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TARgeting the human genome to make gene isolation easy

Michael A. Resnick, Natalay Kouprina, and Vladimir Larionov

Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, NIH, P.O. Box 12233, 110 Alexander Dr., Research Triangle Park, NC 27709

Correspondence to: Michael A. Resnick, Tel: 919 541-4480; Fax: 919 541-7593, E-mail: resnick@niehs.nih.gov

Summary

Considerable information is now available about the human genome and expressed sequences have been identified for most genes. Until recently there was no opportunity to specifically isolate genes or specific chromosomal regions from genomic DNA. We have utilized transformation-associated recombination (TAR) in yeast to isolate genes and specific regions from total human DNA. This has been demonstrated by the direct isolation of complete copies of rDNA, BRCA1, BRCA2 and HPRT genes with high fidelity as yeast artificial chromosomes (YACs). We propose that there are many utilities of TAR cloning including gene therapy and diagnostics.

The Human Genome Project has made great strides in the decade since its inception including the cloning of most of the chromosomal DNA, the identification of unique sequences (sequence tags sites, STS's) approximately every 150 kb and the sequencing of short regions of almost all the expressed genes (expressed sequence tags, EST's). The project is ahead of schedule in that most of the genome will be sequenced within the next 5 to 10 years. In addition to understanding chromosome organization, this vast amount of information is leading to the isolation of genes that correspond to specific diseases, particularly through positional cloning. Furthermore, the genetic makeup of humans is better understood because of sequence relatedness between species.

Until now there has been little opportunity to utilize the information being generated to isolate specific large regions (i.e., greater than 10 to 20 kb) or genes directly from total genomic material. Virtually all cloning of chromosomal DNA from humans, or any organism, has involved the isolation of random DNA fragments into vectors through several steps of enzymatic treatment plus ligation and the subsequent transfer into the desired bacterial or yeast host. The isolation of specific DNAs would provide a variety of opportunities, including studies of human polymorphisms, clinical diagnosis, gene therapy and the filling-in of gaps in sequenced regions. However, the only available enrichment procedure has been the physical isolation of entire chromosomes (McCormick et al., 1993). Even then, the subsequent cloning of human DNAs has involved random DNA fragments.

Over the past year a new approach has emerged that is providing for the specific isolation of genes and regions directly from total human DNA. The approach draws upon several features of the yeast *Saccharomyces cerevisiae.* The first is that during transformation, yeast can take up several small and large molecules (Rudolph et al., 1985; Larionov et al., 1994). Secondly, intermolecular, as well as intramolecular, recombination is highly efficient during transformation between homologous, as well diverged DNAs (Larionov et al., 1994; Ma et al., 1987; Mezard et al., 1992). This includes double-strand break recombination between broken molecules. Thirdly, human DNA contains sequences (about 1 per 20-30 kb) that can function as origins of replication (*ARS*-autonomously replicating sequence) in yeast (Stinchcomb et al., 1980). These features have provided for the development of a novel method based on transformation-associated recombination (TAR) to target the isolation of specific DNAs from total human DNAs.

As described in **Figure 1**, genomic DNA is presented to yeast along with a molar excess of vector containing a selectable marker, a centromere (CEN) to assure production of a single copy of the cloned material and targeting sequence *hooks* A and B (the original circular plasmid is linearized at a site between A and B). [The TAR procedure simply involves the presentation of gently prepared human DNA, originally isolated in low-melt agarose plugs, to competent yeast spheroplasts along with vector DNA.]

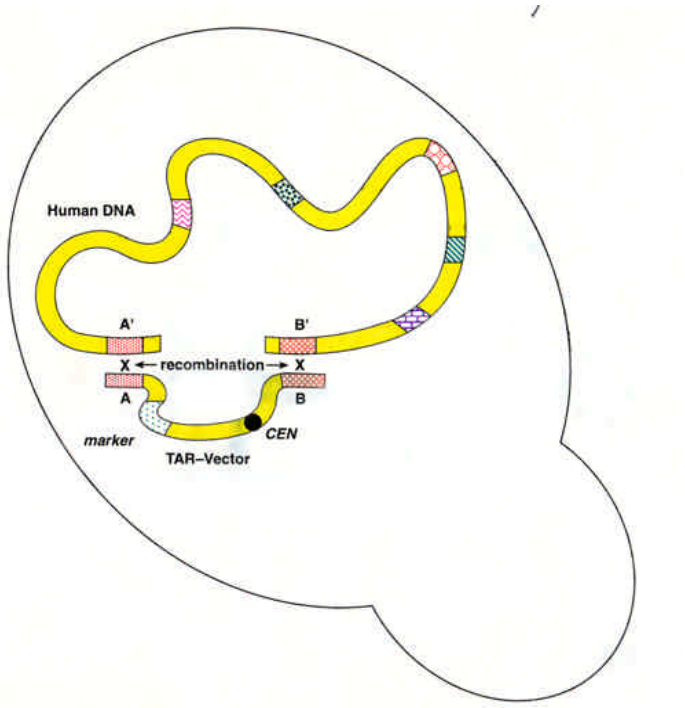

Figure 1. Model of TAR cloning to generate circular YACs. Human DNA is taken up by a yeast cell along with linearized vector DNA. The vector contains a centromere and a marker for selection. If the human DNA contains segments corresponding to the segments--*hooks*--A and B on the plasmid, recombination will lead to the establishment of a circular YAC. Propagation of the YAC depends on the presence of a yeast ARS-like sequence in the human DNA. The various blocks could be diverged repeats, such as *Alu*'s or *LINES*.

The minimum size of the hooks required for TAR cloning appears to be less than 150 bp (Larionov et al., 1996). If a human fragment containing a sequence A' and B' winds up in the same cell as the vector, recombination between the cut plasmid and the fragment will generate a circular yeast artificial chromosome (YAC) which can be selected using the plasmid marker. Because the plasmid has no yeast replication origin, sequences in human DNAs capable of functioning as *ARS*'s in yeast provide for the propagation of the YAC. Thus, the isolation of human DNA is essentially accomplished by marker rescue through recombination. The generation of YACs with large human segments was proposed to be due to preferential double-strand break repair at or near ends of molecules rather than internal regions (Larionov et al., 1996a). [The original model for double-strand break repair (Resnick, 1976) has now had many applications and refinements that extend from the repair of radiation-induced breaks, natural breaks and gap repair of incoming molecules (Orr-Weaver et al., 1983) to gene replacement in mammalian cells (Cappechi, 1988) and the development of knockout mice and now TAR cloning.]

The opportunity to TAR clone human DNA was suggested from experiments (Larionov et al., 1994) in which it was shown that during transformation there was efficient recombination between an incoming plasmid with an *Alu* and an incoming human yeast artificial chromosome (YAC) that contained several *Alu*'s . With this in mind, transformation-associated recombination was explored as an alternative means of generating *linear* YAC libraries containing large fragments of chromosomal DNA (Larionov et al., 1996). Subsequently, the original scheme--which does *not* involve restricting or ligating DNA-- was modified to yield circular YACs (Larionov et al., 1996b) as described in **Figure 1**.

The efficiency and selectivity of TAR cloning was initially demonstrated by the specific isolation of human DNA from a radiation hybrid rodent cell line containing a 5 Mb human chromosome fragment that had the Ku80 gene (Larionov et al., 1996b). A circularizing TAR vector was used that had the same human *Alu* for the targeting A and B hooks (see **Figure 1** and **Table 1**). Approximately 25% of the transformants for the vector marker had YACs containing human DNA and most were greater than 150 kb. Based on the relative number of YACs isolated containing rodent DNA, this corresponded to a nearly 5000-fold enrichment (Larionov et al., 1996b) over the 0.1% human DNA present in the hybrid cells.

These results led to the demonstration that TAR cloning could be used to isolate a specific human gene (Larionov et al., 1997), the breast cancer gene BRCA2. Although it had been sequenced, no complete BRCA2 gene had been isolated either as a YAC or a BAC (a bacterial artificial chromosome in *E. coli*). To do this, the TAR vector with hooks of approximately 500 bp each of the promoter sequence and the noncoding region of the last exon (see **Figure 1**) was presented to yeast cells along with total DNA isolated from human fibroblasts. About 1 in 300 transformants (Larionov et al., 1997 and unpublished) selected for the vector marker also contained

Table 1. Specific isolation of human DNA by TAR cloning

DNA cloned and source	Hook A	Hook B
100 Mb Chromosome 16 in a monochromosomal hybrid [6,7]	consensus *ALU*	BLUR13 *ALU*
5 Mb Ku80 in a radiation hybrid [7]	consensus *ALU*	BLUR13 *ALU*
43 kb rDNA unit in total human DNA [10]	non transcribed spacer	BLUR13 *ALU*
90 kb BRCA2 in total human DNA [4]*	5' upstream sequence	3' downstream sequence
82 kb BRCA1 in total human DNA * (unpublished)	5' upstream sequence	3' downstream sequence
70-350 kb HPRT in total human DNA * (unpublished)	BLUR13 ALU	3' downstream sequence

* Up to 1% of the yeast transformants had the gene of interest. The genes were identified through pooling of transformants, PCR analysis, followed by isolation of clones.

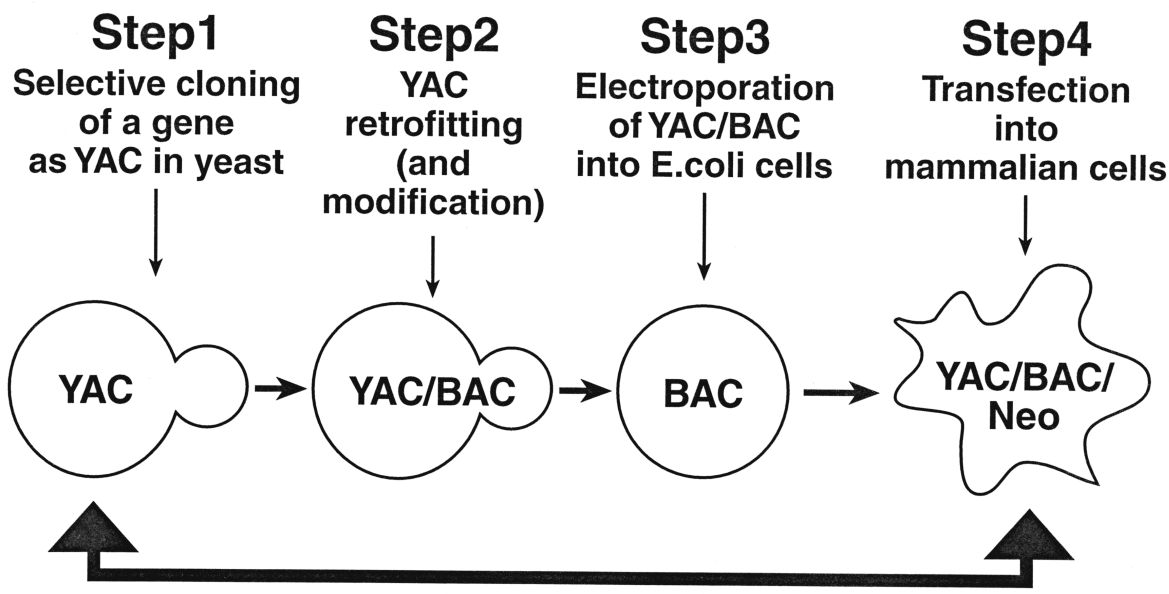

Figure 2. A TAR cloning cycle for the specific isolation of human DNA and its reintroduction into mammalian cells. Human DNA can be specifically isolated in yeast by TAR cloning, modified for transfer to bacteria and then transferred to mammalian cells. Alternatively, the TAR vector can contain sequences that would enable selection in mammalian cells enabling direct transfer from yeast.

the BRCA2 gene and these could be easily identified by PCR analysis (see **Table 1**). Further physical analysis established that several independent copies of the complete gene had indeed been isolated.

The utility of TAR cloning for the specific isolation of human genes has now been demonstrated further for the BRCA1 and the HPRT genes (unpublished) and the human ribosomal RNA gene family (Kouprina et al., 1997). As shown in Table 1, specific isolation can be accomplished with one hook that is unique to the gene(s) being isolated and the other hook being a common repeat. The numbers of unique genes isolated per μg of total DNA presented to yeast were comparable for the BRCA1 and the HPRT genes. Thus, direct gene isolation is now possible using information derived from only a small portion of a gene.

Because only a few weeks are required once the vectors are built, TAR cloning provides new opportunities for investigating genes and chromosomal regions directly from

individuals. Previously, isolation of specific chromosomal regions would have required the development of a library for each person studied followed by extensive analysis to find the region of interest. These features suggest that TAR cloning can open the way to clinical investigations of whole genes or large chromosomal regions since, for example, only 10 to 20 ml of blood would be needed for the isolation of a specific gene.

Another novel utility--referred to as *radial* TAR cloning--derives from the isolation of the rDNA and the HPRT genes with a vector that has a unique sequence hook and an *Alu* repeat hook (A and B, respectively, in **Figure 1** and **Table 1**). YACs are generated that extend from the unique position to various *Alu*'s. By changing the orientation of the unique hook, a radial series of YACs is developed that surround the unique sequence. There are many applications that include isolating a unique region surrounding a particular STS or EST site. In addition chromosomal changes such as amplifications and translocations in individuals become directly accessible with TAR cloning once a chromosomal sequence is identified. Radial TAR cloning also provides the opportunity to clone a region lacking an ARS-like sequence since the hook with the common repeat enables the isolation of chromosome fragments that are sufficiently large that they are likely to contain such a sequence.

The TAR cloning can be used in a cycle that provides for specific human DNA isolation and reintroduction, as described in **Figure 2**. Once DNA is isolated as a circular molecule it can be modified and even retrofitted with bacterial artificial chromosome sequences and mammalian selectable markers such as neomycin (NEO) or hygromycin resistance (or alternatively the original TAR vector could contain these sequences) using recombination methods standard to yeast (Larionov et al., 1996b, 1997). The YAC/BAC can than be transferred into *E. coli* in order to obtain large amounts of this DNA and it could subsequently be introduced into human cells. (Large circular molecules may be isolated directly from yeast, so that the step involving transfer to *E. coli* could be eliminated.) This approach is being applied to the BRCA2, BRCA1 and HPRT genes initially isolated as YACs. The subsequent YAC/BACs are reintroduced into mammalian cells using the NEO marker for selection. Since, as recently shown for HPRT, most of the isolated genes are functional when transferred to mammalian cells (in preparation), the cycle of human DNA isolation and reintroduction can be accomplished with high fidelity.

The tremendous success of the human genome project has relied on the development of new approaches. The information generated can be applied to many areas including functional genomics, investigations of genetic diseases, gene manipulation and gene therapy. TAR cloning is one of the new tools that will make our chromosomes more accessible.

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