibodies and subsequent in situ hybridization experiments proved that the minichromosomes were derived from the dicentric chromosome. In both cell lines, the majority of the hybridization signal was found on the minichromosomes but traces of CM8 or λ sequences were detected at the end of a monocentric chromosome (Fig. 6). In EC3/7C5 and EC3/7C6 cell lines (140 and 128 metaphases, respectively), no dicentric chromosome was found and minichromosomes were detected in 97.2% and 98.1% of cells, respectively. These cell lines provided direct evidence that the extra centromere was functioning and was capable of maintaining the minichromosomes.

Stability of Transformed Cell Lines. Immunofluorescence analysis of EC3/7 cells (103 metaphases) cultured for 46 days in nonselective medium showed that 80.6% of the cells contained either a dicentric chromosome (60.2%) or a minichromosome (20.4%). Subsequent hybridization with biotin-labeled probes proved the presence of “foreign” DNA in the additional centromeres (data not shown). These results indicated that no serious loss or inactivation of the extra centromeres occurred during this period of culture under nonselective conditions.

To estimate the copy number of the integrated human DNA in the extra centromere, DNA of the EC3/7 cell line and human lymphocyte DNA were digested with restriction endonucleases and probed with subfragments of the CM8 insert in a Southern blot hybridization experiment. By comparing the intensity of the hybridization signal on EC3/7 DNA to that of the human DNA, the minimum number of integrated human sequences in the additional centromere was estimated to be >30.

Fig. 5. Colocalization of the integrated DNA sequences with the centromeric region detected by immunostaining with anti-centromere serum. (A) DNA staining with propidium iodide. (B) Indirect immunofluorescence with anti-centromere serum. (C) Subsequent in situ hybridization with biotin-labeled APH-II probe to the same dicentric chromosome of the EC3/7 cells. Arrowheads show the extra centromere of the dicentric chromosome.

Fig. 6. Indirect immunofluorescence with anti-centromere serum (A) and in situ hybridization of biotin-labeled CM8 sequences (B) to metaphase of EC3/7C5 cell line carrying stable minichromosome. Arrowheads show the minichromosome and traces of CM8 sequences left at the end of the formerly dicentric chromosome. Counterstaining was with propidium iodide.
DISCUSSION

We have described a transformed mouse cell line (EC3/7) that carries a dicentric chromosome or, less frequently, a minichromosome. The additional centromere of the dicentric chromosome and the centromere of the minichromosome were formed in vivo after the cotransfection of mouse cells with human putative centromeric DNA and a dominant selectable marker (APH-II). The presence of human DNA, the APH-II gene, and the vector sequences in the additional centromeres was demonstrated by in situ hybridization. These sequences colocalized with the immunostained centromeric region, as visualized by indirect immunofluorescence with anti-centromere antibodies. As a rule, one of the centromeres of dicentric chromosomes is functionally inactive (24). In spite of this general phenomenon, three lines of evidence suggest that the extra centromeres in the EC3/7 cell line are functioning. (i) The extra centromeres bind anti-centromere antibodies from LUB81 serum. This anti-centromere serum, which contains four anti-centromere antibodies (7), recognizes only active centromeres. This is in agreement with the previous finding (25) that centromere proteins can only be detected by immunostaining at active centromeres. (ii) Minichromosomes containing the newly formed centromere regularly appeared in EC3/7 cells. The functioning additional centromere probably leads to chromosomal breakage during mitosis and, therefore, minichromosomes are the break-off products caused by the extra functional centromere. (iii) Two stable cell lines were isolated by subcloning EC3/7 cells. In these cell lines, the extra centromere was separated from the mouse chromosome and formed a stable minichromosome that was maintained by the functioning extra centromere of EC3/7 origin.

It has been suggested that the inactivation of centromeres might occur by an alteration in chromatin conformation (25) such as heterochromatinization. However, this process for EC3/7 cells carrying the centromere-linked APH-II gene would be suicidal under selective conditions. In this respect, the proximity of the APH-II gene to the centromere safeguards the maintenance of the functional centromere.

Calculations made on the basis of the sizes of ACM8 and ΔgtWESneo constructions and their estimated copy numbers suggest that the integrated DNA may exceed a million base pairs in length. However, rearrangements within the sequences used for transfection may alter this expected size. In fact, hybridization of the extreme ends of the CM8 insert to EC3/7 DNA suggests a significant rearrangement of these sequences in the extra centromere (unpublished data). At this time we cannot rule out the possibility that mouse DNA sequences might be involved in the formation of the additional centromeres. Consequently, we think that it is premature to conclude that the isolated human DNA fragment present in the additional centromere has the sole capacity for the formation of a functional centromere. In spite of this, the cell line carrying a centromere-linked dominant selective marker offers a unique opportunity to isolate the components required in a functional centromere of a mammalian chromosome. Furthermore, these cell lines may provide a step toward the construction of an artificial mammalian (human) chromosome.

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