Evolutionary Formation of New Centromeres in Macaque

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A systematic fluorescence in situ hybridization comparison of macaque and human synteny organization disclosed five additional macaque evolutionary new centromeres (ENCs) for a total of nine ENC. To understand the dynamics of ENC formation and progression, we compared the ENC of macaque chromosome 4 with the human orthologous region, at 6q24.3, that conserves the ancestral genomic organization. A 250-kilobase segment was extensively duplicated around the macaque genome sequencing consortium. For a comprehensive marker-order comparison revealed that seven macaque/human homologs (chromosomes 6/5, 8/8, 11/12, 17/13, 19/19, 20/16, and X/X, respectively) were colinear when the position of the centromere was excluded. However, human chromosomes 7/21, 14/15, and 20/22 form syntenic associations as part of three compound macaque chromosomes (3, 7, and 10, respectively). Differences in marker order between macaque and humans were accounted for by 20 chromosome rearrangements. Retention fluorescence in situ hybridization (FISH) experiments with additional BAC clones more precisely defined rearrangement breakpoints (table S1). A summary of all results is graphically displayed at www.biologia.uniba.it/macaque. Tables 2S and 3S provide a comprehensive list of the ~600 clones that were used in FISH experiments, from the perspective of both macaque and human chromosomes, respectively.

This comprehensive marker-order comparison revealed that the centromeres of many orthologous chromosomes were embedded in different genomic contexts. To distinguish whether ENC events had occurred in the human or macaque ortholog, or in both, we took into account previous reports that attempted to establish the ancestral form for each chromosome (2, 3, 6–15). Most of these papers use a different macaque chromosome nomenclature; here we follow the nomenclature used by the macaque genome sequencing consortium. For a comparison, see www.biologia.uniba.it/macaque.) The

Table 1. Macaque chromosomes with neocentromeres. The two noncontiguous positions defining, in human, the ENC of chromosome 1 are due to the colocalization of the ENC with a macaque-specific inversion breakpoint.

<table>
<thead>
<tr>
<th>MMU (HSA)</th>
<th>Clones</th>
<th>Position of the neocentromere on the human sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1)</td>
<td>RP4-621015</td>
<td>chr1:226,810,735–226,866,653</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>RP11-572K18</td>
<td>chr1:160,918,751–161,035,790</td>
<td>Present study</td>
</tr>
<tr>
<td>4 (6)</td>
<td>RP11-474A9</td>
<td>chr4:6,145,651,644–145,845,896</td>
<td>Present study</td>
</tr>
<tr>
<td>13 (2p)</td>
<td>RP11-722G17</td>
<td>chr13:86,622,638–86,827,260</td>
<td>Present study</td>
</tr>
<tr>
<td>15 (9)</td>
<td>RP11-542K23/RP11-64P14</td>
<td>chr15:9,124,189,785–124,493,134</td>
<td>Present study</td>
</tr>
<tr>
<td>17 (13)</td>
<td>RP11-543A19/RP11-527N12</td>
<td>chr17:13,61,11,769–62,699,203</td>
<td>Present study</td>
</tr>
<tr>
<td>18 (18)</td>
<td>RP11-61D1/RP11-289E15</td>
<td>chr18:150,155,761–50,526,34</td>
<td>Present study</td>
</tr>
</tbody>
</table>
results of this analysis confirmed the previously published results and exposed five macaque ENCs (Fig. 1 and Table 1). In total between macaque and human, there are 14 ENCs; 9 ENCs occurred in the macaque lineage [MMU1 (1), 2 (3), 4 (6), 12 (2q), 13 (2p), 14 (11), 15 (9), 17 (13), and 18 (18) (corresponding human chromosomes in parentheses)], and 5 occurred in the human lineage [HSA3 (2), 6 (4), 11 (14), 14 (7a), and 15 (7b) (corresponding macaque chromosomes in parentheses), where HSA denotes Homo sapiens]. The newly discovered macaque ENCs were found on MMU1 (1), 12 (2q), 13 (2p), 15 (9), and 18 (18) (corresponding human chromosomes in parentheses). In this context, all macaque centromeres, including the nine ENCs, harbor very large arrays of alpha satellite DNA (16) (fig. S1). One possibility is that after their emergence, new macaque centromeres were rapidly stabilized by acquiring alpha satellite DNA.

Human chromosome 6 and the macaque homolog, MMU4, both have ENCs. The ancestral centromere for both species was located at HSA6p22.1 (9) (Fig. 2), and the new macaque centromere is located at HSA6q24.3. A comparison of the HSA6q24.3 region [chr6: base pair 139,100,001 to 149,100,000; University of California Santa Cruz (UCSC) March 2006 release] with the orthologous regions of dog, rat, mouse, and opossum genomes, by careful inspection of the specific alignment “Net” in the UCSC genome browser (http://genome.ucsc.edu), showed that a reasonable assumption was that the human region closely resembled the ancestral condition. We reasoned that a detailed comparison of the organization of the MMU4 centromeric-pericentromeric region with the organization of the human counterpart at 6q24.3 might allow us to examine hypotheses of the formation and progression of ENCs.

Human BAC RP11-474A9 (L2 in Fig. 2) mapping at chr6:145,651,644 to 145,845,896 yielded an apparently splitting signal around the MMU4 centromere (9). It was therefore considered to be the probable seeding point, and the FISH analysis of flanking markers was consistent with this conclusion (Fig. 3). The construction of a BAC contig spanning the MMU4 centromere started, therefore, from the L2 locus and is reported in detail in the supporting online material (SOM) text. To briefly summarize this construction, appropriate human sequence tagged sites (STGs), mapping within 1 megabase from both sides of the MMU4 centromere, were used to screen high-density filters of the macaque BAC library CH250, segment 1 (http://bacpac.chori.org). FISH analysis of these BACs showed that some of them were duplicated on both sides of the MMU4 centromere. The sequencing of BAC ends and of appropriate polymerase chain reaction (PCR) products and FISH

Fig. 1. All macaque (left) or human (right) chromosomes harboring ENCs. Quinacrine mustard banded macaque (left) and human (right) chromosomes are also displayed. Details are reported at www.biologia.uniba.it/macaque. Some macaque chromosomes are shown upside down to facilitate comparison in accordance with the orientation in the whole-genome assembly. The Ns in red circles flank ENCs; the As in black circles flank ancestral centromeres. Synteny block conservation is indicated by the color. The arrows on the left of some macaque synteny blocks indicate that these blocks have opposite sequence polarity, with respect to the corresponding block in humans. These annotations, obviously, are affected by the upside down position of some chromosomes. The Human Genome Project has assumed as “+” sequence polarity the 5′→3′ strand that starts from the tip of the short arm of each chromosome. The assumption that centromeres were conserved leads to a violation of this rule for macaque chromosomes 1 (1), 2 (3), 4 (6), 10 (20/22), 13 (2p), and 18 (18) (corresponding human chromosomes in parentheses).
experiments on stretched chromosomes (Fig. 3D) allowed for the construction of a contig defining the duplicated pericentromeric region. In summary, seven imperfect copies of a 250-kb segment, mapping at the seeding point, were duplicated both proximally and distally to the MMU4 centromere. The global tiling path and the detailed organization of the central duplicated region are shown (Fig. 4, A and B). Duplicated regions appear to be co-oriented with respect to each other and to the human sequence assembly. Unexpectedly, a high proportion of BAC ends of duplicated clones were alphoid in nature (30 out of 46). These alphoid sequences showed a monomeric structure that is typical of peripherally located alpha satellite sequences (table S8). Small, repeated inversions in the duplicated regions might be hypothesized to account for these findings. This hypothesis, however, clashes with the apparent co-orientation of the duplicated blocks.

Data from human pericentromeric regions have shown that the ratio of inter- versus intrachromosomal duplications is about 6:1 (1). We previously suggested that duplications of the ENC of macaque chromosome 17 (human 13) were intrachromosomal only (3), but in that case only human probes were used, which precluded any firm conclusion on the absence of interchromosomal duplications. Our current results show that the MMU4 pericentromeric duplications detected by FISH were strictly intrachromosomal and originated only from the ENC seeding point. We have also shown that centromeres of human chromosomes 3, 6, 11, 14, and 15 are ENCs (6, 9–11). Chromosomes HSA3 and HSA6 match the pattern we found on MMU4, whereas human chromosomes 11, 14, and 15 accommodate large blocks of interchromosomal duplications (1). A careful analysis of the evolutionary history of the latter chromosomes, however, showed that it was very likely that large blocks of segmental duplications were already present or simultaneously seeded in the ENC region (10, 11). It could therefore be hypothesized that a novel centromere triggers only local duplication activity, whereas interchromosomal duplications are triggered by distinct forces, probably linked to intrinsic properties of specific sequences (17, 18). However, until further cases are studied we cannot rule out that the duplications we detected on MMU4 are simply macaque-specific.

The corresponding human region in proximity to the L2 marker was investigated for gene content. A relatively large region (780 kb) harboring the MMU4 centromere has not been annotated in the UniProt, RefSeq, and GenBank mRNA databases. The two closest genes on opposite sides, UTRN (chr6:144,654,658 to 145,209,657) and EPM2A (chr6:145,988,133 to 146,098,299), are 778 kb apart. Heterochromatin supposedly silences encoded genes (19). Genes mapping to regions where a centromere repositioning occurred might be at risk of silencing, but recent reports have indicated that a neocentromere by itself does not repress gene expression (20–22). The gene silencing might be attributed to the successive heterochromatization of the region. The average gene content in the human genome is about 1 gene per 100 kb (www.ncbi.nlm.nih.gov). Human chromosome 6 contains about 1272 genes, on average 1 gene every 131 kb (23). Consequently, six genes would be expected in the 778-kb gene-desert area. A similar gene-desert area was also found around the ENC of the Old World monkey chromosome homologous to human chromosome 13 (3). Our data appear to support the hypothesis that the absence of genes in the ENC seeding region can play an important role in ENC maintenance and progression. Analyses of additional ENCs and their corresponding regions in the human genome will be required to determine whether this is a stochastic occurrence or whether it represents a prerequisite for novel centromere survival. This hypothesis initially appears to be contradicted by the presence of active genes at the centromeres of rice chromosomes 8 (24) and 3 (25). However, Nagaki et al. and Yan et al. suggest that these two rice centromeres may represent ENCs that are still acquiring the full heterochromatic organization that is typical of normal centromeres, and the analysis of the fully sequenced Arabidopsis genome strongly supports the view that the absence of gene expression in centromeres is also a general rule in plants. Alternatively, it could be hypothesized that the heterochromatization process pushes the surrounding genes to pericentromeric regions without affecting their expression.

Ferreri et al. (26) reviewed the various hypotheses formulated to explain ENC and clinical-neocentromere emergence. One hypothesis proposes that the centromere seeding event is essentially epigenetic in nature and is sequence independent (27). Another hypothesis considers the seeding regions to be domains with inherent latent centromere-forming potentiality (11, 28). A third hypothesis suggests that rearrangements trigger neocentromere seeding through chromatin repatterning (11). Roizes (29) has suggested that damage to a centromere, like retroposon insertion, could trigger the emergence of evolutionary neocentromeres. All of these hypotheses consider clinical neocentromeres and ENCs to be strictly related.

An unexpected finding is the high number of ENCs in recent human and Old World monkey evolution. In the 25 million years since macaque and human divergence, 14 ENCs have arisen and become fixed in either the human or the macaque lineage. It is difficult to escape the conclusion that ENCs had a considerable impact on shaping the primate genome and that they are fundamental to our understanding of genome evolution. Knowledge of centromere repositioning, for instance, provides a cogent explanation for the unusual clustering of human clinical neocentromeres at 15q25, the domain of an inactivated ancestral centromere (11). Despite their relevance, ENCs have never been identified on the basis of sequence analysis alone. Indeed, the extensive pericentromeric duplication we report has not been identified in the macaque genome assembly, reinforcing the opinion

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**Fig. 2.** A reconstruction of chromosome 6 evolution in primates (modified from (9)). The letters indicate the specific BAC clones used in the study (9) and reported in table S9. PA, primate ancestral; CA, Catarrhini ancestral; and A.C., ancestral centromere. The letter N in a red circle represents a novel centromere. A square bracket encompasses the Old World monkey–specific (macaque included) inversion of K-L-M markers, with respect to the human form. MMU4 is upside down, with respect to the correct position as reported in Fig. 3, to allow for an easy synteny comparison. L2, human BAC RP11-474A9.

**Fig. 3.** (A to O) Partial metaphases showing examples of FISH experiments on macaque and human chromosomes, with the use of the non-duplicated BAC clones (A) CH250-209I15 and (B) CH250-215I15 and the duplicated clone (C) CH250-284C24. (Mapping details are given in Fig. 4. A and B) (D) Partial metaphases showing FISH on stretched chromosomes with the use of the duplicated BAC clones CH250-188G18 (red) and CH250-67B18 (green), containing alphoid sequences. The arrows indicate MMU4.
that an integrated, multidisciplinary approach is needed for high-quality genome assembly and for comparative genomics (30).

The present data extend the link between segmental-duplication bias and centromeres to additional primate species. The homology and shuffling of sequences creates substrates for evolutionary innovation (the birth of new genes) and instability (via non-allelic homologous recombination). Lastly, the contig assembly we have constructed represents a framework for the complete sequencing of the pericentromeric region of MMU4 ENC through a direct sequencing of BAC templates, as opposed to whole-genome shotgun sequencing.

References and Notes
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17. J. E. Horvath et al., Genome Res. 15, 914 (2005).
31. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material
www.sciencemag.org/cgi/content/full/316/5822/243/DC1
Materials and Methods
SOM Text
Figs. S1 and S2
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References
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