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Spacer alterations which increase the expression of porcine growth hormone in *E. coli*

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Full-length porcine growth hormone (PGH) cDNA clones were isolated from a porcine pituitary cDNA library. When the coding portion of the PGH gene was cloned into an *E. coli* expression vector downstream from the powerful *trc* promoter, high levels of mRNA, but no protein were detected. Mutation directed by an oligodeoxynucleotide primer altered 5′-non-coding sequences and raised the level of PGH produced from undetectable to 15% of the total cellular protein. Alteration of four codons infrequently used by *E. coli* in the 5′-end of the gene produced no further increases.

Translational efficiency; Oligonucleotide-directed mutagenesis; Ribosome binding site; Codon alteration; Growth hormone; (Porcine pituitary)

1. INTRODUCTION

A number of recent papers have described difficulty in obtaining high levels of eukaryotic protein expression in *E. coli*, despite the presence of high levels of mRNA produced from powerful promoters, such as λ PL [1,2]. The low level of protein results from inefficient translation as alterations which yield increased levels of protein do not alter mRNA levels [1]. The most common way production increases are engineered is by synthesizing (or mutating) the 5′-coding region of the gene, incorporating codons that either (i) mimic those translated with high efficiency by *E. coli* [1,3,4] or (ii) inhibit the formation of secondary structures in the RNA [2,5].

This paper describes a mutation, 5′ to the coding region, which increased the production of porcine growth hormone (PGH) in *E. coli* by greater than 100-fold. Alteration of codons infrequently used by *E. coli* in the 5′-end of the gene to those used in highly expressed *E. coli* proteins, produced no significant increase in levels in constructs with either the altered or normal 5′-flanking sequences. The 5′-non-coding alteration was carried out using a single oligodeoxynucleotide primer and was thus considerably quicker and easier than codon replacement by partial gene synthesis or extensive mutagenesis, which in itself may not overcome translational problems [2].

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2. MATERIALS AND METHODS

2.1. *Oligodeoxynucleotides*

All oligodeoxynucleotides and linkers were synthesized by Biotechnology Research Enterprises SA (BRESA). The oligonucleotide sequences were as follows: GH.25, 5′-dCAGCCAGTTGGTGCAGGTGCTGGGC-3′; GH.27, 5′-dGCCATCTTCCAGCTCCCGCATCAGGGC-3′; GH.30, 5′-dGGGCATGGCTGGGAACATGGTCTGTTTCCT-3′; GH.38, 5′-dCATGGCTGGGAACATATATTACCTCCTGTGTGAAATTG-3′; GH.34, 5′-dCGGCGTTGGCGAACAGGCTGGACAGCGGCATGGC-3′.

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2.2. *cDNA cloning*

Poly(A)$^+$ RNA was isolated from porcine pituitaries by the method described in [6] and two rounds of enrichment through an oligo-dT column. The RNA was then converted to cDNA by the RNase H method [7] and cloned into pUC19 [8] after the addition of synthetic *Eco*RI linkers.

Colonies containing PGH sequences were identified by colony hybridization as described [9]. The hybridization probe used was a 27 base synthetic oligodeoxynucleotide (GH.27) which is complementary to PGH mRNA [3]. One positive, pPG.3, had an insert of 820 bp which was completely sequenced [10] after subcloning a number of restriction fragments into M13 mp18 and mp19 vectors.

The insert of this plasmid contained a full-length cDNA consisting of a 5′-untranslated region of 41 bp, a coding region of 648 bp and a 3′-untranslated region of 105 bp ending with a 14 bp poly(A) tail. The open reading frame codes for a 216 amino acid prehormone, identical to the partial length PGH amino acid sequence deduced by Seeburg et al. [3]. The sequence missing from the cDNA clone of Seeburg et al. [3] is illustrated in fig.1 (clone pKTGH).

3. RESULTS

To obtain expression of PGH in *E. coli* a *Pst*I fragment was isolated from the replicative form (RF) of the M13 phage containing the *Eco*RI insert from pPG.3 and cloned into pKT52, a high copy number expression vector, kindly provided by J. Shine, containing the powerful *trc* promoter [11], regenerating the full sequence of the pre-hormone (fig.1). Following transformation into a *lac* I^q host such as JM101, cells containing this plasmid, pKTGH, should produce pre-PGH when induced with IPTG. However, no additional protein was visible when extracts from such cells were electrophoresed on SDS-PAGE [12] and stained with Coomassie blue. As our primary aim was to obtain expression of the mature form of PGH, this lack of expression was not pursued.

Oligodeoxynucleotide-directed mutagenesis was used to remove the sequences coding for the 'pre' region of the hormone. An *Eco*RI fragment containing the *trc* promoter fused to the PGH cDNA, was isolated from pKTGH and cloned into mp19. Single-stranded DNA isolated from this phage was then used in mutagenesis reactions. To remove the 75 bp coding for amino acids 2–26 a 30 base oligodeoxynucleotide, GH.30, complementary to 15 bases either side of the required deletion was used. Following mutagenesis [13] the reaction mixture was transformed into JM101 and phage containing the required deletion were identified by plaque hybridization, followed by nucleotide sequencing using the PGH-specific 25 base oligodeoxynucleotide, GH.25 as primer. RF DNA was isolated from one of the phage containing the

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                        RBS
              +24
pKTGH     ...CACAGGAAACAGACC ATG GCT GCA GGC CCT CGG ACC TCC GTG CTC CTG...
                             met ala ala gly pro arg thr ser val leu leu

pGHX.1    ...CACAGGAAACAGACC ATG TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT...
                             met phe pro ala met pro leu ser ser leu phe

pGHXS.4   ...CACAGGAGGTAATAT ATG TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT...
                             met phe pro ala met pro leu ser ser leu phe

pGHXC.1   ...CACAGGAAACAGACC ATG TTC CCA GCC ATG CCG CTG TCC AGC CTG TTC...
                             met phe pro ala met pro leu ser ser leu phe

pGHXSC.4  ...CACAGGAGGTAATAT ATG TTC CCA GCC ATG CCG CTG TCC AGC CTG TTC...
                             met phe pro ala met pro leu ser ser leu phe
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Fig.1. Nucleotide sequence of construct RBS/spacer regions. Spacer and codon alterations have been underlined.

correct deletion and the *Eco*RI insert purified and subcloned back into the larger *Eco*RI fragment of pKTGH. The modified nucleotide sequence of this clone, pGHX.1, is shown in fig.1. When extracts from induced cells containing this plasmid were analysed by SDS-PAGE, no additional protein of the expected M_r corresponding to methionyl-PGH (m-PGH, M_r 22000) was detected. A number of reports have indicated that sequences between the RBS and the initiator ATG, hereafter called the 'spacer', can influence translation efficiency [4,14–17]. We therefore decided to alter the sequence outside the coding area around the RBS, as the sequence of pKT52 is far from the consensus sequence in this region (see above references).

A 38 base oligodeoxynucleotide (GH.38) was designed to alter the RBS from AGGAAA to a consensus AGGAGG and the spacer from CAGACC to TAATAT, as a number of reports have indicated that AT-rich spacers can increase protein expression [4,15]. Single-stranded DNA containing the small pGHX.1 *Eco*RI fragment was mutagenized as described above and positive plaques selected and sequenced. RF DNA was prepared from phage containing the correct mutation and cloned back into pKTGH. The nucleotide sequence of the altered RBS/spacer of this plasmid is shown in fig.1. Extracts prepared from cells transformed with this plasmid, pGHXS.4, contained high levels of m-PGH (fig.2), indicating that the translational problem had been overcome.

In addition to the RBS/spacer alteration, we investigated the effects of strategic 5′-codon alteration on PGH translation. A 34 base oligodeoxynucleotide (GH.34) complementary to bases 134–168 of the PGH gene, but with 4 base changes which would alter codons 6, 7, 10 and 11 of m-PGH to those favoured by *E. coli*, was utilized (fig.1). Both M13 clones with the original RBS/spacer and the altered spacer of pGHXS.4 were mutagenized and mutants selected, screened and sequenced as described above (see fig.1). The levels of PGH produced by pKTGH (pre-PGH), pGHX.1 (m-PGH), pGHXS.4 (RBS/spacer altered m-PGH) and codon altered forms of the latter two plasmids are illustrated in fig.2. The percentage of total cellular protein expressed as PGH was determined by laser densitometry and ranged from less than 0.1% for plasmids pKTGH and pGHX.1, to approx. 15% for plasmid pGHXS.4.

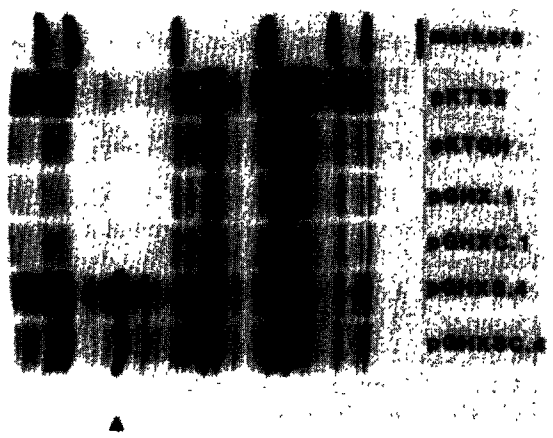

Fig.2. PGH protein production levels. JM101 cells containing expression plasmids were induced with IPTG, then lysed and subjected to SDS-PAGE [12].

The observed increase in PGH production was due to the enhancement of translational efficiency, as PGH mRNA levels were found to be similar in cells containing each of the different plasmids (not shown).

4. DISCUSSION

Our results indicate that the alteration of the RBS/spacer region was all that was required to enhance the translation of PGH mRNA in *E. coli*. The protocol followed differs significantly from those previously used which usually rely on either a number of random changes [2] or the systematic alteration of codons [3]. PGH has also been expressed in *E. coli* by Seeburg et al. [3] who obtained high level expression by replacing the entire 5′-end of the PGH cDNA with a synthetic DNA sequence incorporating codons known to be efficiently expressed. This approach is by no means generally applicable and relies on the nucleotide sequence of the specific promoter/spacer chosen not interacting with the coding region of the gene [2].

The alteration of the RBS/spacer region to a sequence which efficiently interacts with the *E. coli* 16 S rRNA and is AT rich and thereby less likely to interact with coding sequences in a deleterious manner is therefore a useful alternative. The use of a redundant mutagenesis oligodeoxynucleotide could supplement the approach described in this

paper and increase the probability of generating an RBS/spacer sequence which allows efficient translational initiation.

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REFERENCES

[1] Schoner, B.E., Hsiung, H.M., Belagaje, R.M., Mayne, N.G. and Schoner, R.G. (1984) Proc. Natl. Acad. Sci. USA 81, 5403–5407.
[2] Buell, G., Schulz, M.-F., Selzer, G., Chollet, A., Movva, N.R., Semon, D., Escanez, S. and Kawashima, E. (1985) Nucleic Acids Res. 13, 1923–1938.
[3] Seeburg, P.H., Sias, S., Adelman, J., De Boer, H.A., Hayflick, J., Jhurani, P., Goeddel, D.V. and Heyneker, H.L. (1983) DNA 2, 37–45.
[4] George, H.J., L'Italien, J.J., Pilacinski, W.P., Glassman, D.L. and Krzyzek, R.A. (1985) DNA 4, 273–281.
[5] Tessier, L.-H., Sondermeyer, P., Faure, T., Dreyer, D., Benavente, A., Villeval, D., Courtney, M. and Lecocq, J.-P. (1984) Nucleic Acids Res. 12, 7663–7675.
[6] Brooker, J.D., May, B.K. and Elliott, W.H. (1980) Eur. J. Biochem. 106, 27–34.
[7] Gubler, U. and Hoffman, B.J. (1983) Gene 25, 263–269.
[8] Norrander, J., Kempe, T. and Messing, J. (1983) Gene 26, 101–106.
[9] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Brosius, J., Erfle, M. and Storella, J. (1985) J. Biol. Chem. 260, 3539–3541.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Zoller, M.J. and Smith, M. (1983) Methods Enzymol. 100, 468–500.
[14] Gheysen, D., Iserentant, D., Derom, C. and Fiers, W. (1982) Gene 17, 55–63.
[15] De Boer, H.A., Hui, A., Comstock, L.J., Wong, E. and Vasser, M. (1983) DNA 2, 231–235.
[16] Hui, A., Hayflick, J., Dinkelspiel, K. and De Boer, H.A. (1984) EMBO J. 3, 623–629.
[17] Whitehorn, E.A., Livak, K.J. and Petteway, S.R. (1985) Gene 36, 375–379.