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Production of plasmids giving high expression of recombinant DNA-derived ovine growth hormone variants in *Escherichia coli*

O. Caryl Wallis and Michael Wallis

Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, England

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A method for the production of plasmids giving different levels of expression of ovine growth hormone (oGH) variants in *E. coli* is described. The cDNA sequence coding for mature oGH was inserted into the multiple cloning site of plasmid pUC8 and random deletions were then introduced 3′ to the initiation codon. Clones producing GH (with varying N-terminal extensions) were identified by immunological screening. Levels of expression of GH-related protein, measured by immunoassay or on SDS-polyacrylamide gels, varied from over 20% to less than 0.05% of total cell protein. The coding sequence of plasmid pOGHe101, giving very high expression of variant oGH1, was determined.

Growth hormone; Growth hormone variant; cDNA; Expression; Sequence; (Ovine; *Escherichia coli*)

1. INTRODUCTION

A number of authors have reported problems in obtaining high level expression of GH in bacterial systems. Thus, we have previously found only low expression of the cDNA sequence coding for ovine (sheep) preGH in *E. coli* [1] and various authors have reported that the natural sequence coding for the mature form of bGH (which is very similar to that of oGH [2]) is not well expressed in *E. coli* [3–5]. Alterations in the sequence coding for the N-terminus of bGH [3,4], or insertion of additional nucleotides 3′ to the AUG initiation site, forming a 2-cistron expression system [5], increased expression up to 50-fold. Alteration of 5′-non-coding sequences increased expression of porcine GH [6]. These various studies indicate that the sequence 5′ and 3′ to the AUG initiation site has a marked effect on the efficiency of expression of GH. The reasons for this are not clear; formation of secondary structure in mRNA may be important, but cannot explain the observations completely.

In this paper we describe a new approach to the problem of obtaining high level expression of GH in *E. coli*. Rather than studying the effect of introducing a limited number of specific changes, we have produced a range of initially unspecified alterations in the region 3′ to the initiation codon of a gene comprising the mature oGH coding sequence linked to a short leader sequence, in a pUC8-based plasmid [7]. The effect of a large number of sequence changes on expression efficiency could thus be studied rapidly, and those giving high expression could be identified and characterized. A preliminary account of this work has been presented [8].

2. METHODS

2.1. *Bacterial strains, plasmids and enzymes*

E. coli strains JM101 and HB101 F′ were used for expression and sequencing, respectively. Initial plasmids were pUC8 [7], pOGH4 (a plasmid containing the cDNA sequence encoding preoGH [2]) and pJBS633 [9]. Media were those described by Miller [10] with the addition of 50 μg ampicillin per ml when bacteria contained pUC8-based plasmids.

Correspondence address: O.C. Wallis, Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, England

Abbreviations: GH, growth hormone (oGH, ovine GH; bGH, bovine GH); IPTG, isopropyl β-D-thiogalactopyranoside

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DNA modifying enzymes and dideoxynucleotides were obtained from BCL (Lewes, England), Gibco BRL (Paisley, Strathclyde, Scotland) or Amersham International (Amersham, Bucks, England) and were used according to the manufacturers' instructions.

2.2. *Construction of expression plasmids*

The 635 base-pair fragment, obtained by cutting pOGH4 with *Hae*II and *Hin*dIII and containing the coding sequence for mature oGH (fig.1), was ligated into the multiple cloning site of pUC8 between the *Bam*HI and *Hin*dIII sites after conversion of *Bam*HI and *Hae*II sites to blunt ends [11]. After transformation of *E. coli* JM101 [12] the ampicillin-resistant colonies were screened for GH production (see below). Plasmid DNA from these colonies, prepared by the alkali lysis method [11], was treated with *Eco*RI, *Bam*HI or *Hin*dIII. Plasmid DNA from one transformant (pOGHe100), after treatment with *Eco*RI, was subjected to brief digestion with exonuclease *Bal*31, followed by treatment with the Klenow fragment of DNA polymerase I to give blunt ends, before religation with T4 DNA ligase. GH-producing colonies were identified after transformation of *E. coli* JM101 with the ligation mixture. The construction procedure is summarized in fig.1.

2.3. *Screening of clones for GH production*

Colonies were grown overnight on nitrocellulose discs (Schleicher and Schuell, D-3354 Dassel, FRG) on B agar plates [10] and then induced for 4–6 h on B agar plates containing 160 μg IPTG/ml. Bacteria were lysed and proteins bound to the nitrocellulose [13]. Colonies containing GH-related protein were identified by their red/brown colour after treatment with a rabbit polyclonal antibody to bGH followed by donkey anti-rabbit IgG-peroxidase conjugate and then 3,3'-diaminobenzidine/H_2O_2. Further screening was carried out after growth of bacteria in 2TY medium with vigorous aeration at 37°C. When the absorbance of the culture at 600 nm reached 0.6, IPTG (160 μg/ml) was added as inducer. After the induction period bacteria were harvested by centrifugation, washed and resuspended in 0.1 M $NaHCO_3$/NaOH, pH 8.9, broken by sonication for 30 s in an MSE 150w sonicator (amplitude 20) and separated into supernatant and pellet fractions by centrifugation for 2 min at 11 000 × *g*. Proteins in the pellet fraction or in the unfractionated sonicate were analysed by SDS-polyacrylamide gel electrophoresis (12.5%, w/v, acrylamide) [14]. Gels were stained with Kenacid blue and scanned using an LKB Ultroscan XL laser densitometer. Protein bands on gels were subjected to Western blotting and GH-related proteins were detected using antibody to bGH as described above. GH-related proteins in the supernatant fraction were determined by radioimmunoassay [15].

2.4. *Sequencing of plasmid DNA*

DNA from one expression plasmid was digested with *Ban*II and *Pvu*II and the *Ban*II ends converted to blunt ends with T4 DNA polymerase [11]. The sequence of those fragments containing the coding sequence for oGH and the pUC8 derived sequence around the initiation codon was determined by the chain termination procedure [16] after insertion of the fragments between the *Eco*RV and *Pvu*II sites of plasmid pJBS633 [9].

3. RESULTS AND DISCUSSION

The *Hae*II/*Hin*dIII fragment of pOGH4 was ligated between the *Bam*HI and *Hin*dIII sites of plasmid pUC8 in order to insert the DNA sequence coding for mature oGH (without signal peptide) after the initiation codon for β-galactosidase (fig.1). After transformation of *E. coli* JM101 with the ligation products only 8 transformants were obtained and none produced detectable amounts of GH when screened on nitrocellulose discs. Treatment of plasmid DNA from the transformants with *Eco*RI, *Bam*HI or *Hin*dIII showed that all contained *Eco*RI and *Hin*dIII sites but were not digested by *Bam*HI. (It was expected that a *Bam*HI site would be reformed after ligation, giving an in-frame fusion, but this did not occur).

DNA from one transformant was treated with *Eco*RI and then for 30 s–1 min with exonuclease *Bal*31 followed by religation. This procedure should produce a range of short deletions around the *Eco*RI site. Transformation of *E. coli* JM101 with the ligation products led to the production of over 2000 transformed colonies. As the deletions would be expected to be random about the *Eco*RI site only one third of the resulting plasmids would be expected to contain the oGH sequence in the correct reading frame for expression. 19 of the first 92 colonies screened for GH production on nitrocellulose discs gave a positive result and these were studied further after growth in 2TY medium. Table 1 shows the 'GH' content of the soluble fraction from 10 of these transformants sonicated after 2.25 to 21.5 h induction with IPTG. The concentration of 'GH' in these supernatant fractions corresponded to less than 0.05% of the total soluble protein in all cases. Analysis of the pellet fraction on SDS-PAGE after 21.5 h induction showed that a protein band was present in some transformants which was absent from the pellets of control bacteria (fig.2). There were large differences between the clones in the level of expression of these extra bands: in two clones (2 and 6) the band comprised over 40% of the protein in the pellet fraction while in others much lower levels were found. Western blotting demonstrated that the protein in these bands was GH-related, specifically binding polyclonal antibody raised against bGH. When the total proteins from clones 2 or 6 were run on SDS-PAGE the extra bands, which had an apparent M_r

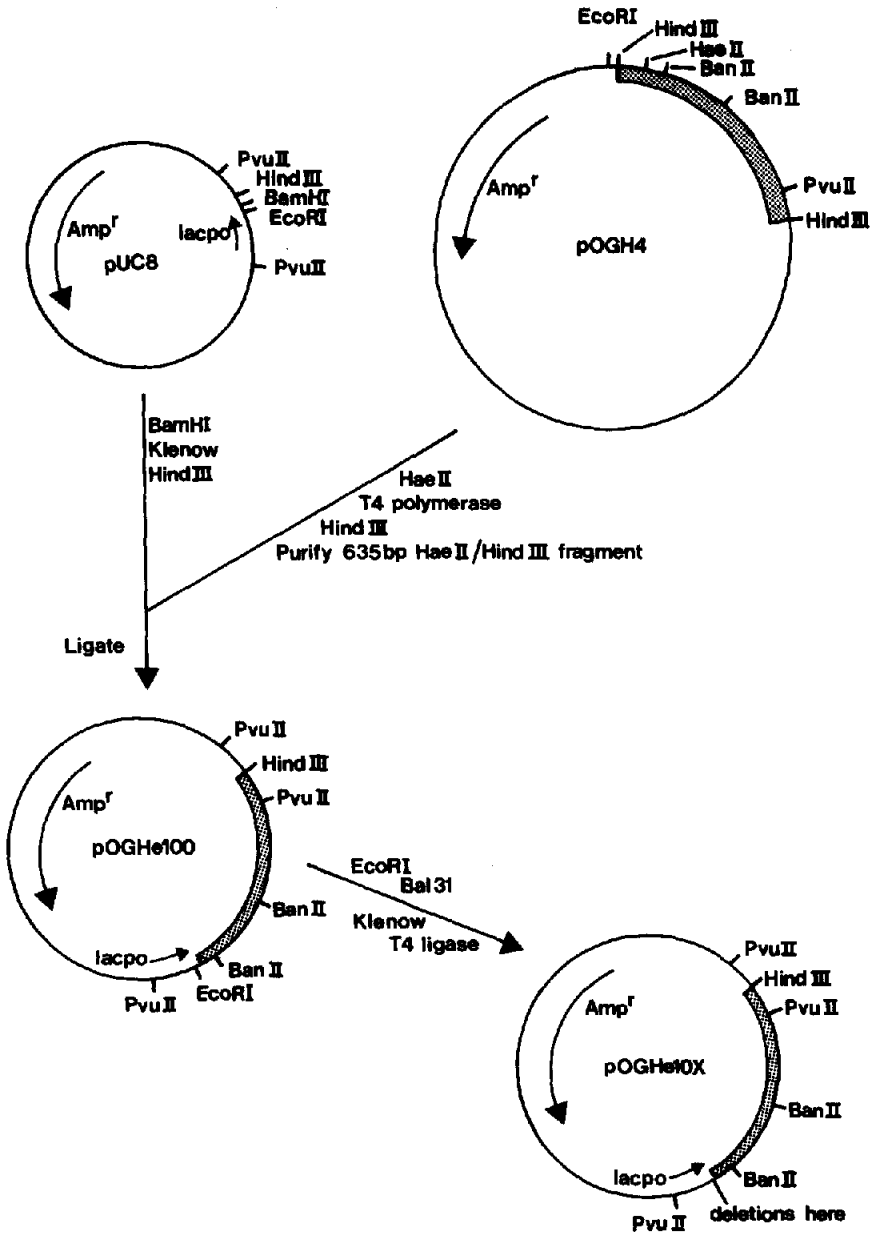

Fig.1. Scheme for construction of expression plasmids. (▭) Bases derived from oGH cDNA. Only those restriction sites used in construction of sequencing or expression plasmids are shown. lacpo, *lac* promoter.

close to that of authentic bGH, were found to constitute over 20% of the bacterial protein. Comparison of the results shown in table 1 and fig.2 indicates that when high expression occurs most of the GH-like protein is present in an insoluble form, presumably as inclusion granules.

Initially clones 6 (fig.2) containing the plasmid designated pOGHe101 and producing GH-related protein oGH1 was selected for further study. The sequence of bases coding for the N-terminus of oGH1, determined by sequencing the appropriate *Pvu*II/*Ban*II fragment from pOGHe101 (see fig.1),

Table 1

Levels of soluble GH variants in *E. coli* JM101 clones containing expression plasmids

Time after induction (h)	Clone number									
	JM101	1	2	3	4	5	6	7	8	9	10
2.25	0[a]	0.64	0.44	0.03	0.82	0	0	0	0.54	0.27	0.03
3.7	0	1.4	1.4	0.29	0.13	0.07	2.6	0	2.0	0.87	0.11
21.5	0	2.5	1.0	0	0	0	1.8	0.10	1.0	0.34	0

[a] A figure of 0 in the table indicates that no significant concentration of GH could be detected under the conditions of the assay

All clones were grown in 2TY medium and induced by addition of IPTG when $A_{600\ nm}$ reached 0.6. Bacteria were broken by sonication after harvesting and the GH content of the 11 000 × *g* supernatant was determined by radioimmunoassay using bGH as standard. The results are expressed as microgram GH in bacterial supernatant per 100 ml culture

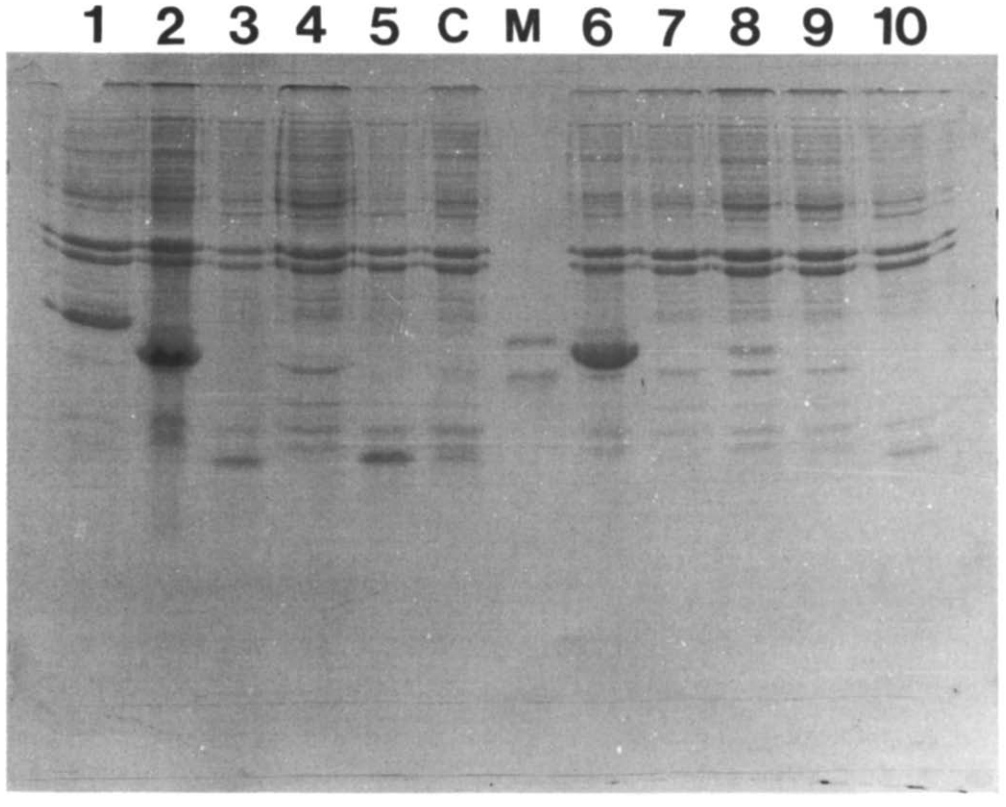

Fig.2. SDS-polyacrylamide gel electrophoresis on proteins from *E. coli* clones. Pellet fractions from broken induced bacteria were run on 12.5% gels, as described in section 2. C, control bacteria (no expression plasmid); lanes 1–10, bacteria clone numbers (+ expression plasmids); M, bGH (M_r 21 800) and ovine prolactin (M_r 22 700) markers.

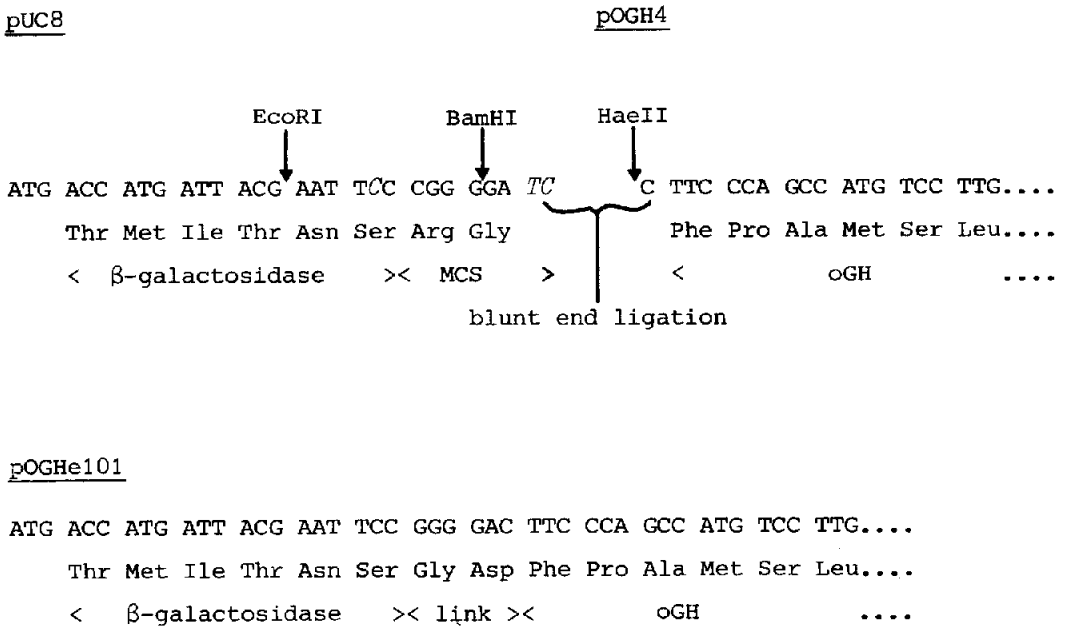

Fig.3. Nucleotide sequences and corresponding amino acid sequences in pOGHe101 and its parent plasmids (pUC8 and pOGH4). Part of the sequences for pUC8 and pOGH4 show the expected sequences of the 3′- and 5′-ends to be ligated to give plasmid pOGHe100 (see fig.1). Bases in italics are missing from the final pOGHe101 sequence. MCS, multiple cloning site. The nucleotide sequence in pOGHe101, corresponding to the codons for the N-terminus of oGH1, was determined as described in the text.

is shown in fig.3 together with the corresponding amino acid sequence. The remainder of the bases coding for oGH1 were found to correspond to the sequence previously determined for oGH [2]. Codons for the N-terminus of β-galactosidase (derived from pUC8) up to and including the *Eco*RI site are complete except that one base has been deleted at this point (presumbly by the *Bal*31 treatment). A further two bases have been lost at the pUC8/pOGH4 junction; the explanation for this is not clear but it explains the failure to obtain expression with the initial fusion plasmid since the oGH sequence would not be in the correct reading frame in this construct. The sequence in pOGHe101 clearly allows a very high level of expression of the GH-related fusion protein. A comparison of this sequence with that in the other expression plasmids showing high or low expression may allow identification of features for efficient protein synthesis in this system. Further screening of the clones obtained in this study and identification of those expressing GH variants in high yield are in progress.

The procedure described here provides a promising method for generating large numbers of expression plasmids and selecting those which give high yields of GH variants. The approach may also be of value in other expression systems. The high level of expression of oGH1 in induced bacteria containing plasmid pOGHe101 allows production of large amounts of this protein for further study. The purification of oGH1 in high yield and a study of its properties will be reported separately.

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REFERENCES

[1] Wallis, O.C., Warwick, J.M. and Wallis, M. (1986) J. Endocrinol. 108, supplement, Abstr. 223.
[2] Warwick, J.M. and Wallis, M. (1984) Biochem. Soc. Trans. 12, 247–248.
[3] George, H.J., L'Italien, J.J., Pilacinski, W.P., Glassman, D.L. and Krzyzek, R.A. (1985) DNA 4, 273–281.
[4] Hsiung, H.M. and MacKellar, W.C. (1987) Methods Enzymol. 153, 390–401.

[5] 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.
[6] Vize, P.D. and Wells, J.R.E. (1987) FEBS Lett. 213, 155–158.
[7] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[8] Wallis, O.C. and Wallis, M. (1987) J. Endocrinol. 112, supplement, Abstr. 273.
[9] Broome-Smith, J.K. and Spratt, B.G. (1986) Gene 49, 341–349.
[10] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[12] Cohen, S.N., Chang, A.C.Y. and Hsu, L. (1972) Proc. Natl. Acad. Sci. USA 69, 2110–2114.
[13] Helfman, D.M., Feramisco, J.R., Fiddes, J.C., Thomas, G.P. and Hughes, S.H. (1983) Proc. Natl. Acad. Sci. USA 80, 31–35.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Ray, K.P. and Wallis, M. (1982) Mol. Cell. Endocrinol. 27, 139–155.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.