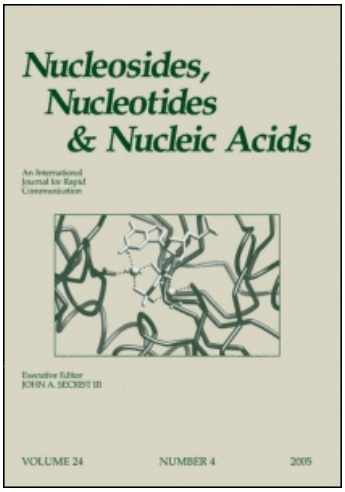

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SYNTHESIS AND EXPRESSION OF SOME GENES FOR HUMAN CYTOKINES

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Abstract. Intronless genes for human mature interleukin 1α (IL1α) and its receptor antagonist (IL1ra) have been synthesized and efficiently expressed in a specially devised bacterial plasmid vector as part of a versatile two-cistron prokaryotic expression system.

Directed protein synthesis is an important source of biologically - factually or at least potentially - active polypeptides. As long as the central dogma of molecular biology stands, it is polynucleotides that are prerequisites for the protein biosynthesis. We devised a PCR-based approach (DNA splicing by directed ligation, SDL) to the synthesis of intronless genes, using *in vitro* amplification of exon segments of genomic DNA followed by their in-phase joining [1]. By means of this and RT-PCR methods we synthesized genes encoding two human proteins of biological importance, interleukin 1α (IL1α) and its receptor antagonist (IL1ra) (both in native and mutated states) fit for prokaryotic expression [1,2].

Attempts to carry out expression of these genes in pDR540 or pGEM1 plasmids failed, apparently because of secondary structure elements interfering with the translation initiation. The expression of the IL1α gene did occur upon the modification of the ribosome binding site (RBS) in pGEM1 by introducing a translation enhancer from the leader mRNA of the T7 phage gene 10 (pGEN vector converted into pGENIL1 upon insertion of the IL1α gene; see FIG.1). To reach a still higher level of expression, a two-cistron system was constructed Its first part, a specially designed artificial minicistron comprising the above-mentioned enhancer, was inserted into pGEM1 to yield the pGMCE (Gem-MiniCistron-Enhancer) vector. Then the target gene (IL1α) was also inserted; it was precisely joined with the minicistron, by means of the SDL method, through a combination of the overlapping termination and initiation codons TAATG (cf.[3]). With the use of

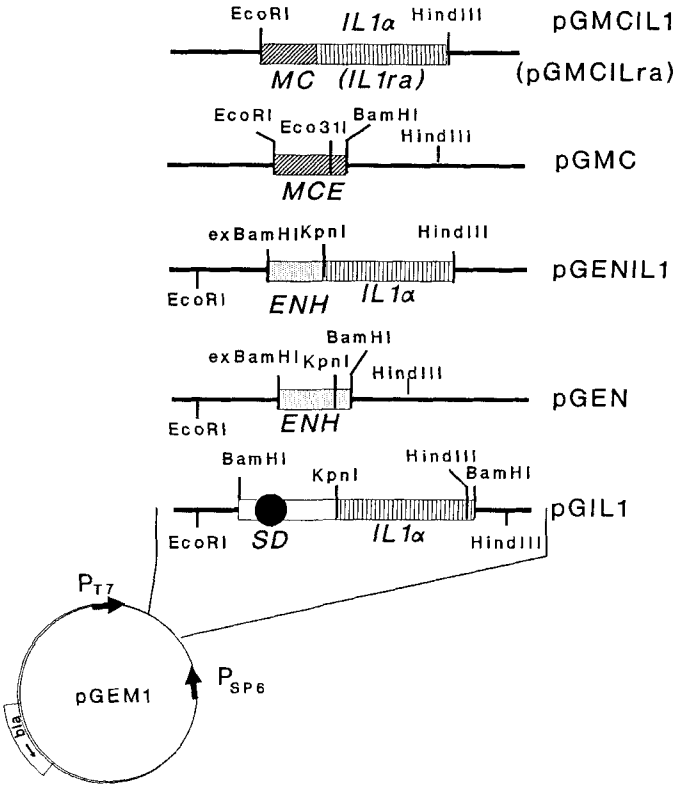

FIG. 1. The expression plasmid derivatives of pGEM1, constructed in the present work. SD - Shine-Dalgarno sequence, ENH - translation enhancer, MC - minicistron

the resulting plasmid pGMCIL1 in the Studier system [4], a high level of expression of the target gene was obtained: IL1α accounted for 33% of the total cell protein (in the case of the one-cistron enhancer-containing construction pGENIL1 the corresponding value was 10%).

This increase in the protein yield could have resulted either from the efficient translation of the first cistron followed by coupled translation of the second cistron, or simply from the optimization of the second translation initiation region (TIR) by an extension of the SD and leader sequences. To distinguish between these two possibilities, we analysed the efficiency of the translation initiation complex formation by the toeprinting technique [5] with the *in vitro* T7 transcripts of the *Hin*dIII-linearized plasmid DNAs. It turned out

that in the case of the two-cistron construction pGMCIL1 ribosomes bound to the second cistron's RBS in the whole mRNA population. The efficient formation of the initiation complex also took place with the one-cistron enhancer-containing mRNA transcribed by T7 RNA polymerase from the pGENIL1 plasmid, though a considerable part of the mRNA remained unbound. With the pGIL1 (pGEM1 carrying the IL1α gene under the control of the T7 promoter) mRNA, which does not contain an enhancer, no toeprint signal was observed. The toeprinting results are in agreement with the calculated secondary structures of the corresponding mRNAs, thus confirming that it is the inhibition of the translation initiation by the mRNA secondary structure elements that prevents the IL1α gene in pGIL1 from being expressed.

The data obtained point out the TIR of the target gene (i.e., the distal TIR) in the two-cistron construction pGMCIL1 to be much more efficient than its counterpart in the one-cistron enhancer-containing pGENIL1. At the same time, these data are insufficient for inferring whether the first TIR in the pGMCIL1 mRNA is efficacious. In fact, as the two initiation codons in the two-cistron mRNA are separated by 40 nt, the ribosome binding to each of the two RBS might well proceed independently. If ribosome binds to the second (distal) site with a high efficiency, the reverse transcription (the first step in the toeprinting technique) is supposed to stop at that site, thus preventing the ribosome binding at the first (proximal) site from being detected.

To diminish the efficiency of the ribosome binding to the second site and thus to reveal its possible binding to the proximal RBS, we carried out the toeprinting with a lower concentration of 30S subunits, approximately equal to that of mRNA. In these conditions, a substantial amount of the unbound mRNA but no toeprint signal corresponding to the first RBS were observed, whereas the ribosome binding at the second site was still efficient. This site is therefore much more active than the proximal one, i.e. the elevated expression level of the target gene was mainly due to the highly efficient translation initiation at the second cistron.

In principle, the pGMCE-based expression system is usable with any intronless gene or its fragment. It only takes the proximal end of the target gene to be flanked with the protruding end 5'AATG (the latter can be universally introduced by means of a restriction endonuclease class IIS yielding tetranucleotide single-stranded ends, or synthetically) complementary to the protruding end 5'CATT of the vector. The 5'AATG end comprises only the initiation codon, setting no limitations on the gene's nucleotide sequence. Distal end of the target gene is to be flanked with a termination codon and a restriction half-site corresponding with the pGEM1 polylinker part retained after cloning the minicistron (*Bam*HI, *Xba*I, *Acc*I, *Sal*I,

*Pst*I, *Hin*dIII sites). In fact, we carried out the cloning and high-yield expression, in the pGMCE vector, of another artificial gene [2], coding for the IL1ra (pGMCIL1ra plasmid; 13% of the total protein in the cell lysate).

This rather high level of expression was obtained despite a stable hairpin (ΔG° = -5.2 kcal/mole) in the second cistron's TIR. The toeprint analysis of this transcript revealed the ribosome binding at the two RBS. The efficiency of the binding at the distal site was considerably lower than in the pGMCIL1 transcript, where this site plays a key role in providing a high yield of the protein. Moreover, in the ratio of the ribosome-bound and non-bound transcripts, which illustrates the RBS activity, the pGMCIL1ra construction falls short even of the one-cistron construction pGENIL1, which makes the protein's yield lower than not only that in pGMCIL1 (see above) but also in pGMCIL1ra itself. These data suggest that the rather high translation level in pGMCIL1ra is due to the activation of the distal TIR through the melting of its secondary structure in the course of the coupled translation from the proximal site.

Efficient expression of two different genes (IL1α and IL1ra) in the pGMCE plasmid indicates that this two-cistron construction is not particularly sensitive to the target gene's primary structure and therefore can be considered as a versatile vector for prokaryotic expression. It functions mainly owing to a highly active distal TIR. If the latter's activity is inhibited by secondary structure, the proximal TIR is supposed to take over, providing for coupled translation and thus keeping a high level of translation of the target gene.

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