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ABSTRACT

The review is concerned with a progress in genetic modification of a mammalian genome *in vitro* and *in vivo* at chromosomal level. Recently three new approaches for the chromosome biotechnology have been developed: Using *Cre/loxP*-system a researcher is able to produce targeted rearrangements of whole chromosomes or their segments or particular genes within the genome, and therefore to modify the set, position and copy number of the endogenous elements of the genome. Mammalian artificial chromosomes (MACs) provide a possibility to introduce into genome relatively large segments of alien chromosome material, either artificially constructed or derived from the genome of different species. Using ES-somatic cell hybrids allows to transfer whole chromosomes or their fragments between different genomes within and between species. Advantages and limitations of these approaches are discussed.

Key words: chromosome biotechnology, *Cre/loxP*, ES cells, MAC.

INTRODUCTION

Genetic modification of a mammalian genome is the most fascinating technology developed in the last two decades. In fact, now this technology allows one to manipulate with genetic materials *in vivo* from individual genes (in case of transgenesis) to whole genome(s) for cloning of animals. For a long time transgenic animal technology was a single approach for genetic modification of a mammalian genome (Chan 1999). Classic variant of transgenesis relies on microinjection of exogenous DNA into the pronuclei of the zygotes. Then the injected DNA can be incorporated into the recipient genome

(Palmiter & Brinster 1986, Gordon 1997). The incorporated exogenous DNA ("transgene") is able to be inherited and, i.e., to be transmitted in the next generation so that to produce animals with novel gene(s). The approach provided a fast progress in the study of gene function and regulation of development (Palmiter & Brinster 1986, Chan 1999). Initially, the approach was applied to mice only but in a short time it became used in the experiments with farm animals as well (Hammer *et al.* 1985, Chan 1999). However, the DNA injected via pronuclear microininjection is usually been integrated into the recipient genome in a random manner. For this reason, expression of transgene varies in a wide range among transgenic animals. Presumably this variation depends upon a site of integration ("position effect") or copy number.

This problem was solved when of the gene targeting approach has been developed (Thomas & Capecchi 1987). This approach allows one to introduce an *in vitro* modified gene via homologous recombination between exogenous DNA and targeting chromosome DNA. This method is in of great use in genome modification of pluripotent embryonic stem (ES) cells. ES cells with modified targeted gene can be combined with early embryos to produce chimeras (see below) which, in turn, are able to generate offspring carrying the modified targeted gene (Robertson 1987). The gene targeting has opened the gate to a new era in transgenic technology named as a site-directed mutagenesis or "knock out" technology (Capecchi 1989). Numerous publications on "knock-out" mice have appeared in the last decade. These animals are in a great use as a tool to studies in gene regulation, as well as in modeling human inherited diseases.

In spit the progress in transgenic technology, development of the technology for making large-scale alternations to the mammalian genome including manipulation with whole or fragments of chromosomes *in vivo* was yet to come. Five years ago such technology has been successfully developed and applied to ES cells *in vitro* (Smith *et al.* 1995, van Deursen *et al.* 1995) and even *in vivo* (Raminez-Solis *et al.* 1995). The technology may be named as chromosome engineering (Raminez-Solis *et al.* 1995) or chromosome biotechnology. More recently two new approaches for the chromosome biotechnology have been developed: one is transfer of whole chromosome or its fragments into a mammalian genome by ES-cell vector, and another is the creating mammalian artificial chromosomes.

The review is concerned with a progress in genetic modification of a mammalian genome *in vivo* at chromosomal level.

Cre/loxP-MEDIATED GENERATION OF SITE-SPECIFIC INTRA- AND INTER- CHROMOSOMAL REARRANGEMENTS IN MAMMALIAN GENOME

Recent development of a new sophisticated approach based on use of *Cre-loxP* system has revolutionized the transgenic biotechnology of animals (Metzger & Feil 1999). The system functions as following manner. The recombination is initiated by *Cre* proteins (cyclization recombinase) binding to 13-bp inverted repeats in the *loxP* sites (a size of *loxP* is 34 bp) and promoting synapsis (or joining) of a pair of sites. *Cre* proteins catalyze exchange between the pair of sites within an asymmetric 8-bp central spacer sequence of *loxP* site by concerted cleavage and rejoining reactions. The recombination between two *loxP* sites inserted in opposite orientation leads to inversion. In case of recombination between two *loxP* sites inserted in direct orientation results in deletion or translocation. Thus, the *Cre-loxP*-system provides a site-specific recombination, and it is predictable on the basis of the relative orientations of the *loxP* sites.

Remarkable merits of the *Cre-loxP* system was firstly in transgenesis. Site-specific recombination mediated by *Cre-loxP* approach allows researchers to control not only site-specific integration and copy number of transgene but also to replace or delete precisely any sequence within a targeting gene (Gu *et al.* 1994, Araki *et al.* 1995, Lasko *et al.* 1996, Babinet 1997). Moreover, the site-specific recombination mediated by *Cre-loxP* has been used to manipulate gene expression in a cell type specific or inducible manner at the stage of development, which need to be investigated (Schwenk *et al.* 1995, Babinet 1997, Metzger & Feil 1999).

The *Cre/loxP* system was somewhat latter applied to induce a variety of chromosome rearrangements in ES cells that can be selected and than used for produce ES-derived mice (Smith *et al.* 1995, van Deursen *et al.* 1995, Raminez-Solis *et al.* 1995). *Cre/loxP*-mediated intra-and interchromosomal rearrangements can generate large deletions, inversions, duplications, and translocations.

A general strategy to produce *Cre/loxP*-mediated site-directed chromosomal rearrangements can be illustrated by an approach developed by Raminez-Solis *et al.* (1995). The authors used, as a selectable gene marker, the *HPRT* minigene (hypoxanthine phosphoribosyltransferase) fused to

a *PGK* promoter (phosphoglycerate kinase-1 gene), intron 2 of *HPRT* gene and poly(A^+) signal. The *HPRT*-minigene was modified by the insertion of a *loxP* site in the *HPRT* intron. This construct is cleaved into two parts; one contained the *PGK* promoter, the exons 1 and 2, the *loxP*- intron 2, and *HPRT* exons 3-6 whereas another contained exons 3-9 and poly(A^+) signal. Thus, both secondary constructs contained a 2 kb overlap including the *loxP* site. Independently these parts do not provide HAT-resistance after transfection of *HPRT*-deficient ES cells. Each of the constructs was fused to positive selectable marker either the neo^+-resistance or puromycin-resistance gene to prepare of two positive selectable cassettes. Both types of positive selectable cassettes were ligated to the large genomic fragments (more than 5 kb) complementary to the genes selected for gene targeting. Raminez-Solis *et al.* (1995) chose for gene targeting the gene pairs located in mouse chromosome 11 with different distances relatively to each others: the $Hoxb1$ and $Hoxb9$ (a distance between the genes is 90 kb), the Gs and the E_2DH (a distance between the genes is 1.0 Mb), the E_2DH and Wnt (a distance between the genes is 3-4 cM), and the *Hoxb* cluster and the E_2DH (a distance is 3-4 cM). Both resulting cassettes were used for transfection of the HPRT-deficient ES cells. After two consecutive rounds of transfection of the ES cells by these resulting cassettes, double-targeted clones were isolated using a selective media; these clones were neo^+- and puromycin-resistance and HAT-sensitive.

To produce the ES cells carrying predicted chromosomal rearrangements, several double-targeted clones were transiently transfected with a *Cre*-expression cassette and placed under HAT selection. Transient expression of *Cre* induced recombination between the *loxP* sites and, as a result, reconstructed a functional *HRPT*-minigene. The reconstructed *HPRT*-minigene allowed the HAT-resistance clones to proliferate. In these clones a rearrangement of chromosome 11 between the *loxP* sites might be took place. Raminez-Solis *et al.* (1995) demonstrated that the *loxP*-mediated recombination in the double-targeted ES cell clones resulted in series of deletions ranging from 90 kb to 3-4 cM, inversions or duplications depending on the orientation of the *loxP* sites. Moreover, four clones carrying the deletion on one homolog and the duplication on another were used for production of chimeric mice by injection of these ES cells into blastocysts. Offspring of some chimeras were hemizygous for this deletion, heterozygous for the duplication or homozygous for the duplication. Most exciting result was that the mice were all fully viable. Thus, the strategy allows to design a variety of chromosome rearrangements *in vitro* and *in vivo* by a *Cre-loxP*-mediated site-directed recombination.

Using similar approach, Smith *et al.* (1995) have produced a chromosomal translocation between chromosome 15 (where the targeted $c-myc$ gene with a *loxP* site was located) and chromosome 12 (where another targeted immunoglobulin heavy chain gene with a *loxP* site was located) in the double-targeted ES cells after their transfection by transient *Cre*-expression. With the use of the *Cre-loxP* system, van Deursen *et al.* (1995) induced a site-specific translocation between the targeted *Dek* gene on chromosome 13 and the targeted *Can* gene on chromosome 2 in mouse ES cells.

Cre-loxP-mediated chromosome rearrangements have been generated not only *in vitro* in ES cells, but also *in vivo*. Special *deleter* strains of transgenic mice have been breed (Schwenk *et al.* 1995). The strains was produced by injection of a DNA construct into the fertilized eggs. The construct contained the *Cre* gene ligated to a cytomegalovirus minimal promoter. Schwenck *et al.* (1995) tested the transgenic mice in crossing to another transgenic mice carrying an allele of the DNA polymerase β gene flanked by *loxP* sites. All offspring had the deletion of the allele in all tissues. Moreover, back-crossing the F_1 generation to wild-type mice revealed that the *Cre*-mediated deletion was present in germ cells since the deletion was transmitted to the offspring. These strains made possible a targeted production of chosen deletions of whole chromosomes, their segments or ever par-

ticular genes. In fact, Lewandoski & Martin (1997) have produced mice with genotype XO by a *Cre-loxP*-mediated site-specific recombination. In the case, mice carrying two *loxP* sites in inverted orientation on Y-chromosomes were crossed with the transgenic females carrying a *Cre* gene that was expressed in the early embryo. Almost all male embryos loss the Y chromosome in early development. The authors suggested to use the approach as a common method to produce mice with deletion in any chromosomes.

More recently, Herault *et al.* (1998) suggested a new modification of the *Cre-loxP*-directed site-specific system. The authors have produced a transgenic mice carrying *Cre*-expessing transgene under the transcriptional control of *Sycp*1 promoter. The gene controls the synaptonemal complex protein-1, which is expressed at an early stage of male meiosis when homologous chromosome pairing occurs. They also generated of two lines of transgenic mice with *loxP* sites at different positions within the *Hoxd* complex and crossed them in order to obtain mice having both loci. Both *loxP* sites were in an orientation opposite to that of the endogenous *Hox* genes. This genetic construct induced meiotic *trans*-allelic recombination and two new haplotypes appeared: one with a duplication of *Hoxd*12 and the other with a deletion of this targeted gene. Essentially the same approach was used by Bunting *et al.* (1999) for targeted self-excision of *Hoxa*3 gene in the germ line.

Thus, *Cre/loxP*-system provides a powerful tool to produce targeted rearrangements of whole chromosomes or their segments or particular genes and by this way to modify structure and function of mammalian genome at large-scale.

MAMMALIAN ARTIFICIAL CHROMOSOMES (MAC) AND CHROMOSOMAL TRANSGENESIS

Initial motivation for creating artificial chromosomes in *Saccharomyces cerevisiae* was the need to elucidate the DNA components necessary for chromosome structure and function. Studies in yeast revealed that at least three components were needed to produce a linear, mitotically stable chromosomes: a centromere, two telomeres and origins of replication. This finding open a way to generation of mammalian artificial chromosomes (MACs).

The importance of MACs is obvious. They provide a unique tool to study the relationships between the chromosome structure and its function in mammalian cells, the organization and function of large genes and gene clusters. They also might serve as a tool of chromosomal transgenesis as they could carry relatively large genes or several genes of interest (Brown *et al.* 2000).

There are two approaches to building MACs: i) to assemble them *in vitro* from cis-acting functional sequences; ii) to produce microchromosomes and modify them *in vivo.*

The first approach has been developed by Harrington *et al.* (1997). They demonstrated that simultaneous transfection of human somatic cells with alpha satellite, telomeric and genomic carrier DNA could lead to *de novo* formation of wholly functional MACs. The resulting MACs were relatively large: 6-10 Mb, which corresponded to $10 - 20\%$ of the size of human chromosomes. These MACs shown to be cytogenetically stable and maintained for dozens of generations in culture.

Series of microchromosomes derived from human X and Y chromosomes have been generated using telomere-associated chromosome fragmentation. The fragmentation was achieved due to targeting telomeric DNA to the centromeric array of alphoid DNA in each of the two possible orientations (Farr *et al.* 1995, Heller *et al.* 1996). Heller *et al.* (1996) introduced several human Y-chromosome derived microchromosomes into mouse ES cells. The success was rather limited, however. Only one of the ES cell lines was able to maintain a 4 Mb microchromosome, which had acquired a functional mouse centromere.

The main problem which restricts the use of MACs is that they can only be manipulated by homologous recombination, which is inefficient in human and mouse cell lines. However, it has been shown that lymphoma-derived chicken cell lines

could be used to overcome this obstacle. Dieken *et al.* (1996) and Brown *et al.* (1996) suggested to transfer human microchromosomes or artificially assembled MACs into lymphoma-derived chicken cells by microcell fusion and then modify them by homologous recombination. After that these chromosomes can be transferred to ES cells by microcell fusion. The resulted ES cells containing these modified MACs then can be used to produce chimeric animals.

TRANSFER OF INDIVIDUAL CHROMOSOMES OR THEIR FRAGMENTS INTO THE GENOME VIA ES-SOMATIC CELL HYBRIDS

High developmental potential is a remarkable and unique feature of ES cells. There are ample data indicating that the ES cells derived from pre-implantation embryos (Robertson 1987) or primordial germ cells (Donovan 1994) are able to retain their pluripotency in *in vitro* culture conditions. The pluripotent capacity of ES cells can easily be revealed by their injection into the blastocoele. In this case, ES cells can contribute to the full range of adult tissues, including the germ line (Papaioannou & Johnston 1993, Hogan *et al.* 1994).

This prompted us to take advantage of the developmental potential of ES cells to reprogram single chromosomes derived from differentiated cells (Matveeva *et al.* 1996, 1998). We chose cell hybridization as a mild method for introduction of intact chromosomes of differentiated cells into the ES cell genome. When we started our fusion experiments of ES cells with splenocytes (Matveeva *et al.* 1996, 1998), it was unknown whether the ES-somatic cell hybrids would retain the pluripotency of the ES partner after close contact with the somatic genome. However, we were encouraged by previous reports that hybrid cells obtained by fusion of embryonic carcinoma (EC) cells with differentiated cells retain certain pluripotent properties (Miller & Ruddle 1977, Andrews & Goodfellow 1980, Gmur *et al.* 1980, Takagi *et al.* 1983, Takagi 1988, Serov *et al.* 1990).

In our experiment of 1996-1997, we isolated 4 clones of hybrid cells which have been obtained by a fusion of the ES cells with the splenocytes of an adult female of DD/c mice. Three clones contained 41-43 chromosomes (at 13-15th passage) whereas one was nearly tetraploid, and all expressed the selectable HPRT marker of the somatic partner. This was unexpected because most hybrid cells obtained so far by fusion of the EC or ES cells with somatic cells showed slow chromosome segregation and were either tetraploid or nearly tetraploid (Takagi *et al.* 1983, Takagi 1988, Tada *et al.* 1997).

The 3 hybrid clones with the near diploid karyotype shared features similar to the ES cells (Matveeva *et al.* 1996). They retained pluripotency including their ability to contribute to the full range of adult tissues (except the germ line, see below) of the chimeras. Moreover, analysis of the chimeras demonstrated that the X chromosome derived from the somatic partner did not segregate during their development, judging by the retained expression of its HPRT marker gene in many chimeric tissues (Matveeva *et al.* 1998). It should be emphasized that hybrid cells encounter non-selective conditions when injected into the blastocoele. The weak segregation of the "somatic" X chromosome suggests that the hybrid cell genome may not discriminate it from the "pluripotent" X chromosome. Thus, the "somatic" X chromosome in the pluripotent hybrid cells had the opportunity to reprogram and revert to the condition characteristic of the X of the pluripotent genome. High pluripotent features and evidence of reprogramming at the imprinting loci were observed in hybrid cells obtained by fusion of embryonic germ cells with lymphoid cells (Tada *et al.* 1997). Summarizing, our data demonstrated that the whole X-chromosome can be transferred from an adult animal into genome another using ES-somatic cell hybrids as a vector.

However, it should be noted that, in our case, there were no mice with the 129/Ola genotype among over 50 chimera offspring (Matveeva *et al.* 1998). The disbalanced of the sex chromosomes and trisomy of some autosomes are presumably responsible for it. Thus, cells with the XXY consti-

tution cannot pass through meiosis and give rise to gametes (Endo *et al.* 1991, Hernandez & Fisher 1999). Similarly, the trisomy might have decreased the proliferative activity of hybrid cells, which eventually conferred a selective advantage to the cells of the recipient embryo.

The origin of trisomy in ES-somatic hybrid cells is unclear. Analysis of chromosome segregation in intraspecific cell hybrids is difficult because the parental chromosomes are indistinguishable by the usual G-banding protocols. However, a set of microsatellite markers enabling the identification of any pair of homologous chromosomes derived from different stocks of mice is now available (Dietrich *et al.* 1992, a database from Jackson Laboratory: www.informatics.jax.org). With this in mind, more recently we have performed analysis of microsatellites for some chromosomes of hybrid clones by PCR-assisted methods (Dietrich *et al.* 1992). The PCR analyses of the hybrid clones demonstrated that they contained the markers for chromosomes 1, 11 and 15 of both parental partners, however HM-1-derived markers were predominantly represented whereas those representing DD/c chromosomes were present in amounts comparable to the number of cells with trisomy for these chromosomes (Serov *et al.* 1999). The data suggest that chromosome segregation of the somatic partner is not complete in the population of the hybrid cells and probably it is a cause of trisomy. However, it should be emphasized that 20-30% of cells had a diploid karyotype, 38 autosomes and XY at the 20-22 passage. Complete chromosome segregation cannot be excluded in these hybrid cells.

Surprisingly, all hybrid clones after the 20th passage showed the predominant presence of X chromosome markers derived from DD/c and not 129/Ola mice. In addition, a specific probe for the murine X chromosome painted only a single chromosome in most hybrid cells. Substitution of the X chromosome derived from the ES cells by "the somatic" X might have taken place in the hybrid cells during their long-term culturing in selective medium (Serov *et al.* 1999).

As mentioned above, it remained unclear whether germ line transmission of the "somatic" X in chimeras is possible or not: The unbalanced karyotype of the hybrid cells probably does not allow them to pass through meiosis. For this reason, our goal is to produce hybrid cell clones with a diploid karyotype, carrying the X chromosome derived from the somatic partner. We hope that such hybrid cells obtained by fusion of the ES cells with somatic differentiated cells would serve as vehicles of individual chromosomes from the genome of one individual to another obviating the sexual process.

Recently Tomizuka *et al.* (1997, 2000) developed another approach to transfer human chromosomes or their fragments into the murine genome by microcell-mediated chromosome transfer (MMCT). A scheme of the approach was following:

i) initially, the researchers have prepared somatic cell hybrids by fusion between human fibroblasts transformed by neo^R-selectable gene with murine established A9 cells. The primary hybrid cells contained a human chromosome carrying a selectable marker and served as donor for preparation of microcells;

ii) then, the investigators transferred human chromosomes from the primary hybrid cells into murine ES by microcell-fusion technique (Tomizuka *et al.* 1997). As a result, they have isolated a set of secondary ES-cell clones containing either human whole chromosome 22 (Hsa22) or fragments of human chromosomes 2 (Hsa2) or 14 (Hsa14);

iii) the resulting ES-cell clones were used to generate chimeras by microinjection the secondary ES-cell into the blastocoele. All ES-cell clones were able to produce chimeras. Moreover, many chimeras showed in most tissues a presence of human chromosomal material. The chimeras were named as trans-chromosomic (Tc) mice;

iv) the Tc chimeras were crossed with non-chimeric mice. Four Tc male chimeras, derived

from hCF(2-W23) clone (genotype 39,XY) carrying a fragment of Hsa2, produced 316 pups, of which 2 showed in their karyotype a presence of a fragment of Hsa2 (Tomizuka *et al.* 1997). Interestingly, among 67 offspring from four Tc female chimeras derived from the same ES-cell clone, 22 carried a fragment of Hsa2.

The transmission of human chromosomal material was possible through the male and female germ line, although its inheritance appeared to be more efficient through the females. It allowed the researches to establish a Tc W23 line of mice carrying a fragment of Hsa2 (a size is $\approx$ 2 Mb) and to observe its transmission for four generations. Also, it should be noted that chimeras carrying whole Hsa22 and a fragment (a size is $\approx$ 50 Mb) of Hsa14 did not transmit the human chromosomal material for the next generations.

More recently Tomizuka *et al.* (2000) have extended the experiments and produced a new Tc line of mice carrying a small fragment (a size is $\approx$ 1.5 Mb) of Hsa14. The authors isolated a subclone (A9/SC20) derived from a A9/14-C11hybrid clone obtained in the first fusion between human fibroblasts with mouse A9 cells (see above). A9/14-11 hybrid cells contained approximately one-fifth of an intact Hsa14 and were used as a donor for MMCT into a female murine ES cell line (TT2F cells, genotype 39, XO). Stability tests under the non-selective condition allowed the researchers to reveal one of the microcell ES hybrid clone (# 21) retained with high stability a fragment of Hsa14. The fragment of Hsa14 showed the presence of 14cen and 14qter sequences without the presence of murine satellites. Twenty chimeras were obtained by microinjection of # 21 ES cells into the blastocoele. The mating of two female chimeras with non-chimeric males resulted 10 of 30 offspring carrying a fragment of Hsa14. According to data of FISH and PCR analyses, the fragment of Hsa14 was quite stable transmittable in the next generations (Tomizuka *et al.* 2000).

The transferred fragments of chromosomes 2 and 14 carried the *Ig* (immunoglobulin) kappa light chain locus (Igk) and the heavy chain locus (*IgH*), respectively. The loci were expressed in proper tissue-specific manner in adult chimeric tissues (Tomizuka *et al.* 1997, 2000). Moreover, in the elegant experiments using double-Tc/double-knock out mice, the researchers demonstrated that the somatic cells of the mice retained both fragments of Hsa2 and Hsa14. A rescue of the defect of *Ig* production was achieved by high level of expression of human *IgH* and *Igk* in the absence of mouse heavy and kappa chains (Tomizuka *et al.* 2000). In addition, the double-Tc/double-knock out mice were immunized by human serum albumin (HAS). The mice produced antibodies against HAS with high titers. One of the HAS-immunized double-Tc/double-knock out mouse served as a donor for production of splenic hybridomas. Analysis of the resulting hybridoma supernatants demonstrated that the hybridomas were able to produce HAS-specific fully human IgG/k antibodies. These data indicated that the double-Tc/double-knock out mice can be used to obtain antigen-specific human monoclonal antibodies with various isotypes. High affinities of the resultant human monoclonal antibodies suggested that the authentic repertoire of fully human Igs was reconstituted in the double-Tc/double-knock out mice (Tomizuka *et al.* 2000). Thus, the study demonstrated that MMCT protocol is an efficient tool for introduction of very large amounts of foreign genetic material into mouse genome.

Recently Hernandez *et al.* (1999) have obtained a set of Tc chimeric mice carrying fragments (sizes from 0.2 to 50 Mb) of Has21. To produce the Tc animals, Hernandez *et al.* (1999) introduced a selectable marker into Hsa21 of human somatic cells. Then, the chromosome was transferred into murine ES cells by irradiated microcell-mediated chromosome transfer. The original MMCT (Tomizuka *et al.* 1997) was modified by irradiation of microcells before fusion with ES cells, in order to fragmentize the targeted Hsa21. Chimeric mice, obtained by injection of the ES-cell hybrids into the blastocoele, maintained the fragments of Hsa21 and expressed

the genes of the human chromosome in many tissues.

CONCLUSION

The three main approaches to chromosome biotechnology are aimed to different but somewhat overlapping target. Using *Cre/loxP*-system we are able to produce targeted rearrangements of whole chromosomes or their segments or particular genes within the genome, and therefore to modify the set, position and copy number of the endogenous elements of the genome. MACs provide a possibility to introduce into genome relatively large segments of alien chromosome material, either artificially constructed or derived from the genome of different species. Using ES-somatic cell hybrids we may transfer whole chromosomes or their fragments between different genomes within and between species. Each of these approaches has its own advantages and limitations. It seems prospective to combine them to solve particular problems.

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