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Cloning of bovine growth hormone gene and its expression in bacteria

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ABSTRACT

A hybrid plasmid was constructed containing β-lactamase gene of plasmid pBR322 and cloned coding sequences of bovine growth hormone (BGH). The constructed plasmid contains all DNA sequences required to encode BGH, and when used as a hybridization probe it detects one growth hormone gene in the bovine genome. The cloned DNA sequences are inserted into the β-lactamase gene in the correct reading frame for BGH synthesis. The hybrid gene is expressed in bacteria and the product, a fused β-lactamase-bovine growth hormone protein, is specifically immunoprecipitated with anti-serum to BGH. Unlike β-lactamase, very little growth hormone containing sequences can be detected in the periplasmic space.

INTRODUCTION

BGH is composed of a single polypeptide of 191 amino acids synthesized initially as a pre-growth hormone containing an amino-terminal extension of 26 amino acid residues (1). Administration of bovine growth hormone (BGH) to dairy cows significantly increases milk production and reduces the amount of feed required per a given output of milk (2). Thus, the availability of a sufficient supply of BGH might be useful in animal husbandry for more economical food production. Since mammalian growth hormones are species specific for their biological activity alternative sources to bovine growth hormone can not be used for this purpose. The goal of this project, therefore, was to construct a recombinant plasmid designed to program bacteria for efficient synthesis of biologically active bovine growth hormone.

Recent developments in recombinant DNA technology have enabled the cloning in bacteria of the natural coding sequences of several mammalian peptide hormones (3-4). In some instances, expression of these DNA sequences in bacteria has been achieved by formation of hybrid proteins, in which bacterial polypeptides are fused with polypeptides encoded by inserted eukaryotic DNA (5). The synthesis in bacteria of a discrete (i.e., non-hybrid)

human growth hormone has also been reported (6). The present study describes the construction and characterization of a recombinant DNA molecule that contains all of the BGH coding sequences and the expression of these sequences in the form of a fused β-lactamase-bovine growth hormone polypeptide. After the completion of this work Miller et al. have reported the molecular cloning of DNA complementary to BGH mRNA (7).

MATERIALS AND METHODS

Construction of hybrid DNA molecules. RNA was extracted from the anterior lobes of bovine pituitary glands, using the procedure described by Kirby (8). Poly(A)-containing RNA was purified by two cycles of oligo(dT)-cellulose chromatography, and double-stranded cDNA was synthesized essentially as described by Seeburg et al. (3). pBR322 was linearized with Pst I and approximately 20 dG residues were added to the 3' end by terminal deoxynucleotidyl transferase under the conditions described by Chang et al. (9). Similarly, dC residues were added to the in vitro synthesized ds-cDNA. The 'dC-tailed' ds-cDNAs were size fractionated by electrophoresis through 1.4% agarose gel and were visualized by autoradiography. Molecules larger than 600 base-pairs were electro-eluted, concentrated by ethanol precipitation, mixed with equimolar amounts of dG-tailed pBR322 vector and dialyzed against 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0. DNAs were annealed by incubation at 70°C for 10 minutes, followed by successive incubations at 46°C, 37°C and 23°C for 1 hr. each.

Transformation and identification of recombinant plasmids. Recombinant DNAs were introduced into E. coli HB101 using the transfection procedure described by Dagest & Ehrlich (10). Transformants were screened for $Amp^{s}Tet^{r}$ phenotype and plasmids containing bovine DNA inserts were identified by in situ colony hybridization (11), using [^{32}P]-labeled cDNA prepared from a total pituitary mRNA as a probe.

DNA sequencing. DNA sequencing was performed as described by Maxam & Gilbert (12).

Analysis of plasmid-encoded proteins in minicells. The minicells producing E. coli strain, p678-54, a derivative of E. coli K12 p678 (13), was transformed with the plasmids indicated in the legend of Fig. 5 by standard methods. A culture of each was grown up to the late log phase in L-broth containing 0.5% glucose and 12.5 μg/ml tetracycline. Minicells were purified by zone sedimentation through 10-30% sucrose gradients and resuspended in

methionine-free M9 salt medium. After 30 min preincubation at 37°C, 250 μCi of ^{35}S-methionine were added and incubation continued for 30 min. The reaction was terminated by addition of sodium-azide to 10 mM. Minicells were pelleted, washed and lysed in SDS sample buffer, and the extracts analyzed by electrophoresis on 12% polyacrylamide-SDS gel. The labeled proteins were identified by autoradiography of the dried gels.

Radioimmunoassay. Aliquots of cell extracts were incubated for 45 min at room temperature with ^{125}I-labeled BGH and rabbit antiserum raised against BGH (kindly provided by the National Pituitary Authority of N.I.H.) in the presence of 50 mM Tris-barbital pH 8.8; 2% BSA, 0.1% Triton X-100 and 0.1% SDS. A suspension of chloroform treated Staphilococcus A cells were then added and incubation continued for 10 min. Tubes were washed extensively with incubation buffer and labeled immunoprecipitates were counted in a gamma counter.

RESULTS

I. Construction and Identification of cDNA Clones

The relative abundance of growth hormone mRNA in a bovine pituitary gland was assessed by subjecting the preparation of poly(A)-containing RNA isolated from the anterior lobe of the gland to in vitro translation. The most abundant mRNA species in the gland were found to be prolactin and growth hormone mRNAs (see Fig. 1C). In some preparations growth hormone specific mRNA constituted up to 20% of the total mRNA activity. Our strategy for obtaining cDNA clones encoding bovine growth hormone was, therefore, to use the entire mRNA population as a template for cDNA synthesis and to select from the population of cDNA clones those containing the BGH gene. The product of double-stranded cDNA synthesis was extended by a short oligo(dC)-tail of 20 nucleotides and was sized on an agarose gel. Material larger than 600 base-pairs was selected in order to enrich for full-length sequences (573 base-pairsare required to encode the 191 amino acids long growth hormone). Two distinct bands were observed in this fraction (data not shown), presumably representing full-length prolactin and GH cDNAs. DNAs were inserted into the Pst I site of pBR322 after extending this cleavage site with 20 base-pairs long oligo (dG)-tails and the recombinant plasmids were used to transform bacteria. Transformants exhibiting Tet^{r}Amp^s pheno-type were selected. To ensure that the chosen clones contained bovine DNA inserts,

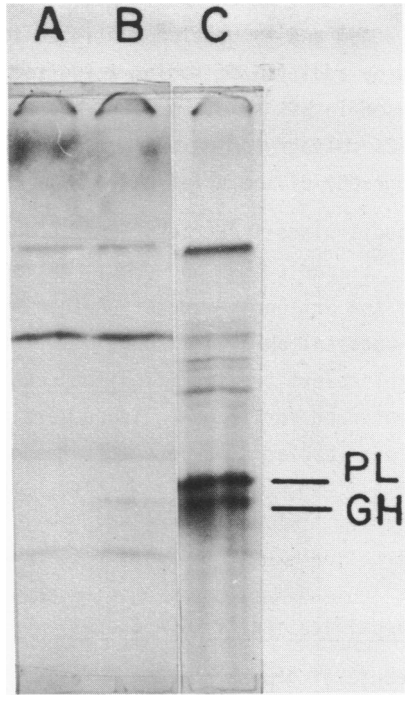

FIGURE 1. Identification of clone D-4 encoded protein by mRNA-hybridization-selection assay. 5 μg DNA of the recombinant plasmid of clone D-4 were heat-denatured, spotted on a nitrocellulose filter (1 cm x 1 cm), air-dried, and baked for 6 hrs at 80°C. Filter-bound DNA was hybridized with 10 μg of total pituitary mRNA. Hybridization conditions, removal of non-hybridized mRNA, subsequent elution from the filter of hybridized mRNA, were carried out as described by Ricciardi et al., (15). Cell-free translation in reticulocyte lysates was performed as previously described (16). ^{35}S-methionine labeled translation products were resolved by electrophoresis through 15% polyacrylamide-SDS gel and autoradiographed. A). No mRNA added. B). mRNA selected by prehybridization with clone D-4 DNA. C). Total pituitary mRNA.

we selected only clones that gave positive signals in an in situ colony hybridization with [^{32}P]-cDNA probe prepared from total pituitary mRNA. The inserts of 60 recombinant plasmids were sized by gel electrophoresis exploiting the fact that the cloning procedure yielded inserts that are excisable by Pst I digestion (14). The restriction enzyme-digestion patterns from inserts larger than 600 base-pairs were analyzed using agarose gels. Two groups of recombinant plasmids each of which produced common restriction fragments were identified. We reasoned that these two groups of clones were most

probably the reverse transcripts of the most abundant mRNA species-prolactin and growth hormone. In order to identify the recombinant plasmids that harbor GH DNA, the candidate DNAs were subjected to an mRNA hybridization-selection (15). This method involves: Hybridization of total cytoplasmic RNA to each of the DNAs in question that have been immobilized on nitrocellulose filters; elution of the hybridization-selected RNAs and; Translation in a cell-free system in order to identify their encoded polypeptides. A typical example is shown in Fig. 1. The mRNA species that were selected by hybridization with DNA from the clone D-4 directed the synthesis of a single polypeptide that co-migrated with authentic GH and, thus, enabled the identification of the insert of clone D-4 as growth hormone DNA. Using this technique, several GH cDNA clones, as well as prolactin cDNA clones, were identified. The remainder of this manuscript is concerned with the clone D-4.

II. Clone D-4 Contains all DNA Sequences Required to Encode BGH

The overall length of the insert of clone D-4 is 820 base-pairs. Figure 2 shows the restriction map of this insert with Pst I and Hinf I. The fragments generated by double-digestion with PstI and Hinf I were isolated and further processed for DNA sequencing. Figure 2 shows a partial DNA sequence of one of these fragments. One of the coding frames of this DNA sequence matches precisely the known amino acids sequences of BGH. This

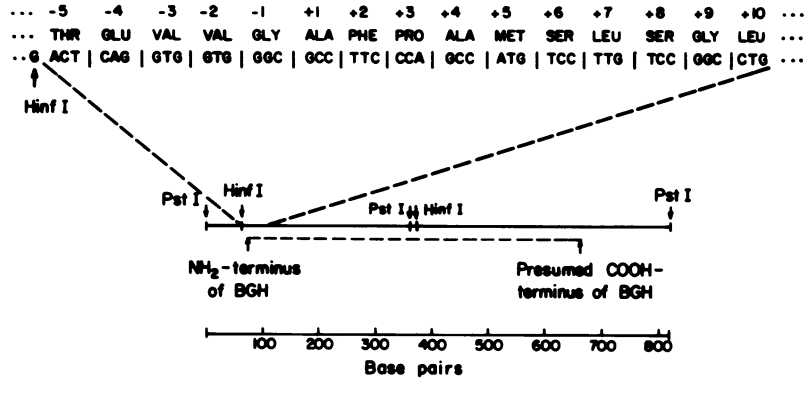

FIGURE 2. Partial DNA sequences of the insertion in clone D-4. Restriction map of the 820 base-pair long insertion in clone D-4 with Pst I and Hinf I is shown. The DNA sequence shown, starting at the Hinf I site, encodes for the 10 $_2$HN-terminal amino acids residues of BGH and for the 5' COOH-terminal amino acid residues of the signal peptide of pre-BGH. The presumed map location of the COOH-terminus of BGH was assigned 573 base-pairs downstream from the triplet that encodes NH_2-terminal Ala of BGH (BGH is composed of 191 amino acids).

constitutes definite evidence for the assignment of clone D-4 as BGH. The DNA sequences at the 5' terminus of clone D-4 (i.e. the 60 bp fragment) revealed that this clone contains the coding information for 23 amino acids of the signal peptide (data not shown). The DNA sequence depicted in Fig. 2 contains the sequence that encodes for the NH_2-terminus of BGH. The map location of the region encoding the first amino acid of BGH is 740 base pairs upstream from the Pst I site marking the other insert-plasmid junction. Since only 573 base-pairs are required to encode BGH, we concluded that all of the DNA sequences required for synthesis of the polypeptide, as well as most, if not all, of the non-translated 3' region of the mRNA are included in clone D-4.

III. Detection of BGH Genes in Bovine DNA

The availability of cloned BGH DNA sequences enables us to probe the bovine growth genome for BGH genes. As can be seen in Fig. 3, all BGH DNA sequences are contained within a single Eco RI or Hind III fragment. Since a single

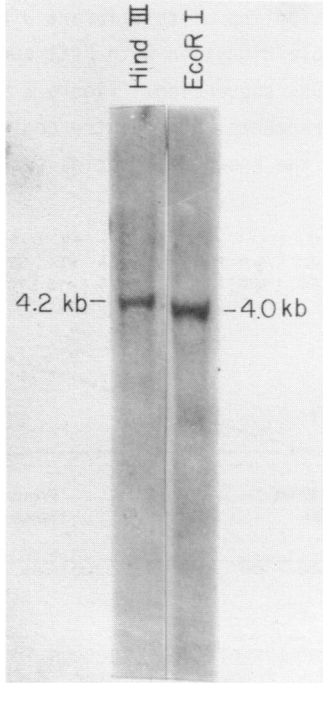

FIGURE 3. Detection of BGH genes in bovine DNA. Samples of bovine DNA (20 μg) were digested completely with the indicated enzymes. DNA fragments were electrophoresed through 0.7% agarose gel, blotted onto nitrocellulose paper, hybridized with clone D-4 DNA that had been labeled by nick-translation (specific activity $1x10^8$ cpm/μg) and autoradiographed.

hybridization band is observed with two different enzymes the possibility of unresolved multiple bands is minimized. This result indicates that there is a single growth hormone gene in bovine DNA, although the possibility of tandem arrangements of more than one gene has not been ruled out. The presence of only one GH gene in bovine DNA eliminates the possibility that the gene, whose mRNA transcript has been cloned, does not encode biologically active hormone.

IV. Orientation of Insertion of Clone D-4

In order that BGH sequences will be synthesized as a fused β-lactamase-BGH polypeptide, the eukaryotic insert has to be in the same orientation as the β-lactamase gene. Since the β-lactamase gene is transcribed counter-clockwise the correct orientation is the one in which the DNA sequences, encoding the COOH-terminus of BGH, resides counter-clockwise with respect to the $_2$HN-terminus of the hormone. Figure 4 shows an experiment designed to determine the orientation of insertion. Since there are two Hinf I sites in the insert,

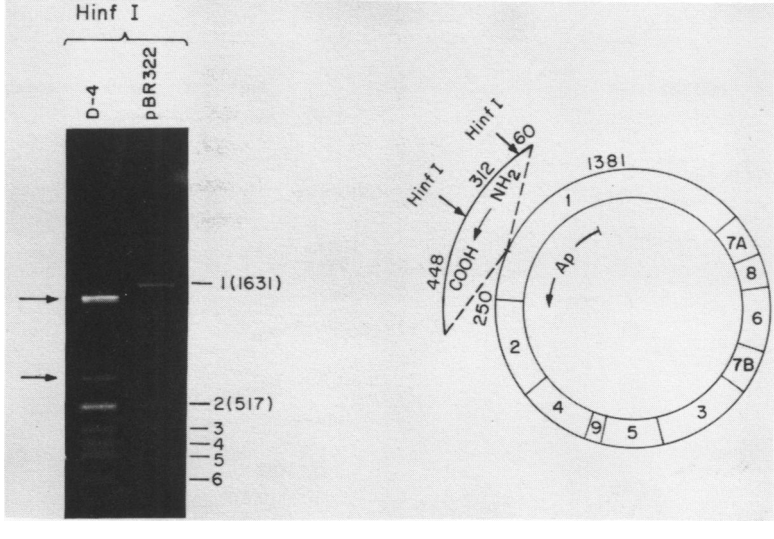

FIGURE 4. Orientation of the insertion in Clone D-4 in respect to the β-lactamase gene. The fragments generated by Hinf I digestion of pBR322 and D-4 DNAs are shown. The Hinf I fragments of pBR322 are numbered according to decreasing size. Fragment No. 1 (which contains the Pst I site of insertion) is replaced in D-4 by three fragments. The junction fragments are roughly 1400 bp and 650 bp (indicated by arrows). These sizes are consistent with the orientation of insertion shown in the scheme, namely, the same orientation as the β-lactamase gene. For comparison, in the case of reverse orientation of insertion, the sizes of the two function fragments would have been 1800 bp and 310 bp, respectively.

digestion of clone D-4 with Hinf I will produce three insert-containing fragments. The size of these fragments depends on the orientation of insertion. As explained in the legend to Fig. 4, the size of the two insert-plasmid junction fragments produced by Hinf I digestion, indicate that in clone D-4 the BGH is inserted in the correct orientation.

V. Expression of a Fused β-lactamase-BGH Protein

Purified minicells were used to study proteins encoded by the recombinant plasmid of clone D-4. Labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and were compared with those encoded by pBR322. As anticipated, the β-lactamase which is synthesized in cells carrying pBR322 plasmid were not made by clone D-4 (Fig. 5A). The recombinant plasmid encodes a unique polypeptide with an apparent 46,000 molecular weight (among several other novel polypeptides). This size is in agreement with the size expected for the hybrid protein. Our cloning scheme did not include any measure to ensure proper phasing. We reasoned that the use of the "d(G)-d(C)-

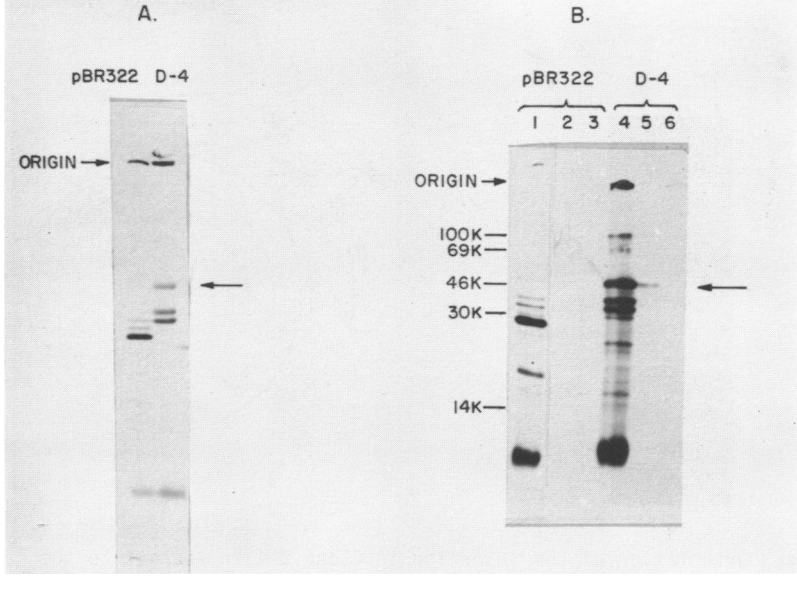

FIGURE 5. Analysis of D-4 encoded proteins in minicells. Analysis of ^{35}S-methionine labeled proteins encoded by plasmids D-4 and pBR322 in minicells was carried out as described under Materials and Methods. A). Total proteins produced by the indicated plasmids. B). Immunoprecipitation: 1,4- total lysates without antiserum; 2,5- antiserum to BGH; 3,6- control non-immune serum. Arrows indicate the 46K protein encoded by D-4 that is immunoprecipitated by anti-BGH.

tailing" procedure will result in randomization of the phasing of insertion and, therefore, clones that harbor BGH DNA in each of the translational reading frames could be selected from the mass populations of recombinant plasmids. The data shown in Figs. 5A and 6, demonstrate that in clone D-4, the BGH is inserted in phase with respect to the β-lactamase DNA. Figure 5 shows that the 46K protein synthesized in clone D-4 is specifically immunoprecipitated by antiserum to BGH. Control experiments show that non-immune serum did not immunoprecipitate the 46K protein and that the anti-BGH serum failed to react with proteins made by pBR322 and with proteins produced by recombinant plasmids harboring prolactin DNA (D-7 in Fig. 6). A fused protein could also be synthesized from an out-of-phase insertion. However, since this protein does not contain BGH amino acid sequences it would not be recognised by anti-BGH. Clone R-41 is an example of an out-of-phase insertion; this plasmid contains a BGH DNA insert and expresses a fused β-lactamase-insert protein (data not shown). This fused protein, however, did not react with anti-BGH (Fig. 6). We quantified the BGH-containing polypeptide produced by clone D-4 by radioimmunoassay, and estimated that approximately 5,000

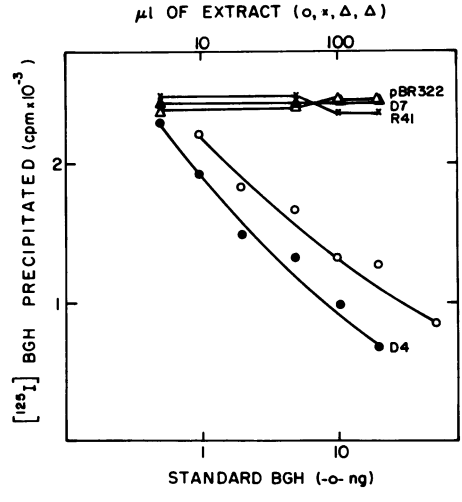

FIGURE 6. Quantifications of BGH-containing protein produced in E. coli. E. coli cells containing the indicated plasmids grown to the late log phase were collected by centrifugation. Protoplasts were prepared by lysosyme treatment and cells were lysed by adding 0.1% Triton X-100 and Na Sarkosyl. Serial dilutions of the cell extracts were analyzed by radioimmunoassay as described under Materials and Methods. D7 is a plasmid containing prolactin DNA (manuscript in preparation). R41 is a plasmid containing BGH DNA sequences.

molecules of BGH were synthesized per cell.

In order to determine whether the fused β-lactamase-BGH polypeptide is secreted to the periplasmic space, we analyzed proteins released from the cells after formation of spheroplasts. Whereas in a pBR322 control sample a large portion of the β-lactamase was released, we could detect very little of 46K protein in the supernatant (Rosner et al., unpublished data). We concluded, therefore, that the fused β-lactamase-BGH-protein is not secreted to the periplasmic space, or that it is rapidly degraded upon secretion.

DISCUSSION

This study describes the successful cloning in E. coli of the DNA sequences essential to program synthesis of the entire BGH. The constructed recombinant plasmid contains BGH coding sequences inserted in an orientation that promotes transcription of a hybrid β-lactamase-BGH mRNA, and in a translational reading frame that allows the synthesis of a fused β-lactamase-BGH protein. The question of whether this hybrid protein has a biological activity remains to be determined. In vivo proteolytic cleavage of a fused bacterially produced protein to give authentic BGH is very unlikely. On the other hand, it has been shown that proteolysis of mammalian growth hormone by several bacterial protease preparations increases its growth activity (17,18). These findings have led to the suggestion that the hormone may have to be modified in vivo post-synthetically to exert is physiological effects, in which case a hybrid protein might still possess biological activity. Alternatively, the hybrid protein could be treated with proteases in vitro to produce the potentiated BGH peptides.

The use of the same cloning procedure employed here (namely "G-C-tailing" and cloning into the Pst I site of pBR322) in other systems has led to the synthesis of structurally discrete and biologically active aukaryotic proteins (9,19). It has been shown that the presence of a translational start codon, a certain distance from the translational control region on mRNA is necessary for the synthetis of structurally discrete proteins. Moreover, it has been demonstrated that the DNA sequence that is created at the Pst I site and the "G-C-tail" has a high degree of homology with the 3' OH end of 16S ribosomal RNA and that it fulfills the structural requirement for initiation of dihydrofolate reductase (19). Therefore, it should be possible to molecularly 'edit' the cloned BGH gene in a fashion that will promote synthesis of a discrete protein.

The fused β-lactamase-BGH protein is not excreted to the peripleasmic space. The rapid turn-over of the protein due to its intracellular location is a likely explanation to the relatively small amounts of protein that was detected. This result is similar to the case of rat growth hormone (5) but is different from the case of the rat proinsulin (20).

Recent work by Talmadge et al., (21) has shown that the presence and precise location of the signal peptide plays a crucial role in the transport of proteins into the bacterial periplasmic space, and that both bacterial and eaukaryotic signals are recognized and processed correctly by bacteria (22). It is possible, however, that the presence of the hydrophobic regions of both signals in the fused β-lactamase-BGH protein is the cause for its failure to be excreted. This possibility is currently being examined by constructing a recombinant plasmid devoid of most of the signal peptide of BGH.

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