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REVIEW ARTICLES

Selective Suppression of Polymerase Chain Reaction

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Abstract—The selective suppression of the polymerase chain reaction and methods based upon it (construction ofcDNA libraries from low amounts of biological material, subtractive hybridization and differential display of mRNA, fast cloning of full-size cDNA, chromosome walking, cloning *in vitro,* and others) are reviewed. These methods display a high effectiveness and, taken together, enable intricate DNA analyses to be performed—from the search for nontrivial sequences to the total sequencing of the corresponding genes.

Key words: polymerase chain reaction suppression, differential gene expression, cDNA subtractive hybridization, mRNA differential display, full-size DNA cloning, chromosome walking

The most important processes in various biological systems (cell differentiation and morphogenesis during embryonic development and regeneration, apoptosis or malignization of cells, etc.) are under the control of specific regulatory genes.[2] To understand the underlying molecular mechanisms, the genes involved in their regulation should be revealed and studied. At present, the majority of methods of molecular biology involved in unraveling such problems are based on PCR, which have made it possible to work with small amounts of biological material. However, the use of PCR requires information on the sequence of the DNA under study. When the sequence is partially or completely unknown, the PCR often encounters difficulties.

The SSP phenomenon, discovered in this laboratory [I], generated a number of highly effective, mutually complementary methods of finding and analyzing new functionally important DNA and RNA sequences when information, complete or partial, on their primary structure is absent. The use of SSP enables one to skip labor-intensive and not very effective methods of physical fractionation of DNA and makes the methods for search and analysis of genetic sequences easier, more rapid, and more reproducible.

SELECTIVE SUPPRESSION OF PCR

SSP consists in inhibiting the amplification of DNA molecules flanked by ITR in PCR with a primer corresponding to the external part of the ITR, provided that the primer is considerably shorter than the ITR (Fig. 1).

This fact may be accounted for as follows. In each PCR cycle, after denaturation upon cooling, intramolecular ITR hybridization (self-annealing) precedes primer annealing, since the temperature of ITR self-annealing is much higher than the temperature of the primer annealing; moreover, only a tiny part of the DNA molecules reaches the temperature of the primer annealing without forming a pan-like structure blocking the site of the primer annealing. Since PCR uses a primer corresponding to the external rather than internal part of ITR, the molecules on which the primer managed to anneal are restored to their original structure after DNA synthesis to allow the retaining of the inhibitory effect in further PCR cycles.

The inhibition of amplification depends on many parameters, of which the following are the major ones:

(1) The difference in the annealing temperatures of ITR and the amplification primer. The ratio of the length and GC-content of the whole ITR and the sequence corresponding to the primer affects the SSP effectiveness considerably. The use of ITR 40-50 bp long with an increased GC-content in its internal (suppression) part and of an amplification primer corresponding to the external 20-25-bp segment of the ITR appears to be optimum.

(2) The length of the ITR-containing DNA molecule. The longer the molecule, the less probable is the encounter and intramolecular hybridization of its termini. Thus, SSP effect does not inhibit, or inhibits sparingly, amplification of very long DNA (the threshold value depends on the conditions and is usually 6-8 kbp).

(3) Primer concentration in PCR. SSP depends on competition between intramolecular hybridization and primer annealing. Therefore, SSP is more effective at low-primer concentrations.

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[2] Abbreviations: ITR, inverted terminal repeat; ODD. ordered differential display; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SSH, suppression subtractive hybridization; SSP, selective suppression of PCR.

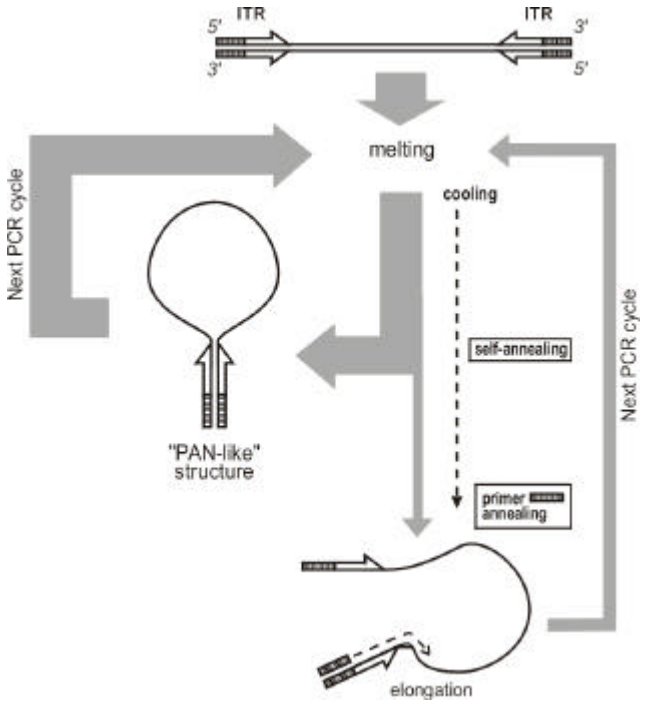

Fig. 1. Effect of inhibition of amplification of ITR-flanked DNA molecules in PCR with a primer corresponding to the external part of ITR (thickness of the arrows reflects the number of DNA molecules participating in this process). Open rectangle, external part of ITR and amplification primer; closed rectangle, internal part of ITR. Each single-stranded DNA molecule resulting from the denaturation stage can either form a pan-like structure or associate with the primer followed by primer extension to give a double-stranded form. Since ITR self-annealing can occur at a much higher temperature than primer annealing, the majority of DNA molecules are involved in self-annealing.

SSP can be used to suppress amplification of an unwanted DNA fraction, which requires suitable long ITR, i.e., suppression sequences, to be introduced into the DNA.

PREPARATION OF ITR-CONTAINING DNA SAMPLES

Two main methods for the attachment of suppression sequences to DNA molecules were developed: (1) ligation of double-stranded DNA fragments with a pseudo double-stranded, or so-called suppression, adapter [2]; (2) PCR with a long (suppression) primer whose 3'-terminal part is also present within the DNA fragments [I]. The first method is highly versatile, since it does not require any special sequences to be introduced into DNA. For example, a DNA sample for ligation can be obtained by the synthesis of double-stranded cDNA or by the treatment of cDNA or genomic DNA with restriction endonucleases. Apparently, blunt-end (rather than staggered-end) DNA fragments are optimum because, in this case, blunt-end suppression adapters can be universally used in ligation. The second technique is effective when an amplified DNA sample is being dealt with so that it harbors known sequences. This can be exemplified by cDNA samples obtained through addition of a homopolimer sequence to the first cDNA strand followed by PCR with a T-primer or C- and T-primers.

STRATEGY OF EMPLOYMENT OF SSP

SSP can be used in the analysis of complex mixtures of DNA fragments (cDNA or fragmented genomic DNA) to suppress an undesirable DNA fraction while retaining exponential amplification of target sequences. By and large, SSP allows the selection of asymmetrically flanked DNA molecules from mixtures with symmetrically flanked ones. Before the discovery of SSP, such a problem could not be solved using PCR.

Three major schemes of SSP employment can be outlined.

The first scheme (Fig. 2a) is based on the addition of one suppression sequence to all DNA molecules, followed by amplification with two primers of which one corresponds to the external part of the suppression sequence while the second one is complementary to the target DNA (gene-specific or oligo(dT)-containing primer). In the course of PCR, DNA molecules bearing the sequence of the second primer are selected. This scheme underlies methods such as the search for the genomic DNA or cDNA sequences belonging to a known fragment [2,3], the construction ofcDNA libraries on the basis of low amounts of total RNA [4], and mRNA ordered differential display [5].

The second scheme (Fig. 2b) is based on an addition of two different suppression sequences to DNA followed by amplification with primers corresponding to their external parts. In the course of PCR, asymmetrically flanked DNA molecules—bearing different suppression sequences at their ends—are selected. This scheme underlies the *in vitro* cloning method [6].

The third scheme (Fig. 2c) is a sophisticated variant of the previous one. Two DNA samples are supplied with different 5'-terminal (but not 3'-terminal) suppression sequences. The samples are then denatured and hybridized together. After filling in the 3'-termini, PCR is carried out with primers corresponding to the external parts of the suppression sequences. As in the second scheme, asymmetrically flanked DNA molecules are selected, but in this case each of these DNA must anneal with the complementary strand from the alternative sample. Such selection of heteroduplexes enables subtractive hybridization of cDNA [I], cDNA normalization [7], and a search for evolutionarily conserved cDNA [8].

SSP-BASED PROCEDURES

Currently, studies on the effects of gene expression on various biological processes often employ the following strategy: (1) construction of cDNA libraries

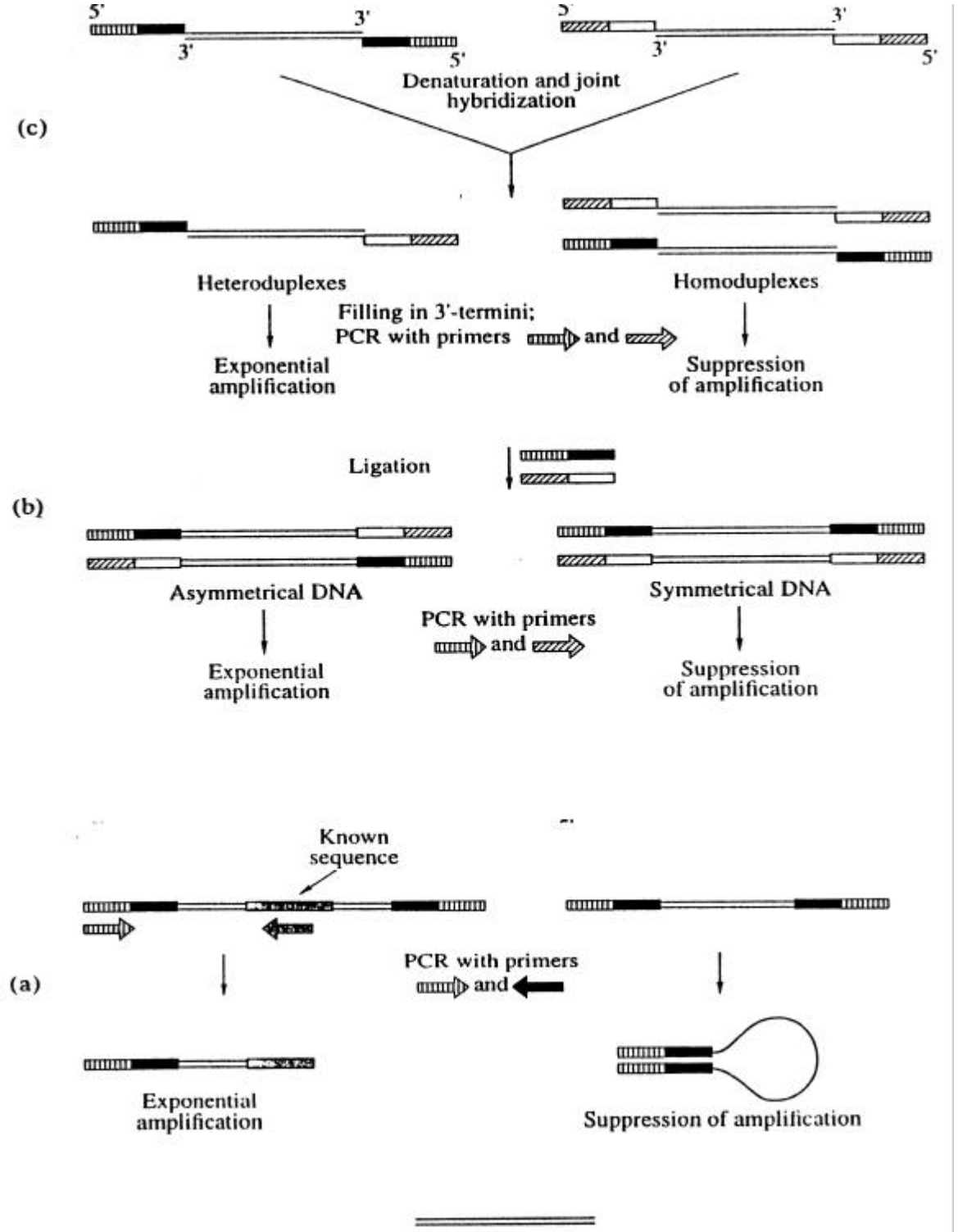

Fig. 2. Three schemes (a, b. and c) of SSP employment (for explanations, see text). Open and closed rectangles, internal parts of suppression sequences; vertically and slantingly hatched rectangles, external parts of suppression sequences.

from biological samples under study; (2) screening of these libraries for genes (more exactly, their fragments) differentially expressed or interesting in other aspects; or (3) preparation of full-size cDNA and genomic copies of the genes of interest. All these stages can be carried out using SSP-based methods that are considered in detail in the following section of this review.

Construction ofcDNA Libraries from a Small Amount of Biological Material

cDNA libraries are needed for a wide range of problems related to the functional and structural aspects of gene expression. The methods for their construction from relatively large amounts of biological material were developed long ago and are now routine genetic

engineering procedures. However, when biological material is scarce and therefore poly(A)$^+$ RNA is insufficiently available (this is the standard situation nowadays), the conventional methods are of no avail. The advent of PCR gave impetus to new methods of constructing cDNA libraries from small amounts of total RNA. The use of PCR, however, requires information on at least a partial sequence of the DNA to be amplified. Since mRNA of the overwhelming majority of genes contain a poly(A) sequence, PCR can be carried out with an oligo(dT)-containing primer (T primer). Exponential amplification needs an artificial sequence to be attached to all cDNA molecules for annealing of a second primer. Several methods for the introduction of such a sequence have been described: (1) addition of a homopolimer sequence to the 3'-terminus of the first strand ofcDNA using terminal deoxynucleotidyl transferase (tailing) [9]; (2) ligation of a synthetic single-stranded oligonucleotide to the first strand of cDNA [10] or to mRNA [11] using T4 RNA ligase; and (3) ligation of a double-stranded oligonucleotide adapter to double-stranded cDNA using T4 DNA ligase [12].

Ligation of double-stranded molecules using T4 DNA ligase is more effective and reproducible than tailing or ligation of single-stranded substrates using T4 RNA ligase [3]. However, method [12], using T4 DNA ligase, has the substantial drawback that it can be applied only to poly(A)$^+$ RNA. If cDNA is synthesized on the basis of total RNA, even the use of a T primer does not prevent the synthesis of a large excess of background cDNA on ribosomal RNA as a template.

On the basis of SSP, a method was developed that involves T4 DNA ligase-mediated ligation of a suppression adapter to double-stranded cDNA, followed by PCR with a T-primer and a primer corresponding to the external part of the adapter (Fig. 2a) [4]. Such PCR is accompanied by selective amplification of the cDNA fraction comprising the T-primer structure. At the same time, SSP inhibits amplification of the rest of cDNA, which is adapter-flanked from both sides. Thus, a cDNA library can be constructed based on a small amount of total RNA without isolating poly(A)$^+$ RNA fraction.

We demonstrated the potentials of this procedure on a model system [4]. The libraries so constructed were subsequently used to reveal and analyze functionally important expressing sequences in various biological systems [13-15]. This method enables representative, essentially full-size, cDNA libraries to be constructed from small (10-100 ng) amounts of total RNA that are of as high a quality as libraries produced by conventional methods from 5-10 μg of poly(A)$^+$ RNA.

Detection of Differentially Expressed Genes

Understanding the molecular mechanisms of biological processes requires the search for and study of genes expressed differentially in these processes. The identification methods for such genes are based on the detection of mRNA molecules that are diversely represented in various tissues and at various stages of a biological process. Changes in composition and content of cellular mRNA can be analyzed in several modes. Currently, two strategies are the most widespread: differential display and subtractive hybridization.

Differential display. The differential display technique was proposed in 1992 [16] and is currently in great demand in searches for differentially expressed genes. This technique is based on the employment of a short oligonucleotide primer that has a low annealing temperature and can direct PCR-amplification of a limited pool of cDNA fragments. Comparative PAGE-analysis of such cDNA samples allows the identification of differentially represented cDNA fragments. Use of an arbitrary primer, however, precludes systematic comparison of the samples by all mRNA types: characteristic sets of DNA fragments are random, and the overwhelming majority of these fragments correspond to the types of transcripts most abundant in the original samples. In addition, use of oligonucleotide primers with a low annealing temperature is accompanied by nonspecific amplification and leads to numerous artefacts. A totally different approach, involving a systematic comparison of samples by all mRNA types and based on the separation of 3'-terminal restriction fragments of cDNA, was proposed by Ivanova and Belyavskii [17]. Its drawbacks are its limited sensitivity and labor-intensive character.

We developed a method of mRNA ODD that is also based on an analysis of 3'-terminal restriction fragments of cDNA [5]. However, to select these fragments, our approach uses PCR rather than physical separation (Fig. 2a) This results in selective amplification of 3'-terminal fragments ofcDNA (from T-primer to the nearest restriction site), which substantially simplifies the method and enhances its sensitivity.

The effectiveness of the ODD method was exemplified by revealing sequences differentially expressed along the anterior-exterior axis ofplanarian [5].

Subtractive hybridization of cDNA. Subtractive hybridization of cDNA is a process of exhaustive hybridization of two cDNA samples named *driver and, tracer*, which is meant to reveal sequences (targets) that are present in tracer but absent from or at a lower level in driver. Subtractive hybridization involves hybridization

of tracer with excess driver, followed by the separation of hybrid molecules from target molecules.

The use of subtractive hybridization of cDNA resulted in the detection of a considerable number of functionally important genes involved in embryonic development, cell differentiation, tumor transformation, and metastasizing. However, the low representativeness of the resulting enriched libraries, moderate enrichment degrees, and labor-intensity of procedures for the purification of the enriched fraction considerably hindered the use of this approach when the amount of biological material is limited and in the identification of genes whose transcripts are sparingly presented in the cell (1 to 10 copies per cell).

Our cDNA subtractive hybridization procedure [1] allows one to overcome the problem of searches for rare transcripts by introducing a step of normalization of concentrations of various transcripts in the cDNA sample under study. This is achieved by using SSP (Fig. 2c), which led to this method being named SSH.

The effectiveness of this procedure was confirmed in model experiments with the use, as a target, of an exogenous viral DNA added to tracer in predetermined concentrations [I]. The method was employed in revealing sequences differentially expressed in various biological processes, such as activation of immune response in a culture of immunocompetent cells [18], alteration of the metastatic potential of tumor cells [19], or regeneration of planarian [15], and for constructing human tissue-specific cDNA libraries [20]. Currently, the SSH technique is in wide use (e.g. [21-25]).

Elements of the proposed technique of subtractive hybridization were successfully employed in the solution of some other problems, for example, constructing normalized cDNA libraries [7] and revealing evolution-arily conserved expressed sequences [8].

Search for 5'- and 3'-Terminal Fragments ofcDNA

One of the most important and technically difficult objectives associated with the characterization of genes is the preparation of full-size cDNA. Conventional methods for detection of genetic sequences (screening of cDNA libraries, cloning of conserved genes using PCR with degenerated oligonucleotide primers, identification of differentially expressed genes using mRNA differential display or cDNA subtractive hybridization) usually allow identification of only a fragment of cDNA. To clone full-size cDNA more rapidly and effectively, a number of methods jointly referred to as RACE were recently proposed for *in vitro* amplification of the ends ofcDNA (for review, see [26]). The majority of RACE methods now known are based on the introduction into the 3'-end of the first cDNA strand of an additional nucleotide sequence, which subsequently serves as the annealing site for a PCR primer. However, the problem of amplification suppression of nonspecific sequences upon use of the RACE strategy for rare genes is even more complicated than in constructing cDNA libraries.

SSP makes it possible to overcome this difficulty. The cDNA sample is ligated with a suppression adapter and then amplified with a primer corresponding to the external part of the suppression adapter and a gene-specific primer (Fig. 2a). Only molecules containing the annealing site for the specific primer, that is, 5'- or 3'-terminal sequences (depending on the direction of the specific primer), are thereby amplified. Amplification of the remainder of the molecules is prevented by SSP.

The effectiveness of this method was tested in model experiments on known genetic sequences [3] and currently is widely used in many laboratories (over 100 papers were published in 1997, e.g. [27-31]).

Search for Promoter Sites (Chromosome Walking)

Analysis of the genomic organization of the sequences isolated and cloning of regulatory regions of genes is important in the structure-functional analysis of genes. However, existing PCR-based techniques of chromosome walking are rather labor-intensive and ineffective. We proposed a novel technique of PCR-walking on genomic DNA based on SSP [2]. To produce an ITR-containing DNA sample, genomic DNA is treated with a restriction endonuclease and ligated with a suppression adapter. Isolation of regulatory regions of the gene under study is carried out in subsequent PCR on the resulting DNA sample with gene-specific and adapter primers according to the scheme for isolation of full-size cDNA. This technique has also been widely used (e.g. [32-37]).

Cloning in vitro

Although the majority of classical genetic engineering methods were recently improved or displaced by more effective PCR-based techniques, traditional methods retained their leading role in the molecular cloning of DNA (cloning in bacterial, phage, and other *in vivo* systems).

Based on SSP, a new method, named cloning *in vitro*, was proposed [6], which allows PCR-amplifica-tion and then sequencing of individual DNA molecules of unknown sequence without cloning *in vivo*. The method of cloning *in vitro* includes the following stages (Fig. 2b): (1) simultaneous ligation of double-stranded DNA fragments with two suppression adapters;

(2) multiple dilution of the resulting sample to adjust the

DNA content in the volume to be used in amplification to single molecules; and (3) PCR-amplification of single DNA molecules using primers complementary to the external parts of the adapters.

The resulting PCR products, named *in vitro* clones, correspond to single DNA molecules. Owing to SSP, these clones are necessarily flanked with sequences of different adapters, which allows one to sequence the cloned DNA fragment by any method suitable for PCR products.

In vitro cloning can be used in solving a wide range of problems of molecular biology in the place of conventional cloning *in vivo*. This method is especially convenient when no more than several dozen clones are needed. We used this approach in the differential screening ofcDNA libraries constructed by subtractive hybridization [38]. In addition, we developed a protocol for rapid preparation of a panel of overlapping sub-cloned to sequence long-stretched (5-kbp) DNA fragments [39].

Thus, the discovery ofSSP has resulted in a number of mutually complementary procedures of analysis of complex DNA samples: these include construction of cDNA libraries from small amounts of biological material, subtractive hybridization and differential display of mRNA for revealing differentially expressed sequences, fast cloning of full-size cDNA, chromosome walking for the cloning of promoter and other genomic regions, and cloning *in vitro*.

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