Construction of a complete bovine growth hormone cDNA

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CONSTRUCTION OF A COMPLETE BOVINE GROWTH HORMONE cDNA

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1 SEPTEMBER 1986

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF REQUIREMENTS
FOR DEGREE OF MASTER OF SCIENCE
DEPARTMENT OF BOTANY
UNIVERSITY OF DURHAM

A mes parents

"I know why there are so many people who love chopping wood. In this activity one immediately sees the results."

ALBERT EINSTEIN

ACKNOWLEDGEMENT

I would like to acknowledge the help of the following with this project: Charlie Shaw, for helpful supervision, Wolfgang Schuch, for providing me with the gene and everyone in lab 20, for all their various advice.

I would like to thank Paul, Graham, Les, Steve and Jo for their moral support, Denise Hughes for the diagrams, and Paul Jackson for his great help before the exams and throughout the year.

I am especially grateful to Andy Ryan without whom this work may have never been carried out to the end. His help in interpreting results and putting them into form was invaluable and his encouragements were greatly appreciated...

I would also like to thank Jean-do, Trisha, Sheryl and Reena who have made this year in Durham so much more worthwhile than ever expected.

CONTENTS

ABSTRACT

The aim of the project was to construct a complete BGH cDNA under control of a plant promoter. This could then be transformed into plants to study its expression.

The N and C-terminus of bGH cDNA were excised from two plasmids constructed by ICI (pBGH 17.2, pBGH 229 respectively), and fused together in PUC8 to obtain a complete cDNA.

The complete bGH coding sequence can be excised from pUC8 by EcoRI+ HindIII digestion and cloned into Binl9, together with a suitable promoter.

In this work, a "complete" bGH cDNA clone was constructed but unfortunately, a potential small deletion at the N-terminus of the cDNA bGH clone was observed. This could subsequently affect correct expression of the gene.

It is not certain wether this deletion occured in the course of this work or through previous manipulation during the construction of pBGH 17.2 at ICI. This question is currently being resolved by ICI.

CHAPTER ONE

INTRODUCTION

1.1 PREVIOUS RESEARCH ON BOVINE GROWTH HORMONE AND BGH EXPRESSION

BGH is synthesized in the bovine pituitary as a protein of 217 amino acids, which is then processed to remove a leader peptide from its amino terminus. It has been shown that depending on the site of cleavage, the resulting mature hormone contains either 191 or 190 amino acids with an alanine or a phenylanine residue respectively at the amino terminus(Li and Ash, 1953; Lingappa et al;1977).

In 1980, a bovine growth hormone cDNA clone was isolated by W.L.Miller in California. Double stranded DNA, complementary to bovine pituitary mRNA, was cloned by insertion into the Pst1 site of pBR322 and recombinant plasmids containing bGH cDNA were identified by hybridisation to a growth hormone cDNA probe, (Miller et al.) A nucleotide sequence analysis published by W.L.Miller gives a full description of the cDNA clone.(see appendix 1)

In 1982, more complete information on the bGH gene was obtained by Woychiychik et al. who isolated the whole bGH gene from a genomic library. It is known that the gene is approximately 1800bp in length and contains four intervening sequences.

A knowledge of the complete genomic sequence was not necessary for researchers who used cDNA clones of the mature bGH and concentrated on obtaining expression of this bGH in

particular systems. Since the late seventies, the idea of producing artificially large amounts of bGH attracted especially those aware of the potential bGH market . The Wall-Street-Journal estimated this market at $ 1.2 billion (27 jun86). Many industries and universities are trying to find an ideal expression system for bGH.

Until recently, it was thought that the bGH gene would probably not be expressed correctly in bacteria which are unable to give the protein the correct secondary structure. However, Gary Gray et al. group reported obtaining authentic bGH by transforming Streptomyces lividans 66 with a plasmid containing the regulatory region of the Streptomyces fradiae aph gene and the mature bGH gene. Radioimmunoassay and immunoblotting (western analysis) were used to show that bGH was present in cells that had been in culture for up to four weeks. No bGH was found in the medium though, and the researchers were disappointed by the low yields obtained. One possible explanation for low expression of the bGH is that there is an inhibitory secondary structure in the mRNA (as with E.coli). It has been suggested that deletion of the first 60 bp of the bGH results in a higher level of expression of the remaining portion of the gene in S.lividans, although the data was not presented (Gary Gray et al.). However, because of the relative ignorance of the sequence requirements for gene expression in Streptomyces, one cannot preclude the possibility that the plasmid containig bGH lacks some information for efficient expression.

More success was obtained in the expression of BGH in eukaryotic systems, and 1985 has witnessed several important achievments. T.V. Ramabhadran et al. in the United States, have obtained high-level expression of bGH in mouse and monkey cells: CV-1 monkey cells were transfected with Simian Virus 40 (SV 40) vectors containing the genomic bGH sequence (2k bp). Replacement of the bGH promoter with the mouse metallothionein-1 (MT) promoter resulted in high-level synthsis and secretion of bGH (whereas the bGH promoter functions poorly in CV-1 cells). The MT-bGH chimeric gene was used to establish permanent-secreting mouse C 127 cell lines (using the bovine papilloma virus as a vector). It was found that bioactive bGH produced by one of the mouse cell lines was secreted at high-levels into the medium: 5ug per 10 cells per day (same reference).

An even greater secretion was obtained by Kopchick by using avian retroviral bovine growth hormone DNA recombinants to direct expression of bGH in mouse fibroblast cell lines: 15 ug of hormone per 10 cells per day was obtained. (Kopchick, et al., 1985)

A further report from Kopchick's group has shown that the cytomegalovirus (CMV) immediate-early (IE) gene regulatory region is three to fourfold more efficient than the Rous sarcoma retroviral long terminal repeat (LTR), in promoting expression of bGH in rat GH3 cells. (F.Pasleau, et al. 1985)

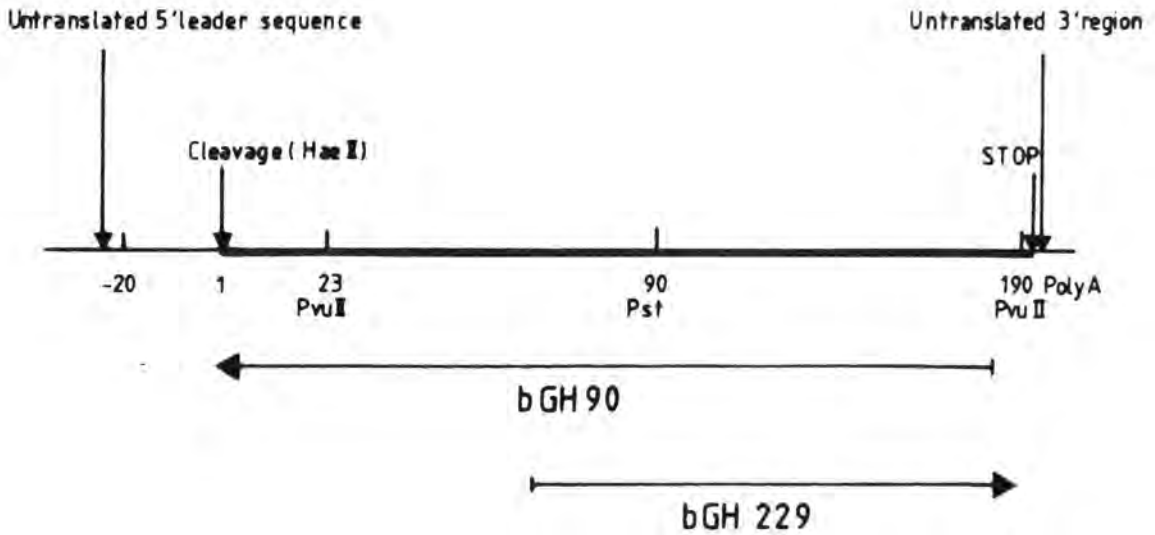
Different animal cell lines, and bacteria have been described and used successfully for expression of bGH, but it remains to be shown that it is also possible to obtain high

yields of bGH by using agrobacterium derived vectors for plant transformation (Michael Bevan, 1984)
Two promoters from NOS (nopaline synthase) and Rubisco (Ribulose-1,5 bisphosphate carboxylase) could be potentially very useful for this purpose.

This project is involved in the long term goal of achieving bGH expression in plants. In the future, it may turn out to be economically worthwhile extracting the hormone from plants rather than from bacteria or animal cells, (animal cells are difficult and more expensive to grow or maintain than plant cells); and perhaps a method may be devised of "protecting" the hormone so that it is not degraded in the gut of the cows that eat it, and the cows will be able to benifit directly from the hormone.
The experiments carried out during this project are continuation of work initiated in the laboratory of Wolfgang Schuch at ICI in Runcorn.

1.2 ICI S WORK ON THE PROJECT

ICI synthesised a range of overlapping cDNA clones of the bGH gene; their longest clone, BGH 90, is about 820 br and contains extra sequences at the 5' end, but ends 4 bases before the stop codon.(personal communication).
ICI also obtained another cDNA clone, BGH 229, that contains the end of the coding region and that even extends about 30 bases into the 3' non-translated region, but this clone lacks a quarter of the beginning of the coding sequence.


Untranslated 5'leader sequence
Untranslated 3'region
Cleavage (Hae III)
STOP
-20
1
23
PvuII
90
Pst
190 PolyA
PvuII
bGH 90
bGH 229

In order to insert BGH 90 and BGH 229 into plasmids, ICI performed the following constructions:

BGH 90 was cut with HaeII (this cut at the beginning of the mature bGH sequence and somewhere in the pAT153 vector yielding a fragment of about 1 600 bp). Linkers were added to create a BamHI site just in front of the beginning of the mature bGH sequence:

bGH sequence around amino acid 1:

GTG GGC GCC TTC CCA

after addition of linkers:

GGA TCC ATG GGC GCC TTC CCA

The linkers also supplies an ATG codon and a further gly codon as start codons. The fragment was then cloned into an expression vector, pWS1.1(high copy number derivative of R300B.) This plasmid was called BGH 17.2 (see fig 1).

BGH 229 was directly cloned into pAT153 in the Pst1 site. PAT153 is a derivative of pBR322 with a 803 bp deletion in it: its size is 3559 bp. Therefore, it is easy to isolate the c terminus of the bGH gene by a Pst digestion; we then obtain a 400 bp fragment which contains the end of the bGH coding sequence (see map 2).

In order to construct a plasmid containing the whole bGH cDNA clone, the N terminus of BGH 17.2 (280 bp Bam H1 -Pst fragment), and the C terminus of BGH 229 (400 bp Pst1 fragment) will be cloned into pUC8 (Viera, et al. 1982) The

orientation of the C terminal fragment will be determined by digestion of the plasmid with appropriate enzymes.

Once the whole bGH cDNA clone is cloned into pUC8, it can be excised by a EcoRl +hindIII digestion which makes it very convenient to clone into Bin 19 (M. Bevan 1984). The Rubisco promoter or the NOS promoter can be directly inserted into the Eco RI site and plants will subsequently be transformed with this recombinant plasmid.

CHAPTER TWO

MATERIALS AND METHODS

2.1 BUFFERS

2.1.1 10 x Alec s electrophoresis buffer, for agarose gel electrophoresis

97g TRIS

7,4g EDTA

About 1700 mls of water was added to dissolve solids. The pH was adjusted to 7.7 with acetic acid (about 32 mls) and the solution was made up to 2l with distilled water. 0.6 mls of 10 mg/ml of an ethidium bromide solution was added to the 2l.

2.1.2 10 x TBE buffer ,for minigels

216g TRIS

18.6g EDTA

110g Boric acid

made up to 2l with distilled water.

2.1.3 Restriction enzyme buffers ,10 x concentrated

stock solutions	Nacl	TRIS (pH 7.4; 1M)	MgSO4 (1m)	DTT (1M)
Low	0ul	100ul	100ul	10ul
Medium	100ul	100ul	100ul	10ul
High	200ul	500ul	100ul	0

The solutions were made up to 1 ml with sterile water.

2.1.4 Core Buffer ,10 x

NaCl (5M)	TRIS (pH 8)	MgCl2 (1M)	H2O
500ul	100ul	100ul	200ul

2.1.5 10 X Ligation buffer

TRIS (pH 7.5 1M)	EDTA (pH 9; 0.4M)	MgCl2 (1M)	DTT (1M)
66 ul	2.5ul	10ul	10ul

2ul of 50mg/ml BSA was also added as well as 5ul of ATP. The solution was made up to 100ul by adding 4,5ul of water.

2.1.6 Denaturation buffer ,one litre solution

NaCL	NaOH
87.7g	20g

700 mls of distilled water was added.

HCl was added to bring the pH of the solution down to 7.0.

The solution was made up to 1l with distilled water.

2.1.7 Neutralisation buffer

Nacl	TRIS
175.3g	60.6g

The solution was made up to 1l with distilled water and the pH adjusted to 7 with HCL.

2.1.8 20 X SSC

NaCl	Na Citrate
175.3g	88.2g

The solution was made up to 1l with distilled water.

2.2 MINILYSATES

From Maniatis

5mls of medium containing the appropriate antibiotic were inoculated with a single colony and put to incubate overnight at 37 C.

1.5ml of the culture was poured into an eppendorf tube. The tube was centrifuged for 1 minute in an eppendorf centrifuge. The remainder of the culture was stored at 4 C.

The medium was removed by aspiration, leaving the bacterial pellet as dry as possible.

The pellet was resuspended by vortexing inn 100ul of an ice-cold solution of:

50 mM glucose

10mM EDTA

25mM Tris HCL(pH 8.0)

The tubes were stored at room temperature for 5 minutes

200 ul of a freshly made solution of:

0.2N NaOH

1% SDS

was added.

The top of the tube was closed and the contents was mixed by inverting the tube rapidly a couple of times. The tube was stored on ice for 5 minutes.

150 ul of an ice-cold solution of potassium acetate was added. The solution was made up as follows: To 60 ml of 5M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H2O was added. The resulting solution was 3M with respect to potassium and 5M with respect to acetate. The pH of the solution was 4.8.

The tubes were centrifuged for 5 minutes.

The supernatant was added to a fresh tube.

An equal volume of phenol/chloroform was added. The tubes were mixed, centrifuged for 2 minutes, and the supernatant was then transfered to a fresh tube.

Two volumes of ethanol were added at room temperature. The tubes were mixed and stood at room temperature for two minutes. They were then centrifuged for 5 minutes.

The supernatant was removed, leaving the pellet as dry as possible

1 ml of 70% ethanol was added, the tubes were briefly vortexed and centrifuged.

All the supernatant was again removed and the pellet was dried in a vacuum dessicator.

50 ul of TE (pH 8.0) was added together with 1 ul of DNase-free pancratic RNase (20 ug/ml).

10 ul of the solution was removed to a new eppendorf tube. 1.2 ul of core buffer was added and 1 unit of the desired restriction enzyme. The tube was incubated at 37 C for 1-2 hours.

The DNA fragments in the restriction digest were analyzed by gel electrophoresis.

2.3 50 ML PLASMID PREPARATION

A 50 ml culture was grown up overnight from a single colony (in the presence of an antibiotic). The cells were spun for 10 minutes at 8 000 rpm, resuspended in 2 ml of solution I (as for the minilysate), and left at room temperature for 10 minutes. 4 ml of solution II was added.

the corex tubes were mixed and left on ice for 10 minutes. 3 ml of potassium acetate pH4.8 was added. Again, the tubes were left on ice for 10 minutes. They were then spun for 15 minutes at 8 000 rpm. The supernatant was removed and an equal volume of chloroform, phenol was added to it. These solutions were, vortexed, spun at 8 000 rpm for 10 minutes. The supernatant was then removed. 2 volumes of ethanol was added, and the tubes were left at room temperature for 5 minutes. They were centrifuged for 10 minutes at 8 000 rpm. The supernatant was removed. The pellet was washed with 5 ml of 70% ethanol. After centrifugation, the pellet was dried in dessicator, resuspended in 500 ul of TE and 50 ul of RNase was added.

2.4 LYSIS BY ALKALI

"Lysis by alkali" was the method used for extacting bacterial DNA from a 500-ml culture of stransformed bacterias. The reference is Maniatis page 90.

An overnight culture of a colony of transformed bacteria in 5-mls of L-broth was prepared; 4-mls of that culure was grown up to stationary phase in 500-mls of L-broth. When the plasmid was chloramphenicalamplifiable, 6,5 mls of 10mg/ml chloramphenicol solution was added in the culture after 4 hours. the culture was left to grow overnight.

In the morning, the culture was centrifuged at 7,000rpm for 15 minutes, in order to obtain a bacterial pellet.

The bacterial pellet from the 500-ml culture was then resuspended in 10-ml f of solution I containing 5 mg/ml lysozyme, and the solution was left at room temprature for 5

minutes.

Solution I	volume	stock
50 mM glucose	0.9 g/100ml	
25 mM tris-Cl	2.5 ml	1M tris-HCl
10 mM EDTA	4 ml	250 mM EDTA
5 mg/ml lysozyme	50 mg	

(The lysozyme was added just before use)

20-mls of freshly made solution II was added. The mixture was transfered to sorval tubes. The top of each tube was covered with nescofilm in order to mix the contents gently by inverting the tube several times. the tubes stood on ice for 10 minutes.

Solution II	volume	stock
0.2 N NaOH	2 ml	10 N NaOH
1% SDS	10 ml	10% SDS
H2O	88 ml	

5-mls of an ice-cold solution of a 5M potassium acetate (pH:4,8) was added. Again, the contents of the tubes were mixed gently, and the tubes were left on ice for 10 minutes.

The solutions were centrifuged on a Sorvail RCB50 at 19,000 rmp for 20 minutes at 4 C. The cell DNA and bactrial debris formed a tight pellet on the bottom of the tube.

Equal quantities (about 18 mls) of supernatant was transfered into 30 ml corex tubes.

Isopropanol was added to each tube (almost an equal volume). The contents was mixed and the tubes were left to stand on ice for 15 minutes.

The DNA was recovered by centrifugation in a sorvall at 10,000rpm for 30 minutes.

The supernatant was discarded and the pellet was dried

briefly in vacuum a dessicator.

The pellet was dissolved in a total volume of 8mls of TE (pH 8).

The plasmid DNA was purified by centrifugation to equilibria in caesium- chloride-ethdium bromide density gradients.

The gradients were prepared as follows:

For every ml of DNA solution, 1g of solid CsCl was added. the solution was mixed until all of the salt was desolved.

0.8ml of a solution of ethidium bromide (10mg/ml in H2O) for every 10ml of CsCl solution was added.

The solution was transfered to Beckman tubes.The tubes were carefully balanced and centrifuged at 45,000 rpm for 24 hours at 20 C.

Two bands of DNA were visible (the non-chloramphenicolamplifiable plasmid band was only visible under uv light). The upper band consisted of linear bacterial DNA and nicked circular plasmid DNA; the lower band consisted of closed circular DNA.

2.5 PREPARATION OF COMPETENT CELLS AND TRANSFORMATION

Two different methods were used for the preparation of competent cells: the first one is for the immediate use of the cell, the second one allows the cells to be stored at -80 C for subsequent use.

The first transformation method used is the one described by H.J.Fritz.

2.5.1 Preparation of competent cells

An overnight culture of E.Coli (K514) was prepared in 5 mls of L.Broth.

50mls of L.Broth were inoculated with 1ml of o/n culture in a side arm flask. The culture was shaken at 37 C until samples produced an absorbance reading of 0.2-0.25. (takes about 2-2 1/2 hours).

40mls of culture were centrifuged in an M.S.E. rotor, or in a sorvall ss34 rotor, at 7,000 rpm, for 10 minutes at4 C.

The supernatant was discarded, and the pellet was resuspended in a half of volume (20mls) of cold 0.1M Ca Cl .

this was left on ice for 10 minutes, centrifuged at 7,000 rpm for 20 minutes (at 4 C). The pellet was resuspended in 2mls of cold 0.1M Ca Cl .

These "competent bacteria" were left on ice for at least 30 minutes and they were then used for the transformation.

2.5.2 Transformation

100ul of DNA solution was prepared, (10ul plasmid + 90ul 0.1M CaCl2, or 100ul of "ligation mix").

100ul 0.1M CaCl2 was added to another tube (negative control)

200ul of competent bacteria was added to each tube.

The tubes were left on ice for more than an hour for the DNA to penetrate the bacteria.

The bacteria were heat shocked in a water bath at 42-45 C, for 2-3 minutes.

1ml of L-broth was added.

the tubes were incubated at 37 C for over an hour.

Appropriate dilutions were prepared and 100ul was spread out on selective plates then incubated overnight at 37 C.

2.6 FRAGMENT ISOLATION

2.6.1 FREEZE SQUEEZE METHOD

The fragments were cut out from the agarose gel and put into sterile corex tubes. 0.9ml of water was added to the tubes as well as 0.1ml of 3M Na Ac 10mM EDTA. The tubes were then left in the dark for 15 minutes, and occasionally gently shaken. A hole was put in the bottom of small eppendorf tubes and plugged with glass wool. the gel slices were removed from the liquid in the corex tubes, they were cut in order to fit into the small eppendorf tubes, and the eppendorf were placed at -80 c for 15 minutes. The small tubes were then put into large eppendorf tubes and centrifuged for 15 minutes. 5ul of 1M MgCl2 10% Acetic acid was added to the liquid collected in the large tubes. 20ul 3M Na Ac pH 4.8 and 1ml of ethanol was also added, and the tubes mixed. After storage at -80 c for 20 minutes, they were centrifuged for 5 minutes and the pellet was resuspended in 180 ul of TE. 20ul 3M Na Ac, 500ul of ethanol was added. The tubes were mixed, stored at -80 c for 20 minutes and centrifuged again. The liquid was removed, the pellet dried and the DNA resuspended in 10ul of sterile water.

2.6.2 Run into well

A well was cut in front of the band to be isolated. The buffer was drawn off until it was 1 mm below the gel surface. The well was filled with 200 ul of buffer without ethidium bromide. The gel was run until half of the fragment was run into the well and 200 ul of buffer was removed to an eppendorf tube. The well was refilled and the rest of the fragment was allowed to run into the well. 200 ul of buffer was removed and pooled with the 200 ul from the first run. The DNA was phenol extracted, ethanol precipitated and resuspended in TE.

2.7 COLONY HYBRIDISATION

The colonies grown on agar plates were transferred to nitrocellulose filters by placing a filter on each plate and leaving for up to five minutes. The filters were then removed and placed successively on 3MM filter paper soa ked in: denaturation buffer (0.5M NaOH, 1.5M NaCl), for 7 minutes

1M TRIS pH 7.6, for 2 minutes

1M TRIS pH 7.6, again for 2 minutes

neutralisation buffer (1.5M NaCl, 0.5M TRIS pH 7.6),for 4 minutes

The filters were dried on 3MM paper, and then baked in a vacuum oven at 80 c, for 2 hours. After transfer, the plates were incubated at 37 C to allow the colonies to regrow.

The next step was the prehybridisation of the colonies: the following solution was made up: 3 X SSC, 200mg BSA, 200mg

ficoll, 200mg PVP, per litre. The filters were placed in the above solution in a plastic box, and incubated at 68 c for 4 hours.

The hybridisation solution was prepared as follows: 3 X SSC, 200mg of BSA, ficoll, PVP (per litre), 0,5% SDS. The filters were placed in plastic bags. Just sufficient solution to bathe the filters was added as well as the boiled radioactive probe. The bags were sealed, without any air bubbles in them and left to incubate overnight at 68 c.

The probe was prepared shortly before by adding 10ul of nucleotide stoke fer buffer as well as 5ul of dCTP and 5ul of DNA polymerase 1, to 30ul of DNA. This was put through a column with TENS buffer in order to seperate out the DNA from the RNA. The first radioactive peak was collected. The probe was denatured by boiling for 10 minutes and then stored on ice.

After the overnight hybridisation step, the filters were placed in a plastic box with a washing solution (3 X SSC 0.5% SDS). This box was shaken and incubated for 10 minutes with movement at 68 c. This procedure was repeated 4 times (until the washing solution was no longer radioactive). The filters were then rinsed in the same way in 3 X SSC, (3 times only), and dried in the vacuum oven for 30 minutes.

The filters were then placed on a metal plate covered with clingfilm (they were marked with radioactive ink for their identification. The cassettes were exposed to Fuji X ray film for a couple of hours at -70 c (the probe was quite hot).

2.8 SOUTHERN BLOTTING

This technique was described by Southern in 1975.

After electrophoresis, a photograph was taken of the gel. The gel was then transfered to a glass baking dish, soaked in several volumes of 1.5M NaCL and 0.5 M NaOH for 1 hour at room temperature with constant shaking, in order to denature the DNA. The gel was neutralized by soaking in several volumes of a solution of 1 M Tris HCL (pH 8.0), and 1.5 M NaCl for 1 hour at room temperature, with shaking. A piece of Whatman 3MM was wrapped around a stack of glass plates. This support which was longer and wider than the gel was placed inside a large baking dish. The dish was filled with 10X SSC almost to the top of the support and the air bubbles in the paper were smoothed out.

The gel was placed face down on the support. Using a fresh scalpel a piece of nitrocellulose filter slightly larger than the gel was cut. The nitrocellulose filter was floated on the surface of a solution of 2X SSC until completely wet from beneath. The filter was then fully immersed in the 2X SSC for 2-3 minutes. The wet nitrocellulose filter was placed on top of the gel. Any air bubbles were removed. Two pieces of Whatman 3MM paper were cut to the same size as the gel, wet in 2X SSC and placed on top of the nitrocellulose filter. A stack of paper towels (5-8 cm high), just smaller than the 3MM paper was cut and placed on the 3MM paper. A glass plate was put on top of the stack, as well as a 500g weight. Transfer of DNA proceeded in the followin 12 hours. The towels and 3 MM papers were removed, the dehydrated gel and the filter were turned over and put on a dry sheet of 3MM

paper. After marking the position of the slots on the filter, the gel was peeled off and discarded. The filter was soaked in 6X SSC at room temperature for 5 minutes, dried at room temperature on a sheet of 3MM paper, baked for 2 hours at 80 C under vacuum.

The filter was then used in hybridization experiments, (previously described).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 TRANSFORMATION OF E.COLI WITH PLASMIDS BGH 17.2 AND BGH 229

BGH 17.2 and bgh 229 were transformed into K514 (Colson et al. 1965) as previously described.

PWS1.1 is sulfonamide resistant and tetracycline resistant. The bGH fragment was inserted in the tetracycline resistance gene to create pBGH 17.2 which is only sulfanomide resistant (see fig 1). Mueller-Hinton medium plates were made up for E.coli K514 containing pBGH 17.2, Mueller-Hinton being an appropriate medium for sulfonamide resistant bacteria.

PAT153 is ampicillin resistant and tetracycline resistant. The bGH gene is inserted in the ampicillin resistance gene and so pBGH 229 is only tetracycline resistant (see fig 2). L-agar tetracycline resistant plates were made up.

Several dilutions of E.coli K514 transformed with pBGH 17.2 and pBGH 229 were respectively plated out on sulfonamide resistant Mueller-Hinton medium, and tetracycline resistant L-agar plates. The cells were diluted in $MgSO_4$, up to 10 000 times.

The next day a few colonies were purified by replating a few and streaking out for single colonies.

Individual colonies were picked for plasmid preparation.

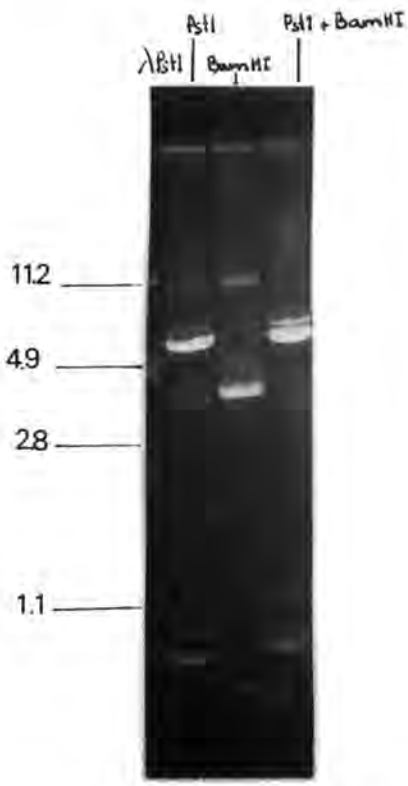
3.2 DIFFCULTIES IN PURIFYING PBGH 17.2

Several difficulties were encountered during the initial stages of this project, i.e. trying to clone pBGH 17.2. After a 500 ml plasmid preparation, little DNA was obtained and never showed the expected digestion pattern, although digestions were repeated with medium buffer and core buffer.

Pst1 should yield the following fragments: 7.7 kbp, 1.375 kbp, 0.8 kbp, 0.36 kbp.

Instead it only yielded the two following fragments: 7kbp, 0.8 kbp.

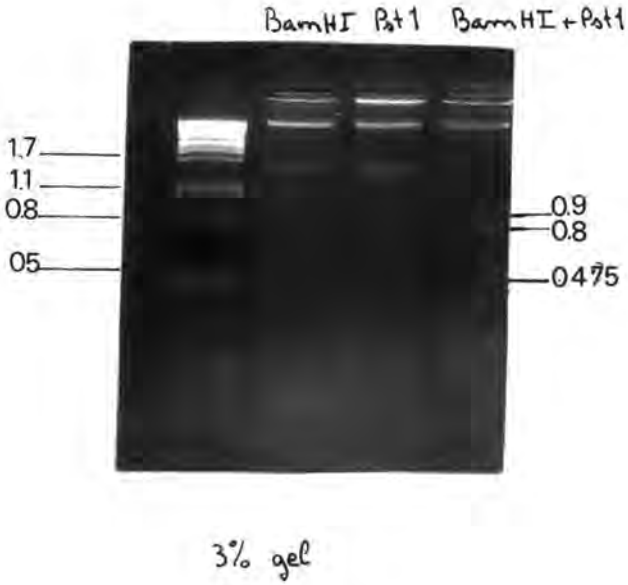
BamHI did not restrict; it should yield: 8.7 kb and 1.54 kbp fragments



Several attemps were made to obtain the expected plasmid and digestion pattern, all of which failed. A fresh isolate of pBGH in E.coli K514 was obtained from ICI Runcorn, as the original plasmid provided could not be transformed

successfully in our laboratory.

A 50ml plasmid preparation of the fresh pBGH 17.2 isolate gave plasmid DNA which showed the expected digestion patterns, as on the following picture.



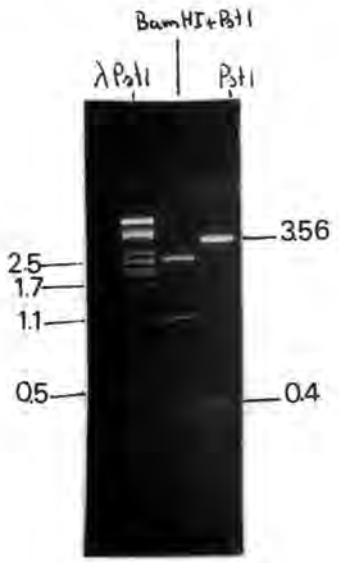
3% gel

An inconvenience of the 50ml preparation method is that relatively little plasmid DNA is obtained (10ug) and a large amount is needed for the isolation of a 280 bp fragment, as shown below.

3.3 PURIFYING PBGH 229

PBGH 229 500-ml plasmid preparation worked without any problems and gave about 100ug of DNA. The plasmid is cloramphenicalamplifiable, and therefore one would expect to get a large yield of DNA. Digestion of pBGH 229 with Pst1 and Bam HI should give a 2 436 fragment as well as 1 123bp and 400bp fragments (see fig 2 and table 2). The picture below shows that Pst1, and Pst1 + Bam HI digestions of the

isolated pBGH 229 gives the expected fragments.



The Pst1 digestion on its own yields the 400 bp fragment which is the 3' end of bGH, and a bigger fragment which is the vector. Pst 1 also yields a very small fragment that can be seen on the gel because of the GC tail upstream of the internal Pst 1 site, that has been added by ICI for the insertion of the bGH c-terminus into pAT153.

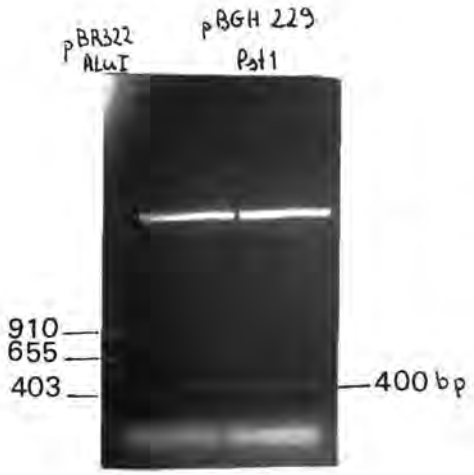
3.4 FRAGMENT ISOLATION

As mentioned in the introduction, the fragments to be isolated were very small: 280 bp and 400bp.

3.4.1 400bp fragment isolation

The 400bp fragment isolation from pBGH 229 was straightforward because a large amount of DNA was available from the 500ml plasmid preparation. "Freeze squeeze"

described in "Materials and methods" was the method used to isolate the 400bp fragment. 200ul of Pstl digested pBGH 229 DNA was loaded onto a 2% agarose gel for the 400 bp fragment to be isolated.



A small amount of the isolated DNA was run on a minigel and a very faint band confirmed the fact that the 400 bp fragment had been successfully isolated.

This fragment could not be directly inserted into pUC8 before the 280 bp fragment, as insertion of the 280 bp fragment requires pUC8 to be digested with Pstl and BamHl, which would result in excision of the 400 bp fragment if already ligated into the vector.

Although the 400 bp fragment from pBGH 229 was isolated before the 280 bp fragment from pBGH 17.2, it was stored in the freezer umtil the 280bp fragment was ligated into pUC8.

3.4.2 280 bp fragment isolation

The 280 bp fragment from the BamHl-Pstl digestion of pBGH 17.2 was very difficult to isolate for two reasons: it is about one fourtieth of the total plasmid DNA, which means that when all the DNA from a 50ml plasmid preparation was loaded onto a 2% agarose gel, only a faint band could be seen. The other difficulty was seperating out the two smallest fragments of the BamHl-Pstl digestion of pBGH 17.2, their sizes differing in only about 80bp. A polyacrylamide, 3% nusieve, or a 2% agarose gel could be used to seperate out these fragments.

The firsts attempts to isolate the 280bp fragment neither gave enough DNA to be seen on a minigel nor to be ligated successfully into pUC8.

A different strategy was therefore attempted.

3.5 DIFFERENT STRATEGY

3.5.1 7.7 bp fragment isolation

Instead of isolating the 280 bp fragment, pBGH 17.2 was digested with Pstl in order to obtain a 7.7 kb fragment which contained the 280 bp fragment but not the 360 bp one. This 7.7 kb fragment was then inserted into pUC8 cut with Pstl, (see fig 4).The strategy was then to remove the unecessary BamHl fragment and recircularise pUC8 with just the 280 bp insert in it.

E.coli JM83 was transformed with pUC8 containing the 7.7kb insert. Five white colonies were obtained on the X-gal

ampecillin selective plates, from several hundred blue colonies – the overall transformation efficiency was therefore low.

3.5.2 ligation of the fragment

Minilysates were prepared of the white colonies, and none of the resulting plasmids seemed to contain the 7.7 kb insert. One of them was clearly only pUC8 with probably a small deletion since the B-galactosidase gene was inactivated, leading to a white colony. The others seem to be pUC8 with inserts of various sizes which may represent portions of the 7.7 kb DNA fragment from pBGH 17.2, perhaps due to deletions. (see two examples on following picture)

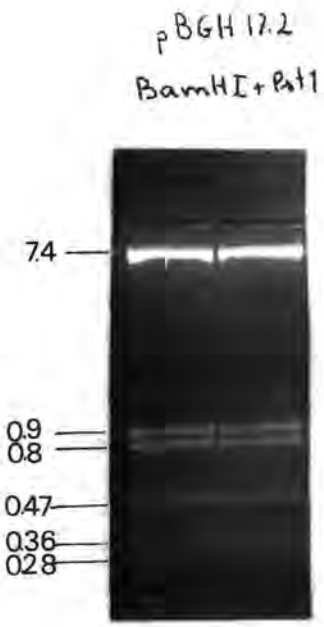
It may be that there is homology between sequences in pUC8 and pWS1.1 which allow recombination and deletion to occur. This may not have happened in a REC A strain (like HB 101) because recombination would have been suppressed, but in E.coli JM83 it appears clearly that pUC8 with the 7.7kb

fragment does not remain stable.

3.6 ISOLATION AND LIGATION OF THE 280 BP FRAGMENT FROM PBGH 17.2 INTO PUC8

Since the insertion into pUC8 of the 7.7 kb Pstl fragment of pBGH 17.2 failed in E.coli JM83, it seemed worthwhile to try again to isolate the 280 bp fragment from pBGH 17.2 and insert it into pUC8.

Two 50ml plasmid preparations of pBGH 17.2 were made. One of the preparation was used to to characterise the plasmid DNA. All the DNA obtained from the other preparation was digested with Pstl and BamHl and loaded on a 2% agarose gel.



The 280 bp fragment was isolated by the freeze squeeze method previously described. Unfortunately there was not enough DNA to run a gel to check e therecovered fragment. The small amount of DNA obtained was ligated into pUC8 t cut with Pstl and BamHl. The ligation efficiency was determined by

carrying out several ligation and transformation experiments. JM83 was transformed with:

1. ligated Pst1-BamH1 cut pUC8

2. pUC8

3. Pst1-BamH1 cut pUC8

4. insert ligated into Pst1-BamH1 cut pUC8

The cells were diluted 10 , 10 , 10 fold and 100ul was plated out onto selective media.

Transformation with uncut pUC8 resulted in twice as many colonies as transformation with ligated Pst1-BamH1 cut pUC8. From this result, it can be deduced that the ligation reaction is working with an fficiency of about 50%.

Transformation with unligated Pst1-BamH1 cut pUC8 gave only a few individual colonies.

Transformation with insert ligated into Pst1-BamH1 cut pUC8 resulted in several hundred colonies of which 10 were white.

Plasmid DNA was prepared from these 10 white colonies by the minilysate method and the DNA was digested with PvuII, in order to obtain fragments of the following size: 2 416 bp, 303 bp, 269 bp, (see fig 5 and table 4).

Only one of the colonies -colony #2- yielded the expected fragments.

3.7 LIGATION OF THE 400 BP FRAGMENT INTO VECTOR AND COLONY HYBRIDISATION

3.7.1 Ligation and digestion pattern of the colonies with the second insert

Enough DNA was obtained from the above minipreparation to cut pUC8+280 with Pstl and insert the 400 bp fragment from pBGH 229.

After transforming E.coli JM83 with pUC8+280+400, cells were diluted up to a hundred times and 100ul plated out on X-gal ampecilin selective plates.

As expected all the colonies on the plates were white and a colony hybridization was attempted in order to determine which contained the 400bp insert from pBGH 229.

Following hybridisation of the colonies of the 100 times diluted plates using radioactively labelled 400 bp fragment as the probe, the resulting autoradiograph indicated no colony containing the insert.

The autoradiograph corresponding to the undiluted plates had several dark spots indicating the presence of colonies containing a plasmid with the 400 bp insert.

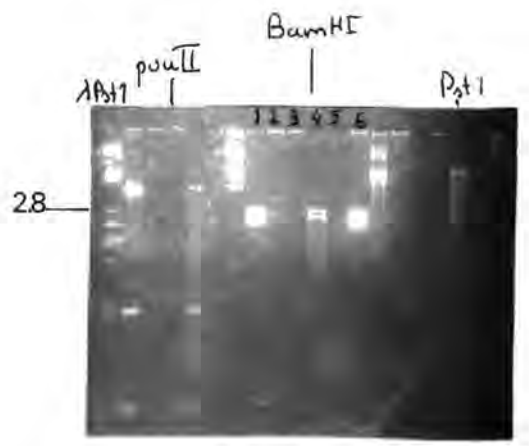
Because the density of the colonies on the plate was so high, it was necessary to replate out the colonies in the area of the dark spots. Four hundred colonies were replated out neatly on five plates and the colony hybridisation was repeated. The autoradiograph showed that many colonies contained the insert.(see the following picture).

Plasmid DNA was prepared from six of these colonies by the minilysate method, and the DNA was digested with PvuII, BamHl and Pstl.

The expected fragments for these digestions were:

PvuII	BamHI	Pstl
2 416	3 588	3 188
494		400
277		
206		

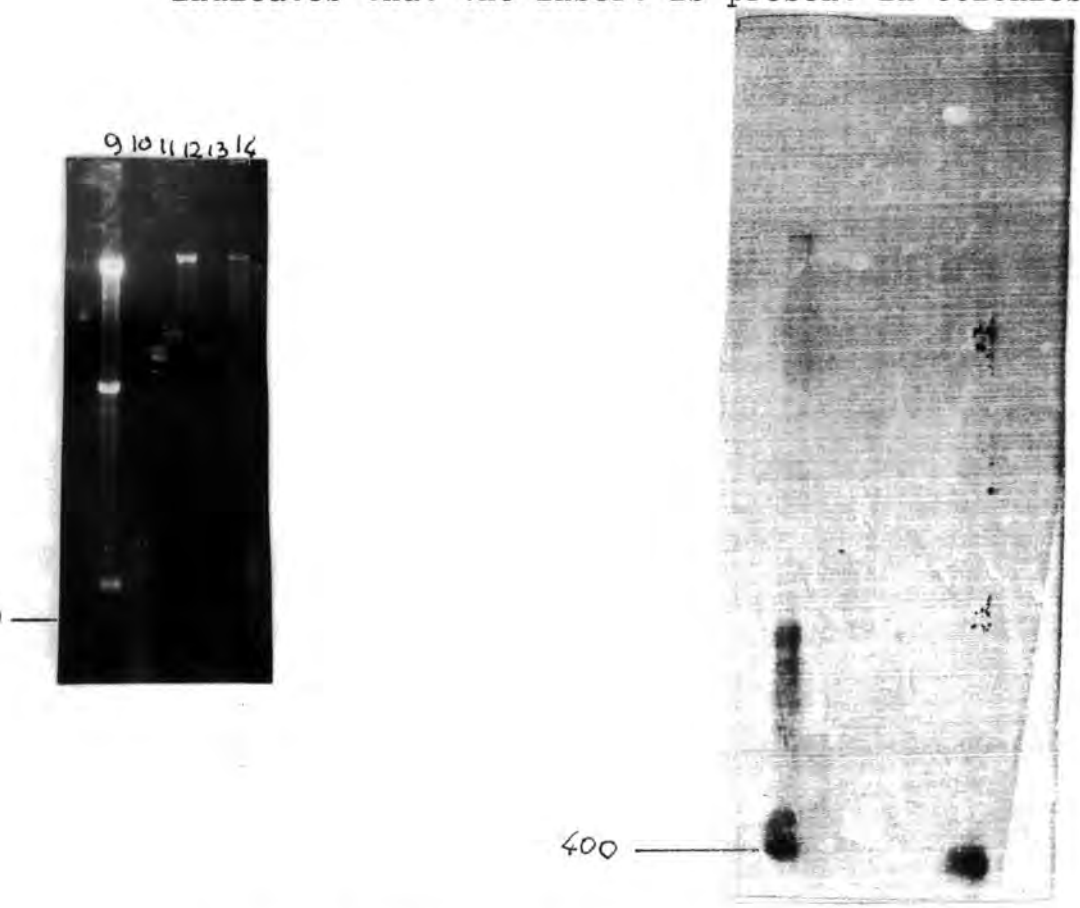
As the following picture shows, the fragments sizes produced differ markedly from those expected. The plasmids are larger than 3.6 kbp.



Eight more colonies were picked out for further minilysates but the digestion pattern obtained with Pstl also showed bigger fragments than expected.

Southern blotting of this last gel was done in order to determine the presence the 400 bp fragment.

Following hybridisation of the southern bolts using radioactively labelled 400 bp fragment as the probe (see methods), the resulting autoradiograph (see folowing picture) indicates that the insert is present in colonies 9 and 14 .



From this experiment, it is clear that the ligation of the 400 bp fragment was successful and the unexpected digestion pattern must be due to the vector which is probably not pUC8+280, as first thought.

3.8 ANALYSIS OF THE VECTOR

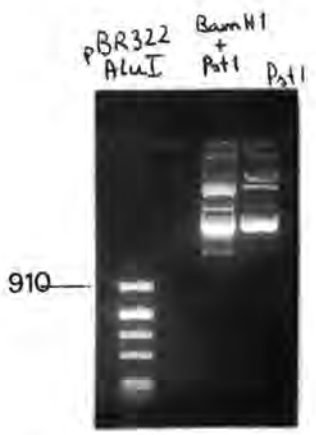
In order to recharacterise the vector, it was necessary to prepare and digest plasmid DNA of the colony thought to contain pUC8+280. This colony was streaked out on a fresh plate. Again it was isolated, grown in culture, and the plasmid DNA extracted. Several digestions of the plasmid DNA (see folowing picture), revealed that the putative single colony was probably a mixed colony :

(a) PUC8 without the BamHI and Pstl sites

(b) PUC8 with a 1000bp insert

Pstl digestion would leave plasmid (a) unrestricted, and linearise plasmid (b).

Pstl+BamHI would leave plasmid (a) unrestricted and release a 1000bp fragment from (b) together with pUC8.

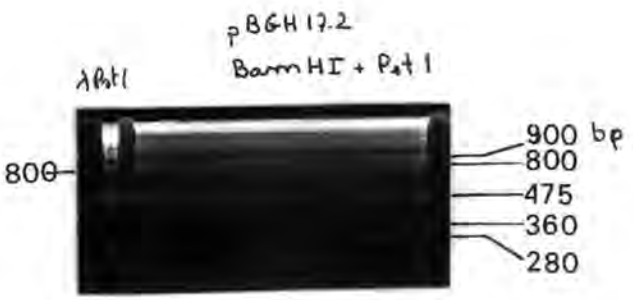


It would be difficult to explain where that 900 bp fragment came from but it is obvious that the 280 bp fragment has not

been successfully ligated into pUC8, so it was necessary to reisolate the 280 bp fragment from pBGH 17.2 and insert it into pUC8.

3.9 REISOLATION AND LIGATION OF THE 280 BP FRAGMENT INTO PUC8

The 280 bp fragment from pBGH 17.2 was freshly isolated using the "run into well" method (see methods). A 3% Nusieve minigel was made in order to clearly seperate out the last two bands of the BamHI+Pst1 digestion of pBGH 17.2 (see following picture)



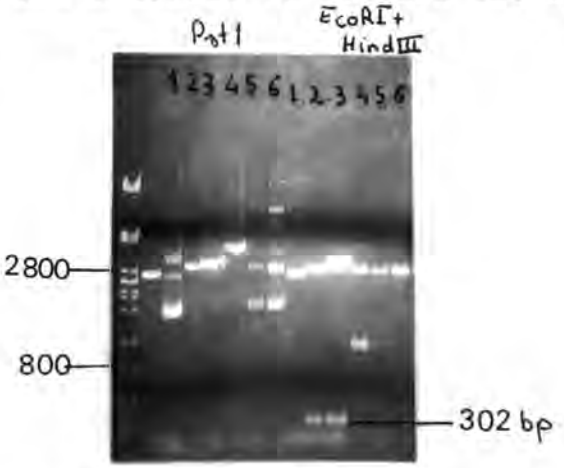
With the "run into well" method, more DNA was obtained than with the "freeze squeeze method". It was resuspended in only 10ul of water in order to carry out the ligation into pUC8 in a total volume of 20ul, according to the method described in Maniatis et al.(1982), in a total volume of 20ul. The reason for changing the total volume from 50 to 20 ul was to enhance the ligation of the 280 bp fragment into pUC8.

After transformation of E.coli JM83 with the recombinant plasmid, and after plating out 100 ul of the undiluted cells on X-gal ampecillin selective plates, 6 white colonies were obtained. The 6 white colonies were screened by minilysates: the plasmid DNA was digested with Pst1 and EcoRI+HindIII.

The expected fragments are:

Pst1	EcoRI+HindIII
2 988	2 686
	302

Colonies 2 and 3 seemed to contain the insert (see following picture)

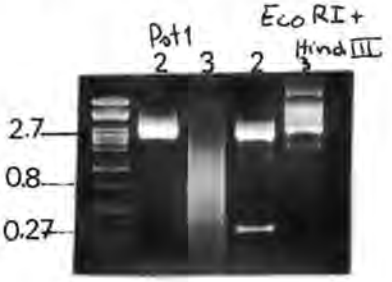


3.10 PURIFICATION PUC8+280

Two 50 ml plasmid preperations were made of colonies 2 and 3.

Pst1 and EcoRI+HindIII digestion of the plasmid DNA of each of the colonies shows that colony 2 contains pUC8+280 whereas colony 3 does not.

The Pst1 digestion of colony 3 may have been contaminated with nuclease (hence the smearing effect). The EcoRI+HindIII digestion did not show the expected results for colony 3 whereas it did for colony 2 (see following picture).



The Pst1 digestion of plasmid DNA of colony 2 shows a band above the 2.7kp triplet of the Pst1 marker and the EcoRI+HindIII digestion shows a band which is slightly smaller than the one obtained with Pst1 digestion of the plasmid, and an other band above the 0.27 kp of the Pst1 marker.

3.11 LIGATION OF THE 400 BP FRAGMENT INTO PUC8+280

PUC8 was digested with Pst1. In order to improve ligation of the 400 bp fragment into pUC8, the vector was phosphatased (according to the method described in "Materials and Methods"), in order to prevent it from religating without the insert. Ligation should only occur if the insert, with a phosphatase group at each end, is the link between the two arms of the vector. The ligation total volume was 20 ul (again in order to enhance ligation of the insert into the vector. Phosphatased treated vector was religated as well as unphosphatased vector in order to calculate the efficiency of the phosphatase treatment.

E.coli JM83 was transformed with:

1. 400 bp insert ligated into Pst1 cut vector (pUC8+280)
2. religated phosphatased vector
3. religated unphosphatased vector.

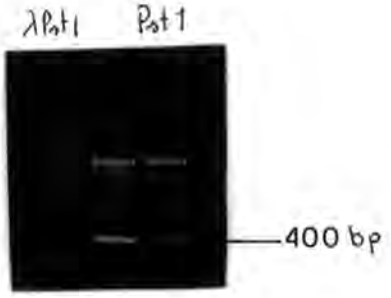
The undiluted cells were plated out on X-gal ampicillin plates.

Transformation with religated unphosphatased vector resulted in several hundred colonies (600-800) whereas transformation

with religated phosphatased vector resulted in only 150 colonies, which means that the phosphatase treatment efficiency is about 80%. Previous calculations showed that the ligation efficiency is about 50%, so one would expect the insert to be ligated into the vector with an efficiency of about 40%.

Because of this fairly high recombination efficiency, it was thought unnecessary to do a colony hybridisation and 30 colonies were screened by minilysates. Out of these 30 colonies, only one contained the insert, which means that the efficiency of insert ligation into the vector was overestimated. The reason for this is not known, althought it may be due to residual phosphatase activity which may still have been present along with the phosphatase vector.

A Pst1 digestion of pUC8+280+400 gives evidence of the 400 bp fragment successfully ligated into the vector:



3.12 ORIENTATION OF THE 400 BP FRAGMENT

The orientation of the 400 bp fragment can be determined by pvuII digestion (see fig 6 and table 4)

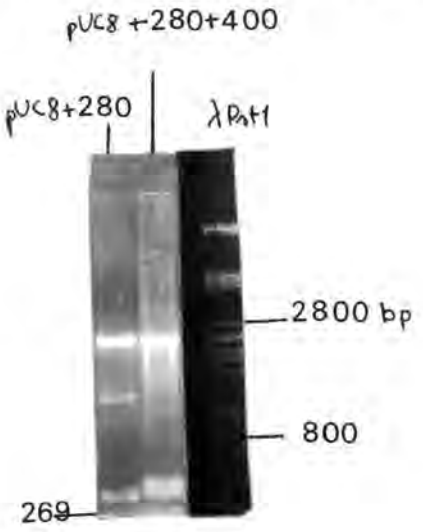
If the 400 bp fragment is in the correct orientation, a pvuII digestion of pUC8+280+400 should yield the following fragments:

2 416 bp, 494 bp, 269 bp, 209 bp

if not it should yield the following:

2 416 bp, 397 bp, 306 bp, 269 bp

Which is the case here,(see following picture):



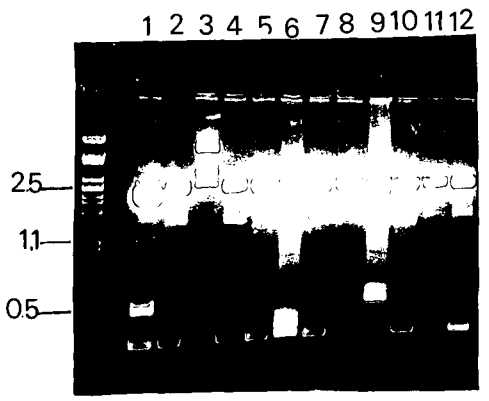
PvuII digestion of pUC8+280 is compared with PvuII digestion of pUC8+280+400: The observed fragments are respectively:

2 420 bp, 303 bp, 230 bp(the latter fragment is probably the 269 bp fragment that runs abnormally).

2 420 bp, 300 bp, 230 bp(the latter fragment is the same one that runs abnormally)

Since the lower bands of these two plasmid, run at the same level, the 400 bp fragment is in the wrong orientation.

PUC8+280+400 was cut again with Pst1 and religated in order to obtain a plasmid with the 400 bp fragment in the correct orientation. After transformation of E.coli JM83 with pUC8+280+400, minilysates of 12 colonies were done. The plasmid DNA of these colonies was digested with PvuII (see following picture):



Colony #1 seemed to be the only one to yield a fragment of about 500 bp and two smaller ones (under 300 bp) This indicates that the plasmid contains the 400 bp fragment in the correct orientation.

A 50 ml plasmid preparation was made of colony 1. The plasmid DNA was digested with:

Pst1+BamHI,

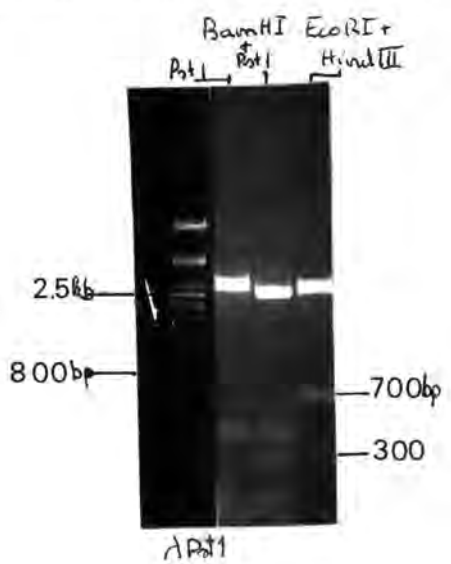
HindIII+EcoRI,

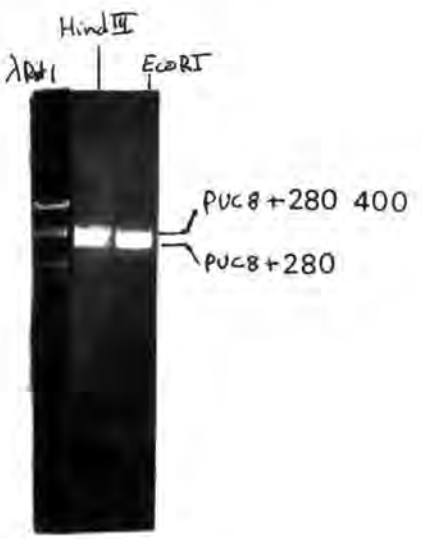
PvuII

The gel indicates that the plasmid DNA obtained was from a mixed colony: one containing pUC8+280 and one containing pUC8+280+400.

HindIII+EcoRI digestion of the plasmid DNA yielded 3 fragments instead of the expected 2: instead of obtaining vector DNA plus an insert of 700 bp, an extra band of about 300 bp could be seen. The 700 bp contains the 280 bp fragment plus the 400 bp fragment and forms the complete bGH; the 300 bp fragment only contains the 280 bp fragment.

Digestion of HindIII and EcoRI on their own show two fragments, one slightly larger than the other: 2 9988 bp, 3388 bp fragments. These are the two linearised plasmids: pUC8+280 and pUC8+280+400.





All future digestions of this plasmid DNA agree with this explanation of a mixed colony.

The fact that the plasmid DNA obtained is of a mixed colony does not affect the continuation of this work. The

next step requires the isolation of the 702 bp fragment and insertion into Bin 19, which can still be achieved with this mixed colony.

3.13 RESTRICTION OF PUC8+280 AND PUC8+280+400 WITH PVUII AND OTHER ENZYMES

3.13.1 Digestion and expected fragments

PUC8+280 digested with the following enzymes should yield the corresponding fragments:

PvuII: 2 416 bp, 303 bp, 269 bp

BamHI+PstI: 2 708 bp, 280 bp

PvuII+PstI: 2 416 bp, 269 bp, 200 bp, 103 bp

PUC8+280+400 digested with the following enzymes should yield the corresponding fragments:

PvuII: 2 416 bp, 494 bp,(303 bp), 269 bp (269 bp), 209 bp

PstI : 2 988 bp, 400 bp

PstI+BamHI: 2 708 bp, 400 bp, 280 bp,(280 bp)

EcoRI+HindIII: 2 686 bp, 702 bp,(302 bp)

PvuII+PstI: 2 416 bp, 294 bp, 269 bp, 200 bp,(200 bp),(103 bp)

PvuII+BamHI: 2 416 bp, 494 bp,(302 bp), 209 bp, 189 bp,(189 bp), 80 bp,(80 bp)

The numbers in parenthesis are due to the mixed colony (PUC8+280 being present in with pUC8+280+400).

3.13.2 Fragments on a 2% agarose gel

on a 2% agarose gel the following fragments are obtained:

For pUC8+280 digested with:

PvuII: 2.4 kbp, 300 bp, 240 bp

BamHI+PstI: 2.7 kbp, 270 bp

PvuII+PstI: 2.4 kbp, 230 bp, 200 bp, 100 bp

For pUC8+280+400 digested with:

PvuII: 2.4 kbp, 495 bp,(300),(a) 220 bp,(220 bp), 220 bp

PstI: 3 kbp, 400 bp

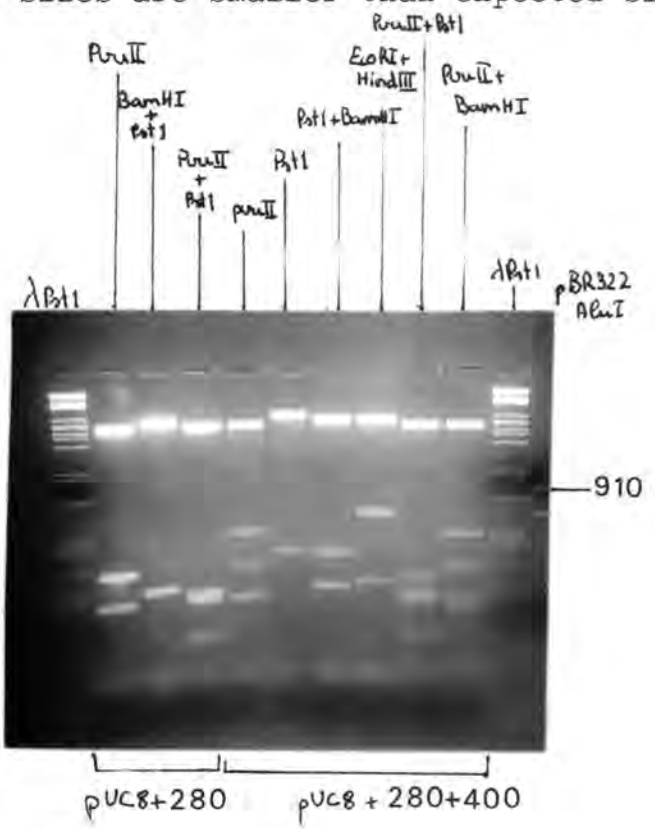
PstI+BamHI: 2.7 kbp, 400 bp,(b) 250 bp

EcoRI+HindIII: 2.7 kbp,(c) 640 bp,(d) (240 bp)

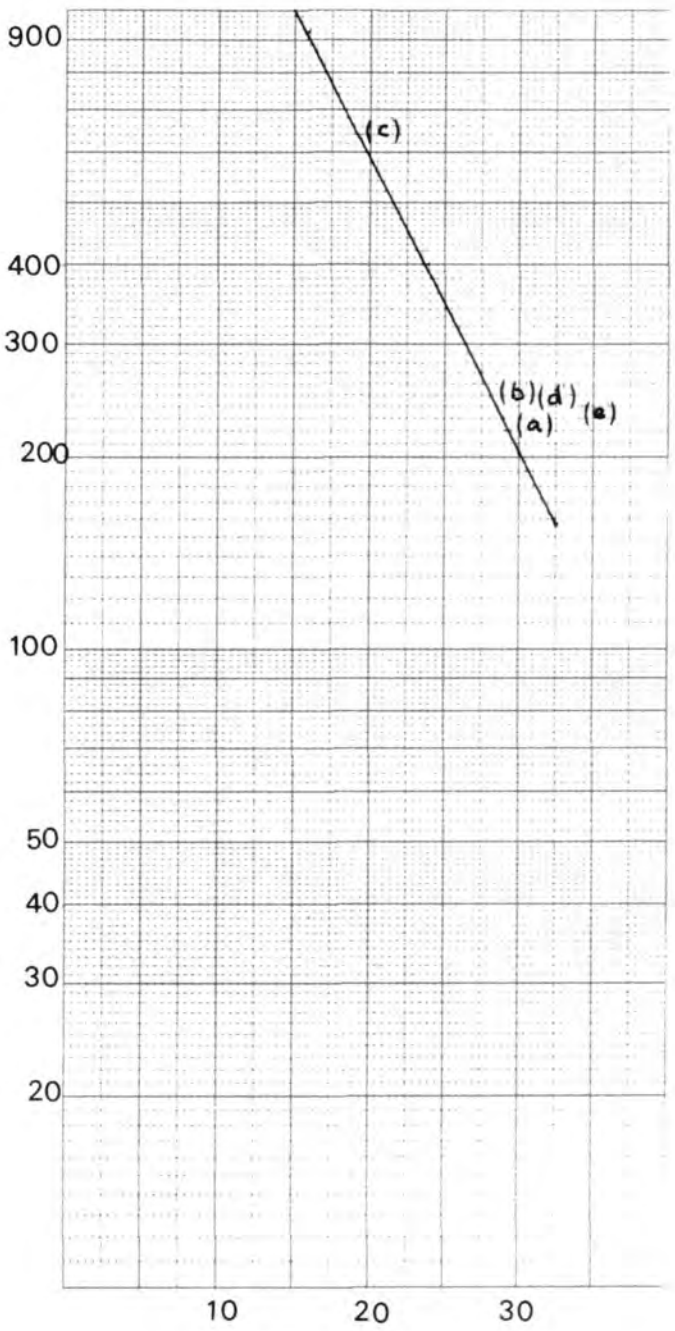
PvuII+PstI: 2.4 kbp, 295 bp,(e) 230 bp, 200bp,(200 bp),(100 bp)

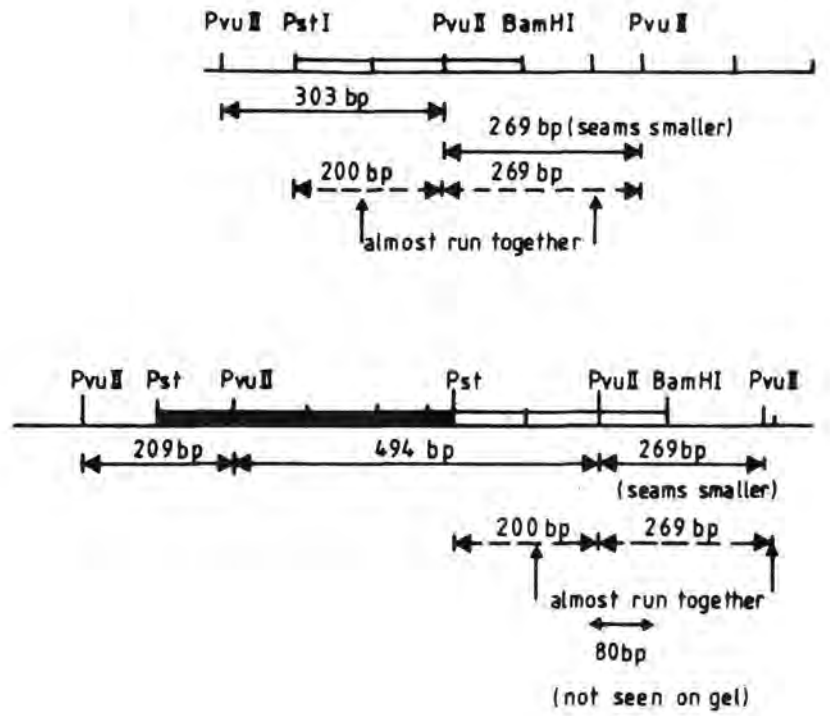
PvuII+BamHI: 2.4 kbp, 495 bp,(280 bp), 220 bp, 185 bp, no 80 bp

The underlined sizes are smaller than expected size.



A graph can be drawn: migration/ molecular weight. Fragments (a),(b),(c),(d) and (e) are represented on the graph.




PvuII PstI
PvuII BamHI
PvuII
303 bp
269 bp (seams smaller)
200 bp
269 bp
almost run together
PvuII Pst
PvuII
Pst
PvuII BamHI
PvuII
209bp
494 bp
269bp
(seams smaller)
200 bp
269 bp
almost run together
80bp
(not seen on gel)

3.14 POSSIBLE DELETION

In comparing the expected sizes with the observed sizes of the fragments, it seemed quite plausible that a deletion of around 30-40 bp could have occured.

Evidence:

1. The 269 bp fragment from PvuII digestion of pUC8+280 seems about 30 bp smaller than expected (observed size 240 bp)

2. The 269 and the 200 bp fragment from the PvuII+Pstl digestion of pUC8+280+40 0 almost run together

3. The 269 and 206 bp fragments from a PvuII digestion of pUC8+280+400 almost run together.

4. No 80 bp fragment can be seen on the gel as one expect with a PvuII+BamHI digestion of pUC8+280+400. The fragment is probably too small to see.

5. The 269 and 200 bp fragments from the PvuII+Pstl digestion of pUC8+280+400 almost run together.

These observations are illustrated below:

The small deletion seems to be between the PvuII and BamHI site...or could it be that that fragment simply runs abnormally?

3.15 CONFIRMATION OF THE 40 BP DELETION AROUND THE HAEII SITE OF PUC8+280

A HaeII digestion of pUC8+280 and pUC8+280+400 revealed that the HaeII site next to the BamHI site in the 280 bp fragment, is missing:

The expected fragments for pUC8+280 were: 1 923 bp, 439 bp, 370 bp, 256 bp

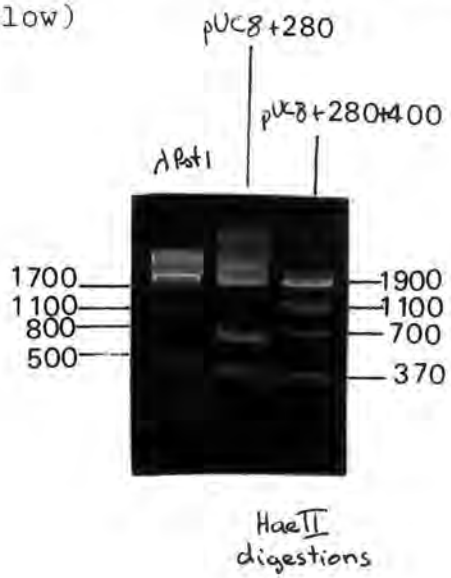
and for pUC8+280+400 they were: 1 923 bp, 839 bp, 370 bp, 256 bp

The fragments of the pUC8+280 and pUC8+280+400 HaeII digestion to be seen on the gel were respectively of about:

1 920 bp, 700 bp, 370 bp

and 1 920 bp, 1 100 bp,(700 bp), 370 bp

(see picture below)



These are precisely the sizes that one would expect if the HaeII site near the BamHI site in the 280 bp fragment was not present. In order to confirm this 30-40 bp deletion around the HaeII site, the 280 bp fragment from pUC8+280+400 was isolated and digested partially with PvuII. A 3% nusieve gel was made in in order to calculate the sizes of the digested fragments (see picture and graph below).

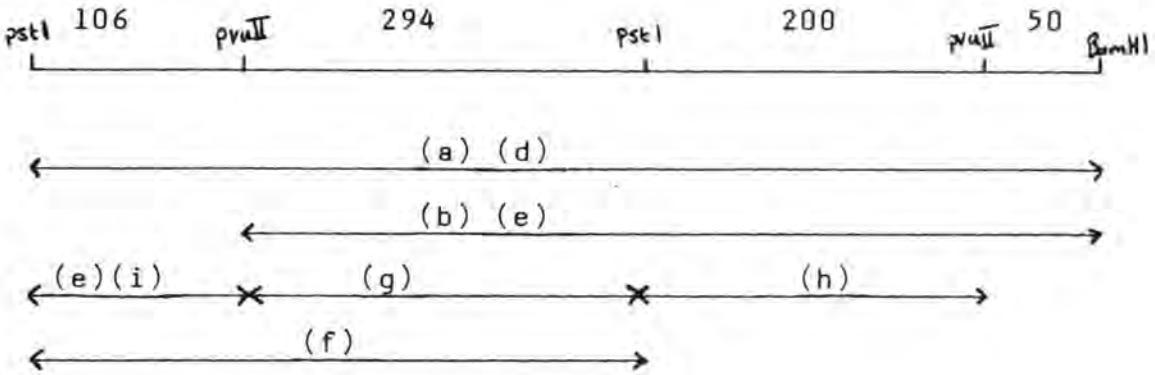
The partial digestions of the 680 bp fragment yield the following fragments:

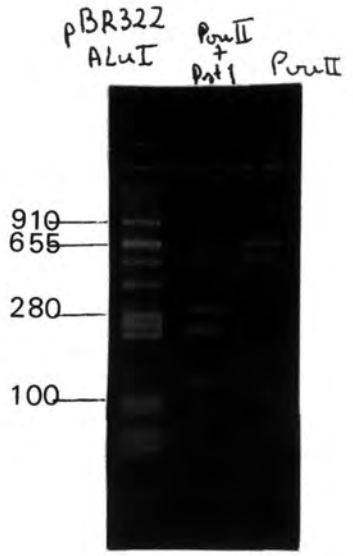
For the PvuII digestion: (a) 660 bp*, (b) 540 bp, (c) 110 bp

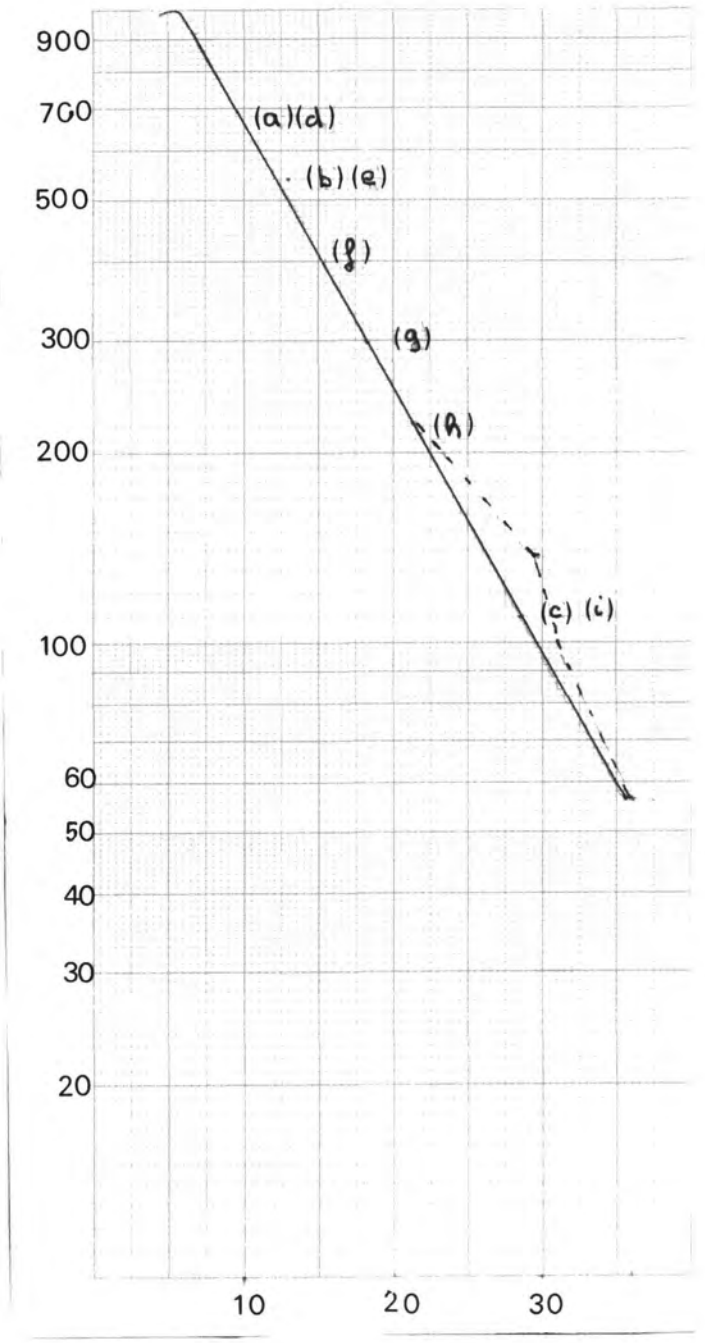
For the PvuII+PstI digestion: (d) 660 bp*, (e) 540 bp*, (f) 400 bp*, (g) 300 bp, (h) 210 bp, (i) 110 bp

* indicate the partial digestion

These results correspond to the following restriction map:




pBR322
AluI
PvuII
+
PstI
PvuII
910
655
280
100

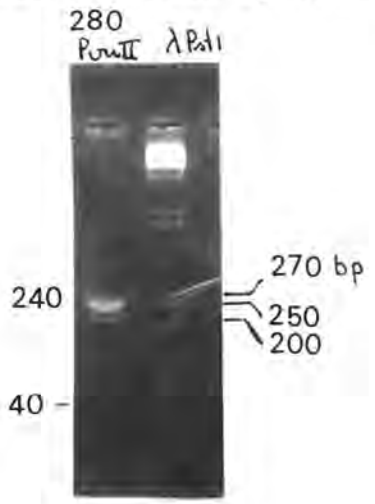

900
700
500
300
200
100
60
50
40
30
20
(a)(d)
(b)(e)
(f)
(g)
(A)
(c) (i)
10
20
30

3.16 WHEN DID THIS DELETION OCCUR?

It is obvious that the deletion occured sometime before the insertion of the 400 bp. PUC8+280 clearly shows the deletion as well as pUC8+280+400. It is quite intriguing that the deletion should not have occured at the far end of the 280 bp fragment. It could be that this deletion had actually come about during ICI's work on addition of linkers to create a BamHI site next to the HaeII site(see introduction).

In order to find out if that was the case or if it arose later, the 280 bp fragment from pBGH 17.2 was isolated, digested with PvuII and compared with the 280 bp fragment of pUC8+280.

Unfortunately, although all of the DNA from a 50 ml plasmid preperation was digested, loaded onto a gel and the Pst1-BamHI 280 bp fragment was isolated, not enough DNA was obtained to see on a gel. A partial PvuII digestion of the 280 bp fragment from pUC8+280 shows that the 280 bp fragment is in fact about 240 bp, and the largest PvuII digested fragment obtained is of about 210 bp (it should be 200 bp). An extremely faint band can be seen: it indicates a fragment of about 30 or 40 bp.(see following picture)



This obviously confirms that the deletion around the HaeII site is of about 40 bp.

Too little time was available to do a DNA sequencing of the 280 bp fragment (actually 240 bp) and ICI proposed to clarify this question about the deletion in the future.

Regretfully, one must come to the following conclusion: since the beginning of the coding sequence is vital for correct expression of the gene(the HaeII site is at the place of the start codon), it is impossible to continue using the construction obtained. Therefore another plasmid must be constructed in the same way so that a complete bGH cDNA clone is obtained.

CHAPTER FOUR

CONCLUSION

Several techniques and strategies were tried in order to insert the N-terminus of bGH into pUC8.

After several attempts, the 280 bp fragment from pBGH 17.2 was isolated and ligated into pUC8 successfully, together with the 400 bp fragment from pBGH 229.

Useful PvuII restriction sites in the bGH clone enabled the determination of the orientation of the 400 bp fragment. Since the 400 bp fragment had first been inserted in the incorrect orientation, it was cut and religated and a recombinant with the 400 bp fragment in the correct orientation was then obtained. Thus the N-and C-terminus of the bGH clone were fused together.

However, the cDNA clone was not complete since a deletion of about 40 bp was observed around the HaeII site at the beginning of the coding sequence. This deletion not only implies that the reading frame may be modified but it is not certain whether the bGH sequence would be translated at all, since the initiation codon (at the HaeII site) may also be modified.

CONTINUATION OF THIS PROJECT

A complete bGH clone would have to be constructed and the beginning of the coding sequence of this clone should be checked.

Once this is achieved, the bGH cDNA clone can be inserted into Bin 19 (Bevan 1984). Bin 19 contains polyrestriction

sites, similar to pUC8, located in the lac DNA, in order to identify the colonies with the foreign DNA. The bGH clone obtained from the previously described construction is located on an EcoRI-HindIII fragment. Bin 19 would be cut with EcoRI-HindIII and the bGH clone could easily be ligated into the vector (fig 8). The relatively small size of the vector allows a quite efficient ligation.

The recombinant plasmid would then be cut with EcoRI and the Rubisco promoter, isolated by a EcoRI digestion of pDUB1111, would be inserted into Bin 19+bGH (fig 9).

Bin 19+ rubisco+ bGH would then be mobilised to A.tumefaciens and transfered to plant cells in infection of leaf. It is likely that to achieve regeneration of plants carrying he construct could take up to 6 months.

A recent report by Barta et al., shows that expression of a nopaline synthase-human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue has been obtained. The hGH gene was a genomic sequence and although the gene was transcribed, the mRNA was not processed to remove the intervening sequences (introns).

However, it was demonstrated that hGH pre-mRNA isolated from plant cells could be spliced in a He La cell nuclear extract, indicating that the hGH pre-mRNA was functional.

In the same way, bGH mRNA could be obtained by expressing the rubisco-bGH chimeric gene in plants; as mentioned earlier, the Rubisco promoter is one of the most efficient in plant cells (Fluhr et al. 1986), implying that a new system for producing bGH could be developped.

Appendix

Table 1

Nucleotide sequence of a bovine qrowth hormone mRNA:

-20 -10

CGG ACC UCC CUG CUC CUG GCU UUC GCC CUG CUC UGC CUG CCC UGG ACU

1 10

CAG GUG GUG GGC GCC UUC CCA GCC AUG UCC UUG UCC GGC CUG UUU GCC

20

AAC GCU GUG CUC CGG GCU CAG CAC CUG CAC CAG CUG GCU GCU GAC ACC

30 40

UUC AAA GAG UUU GAG CGU ACC UAC AUC CCG GAG GGA CAG AGA UAC UCC

50 60

AUC CAG AAC ACC CAG GUU GCC UUC UGC UUC UCC GAA ACC AUC CCG GCC

70

CCC ACG GGC AAG AAU GAG GCC CAG CAG AAA UCA GAC UUG GAG CUG CUU

80 90

CGC AUG UCA CUG CUC CUC AUC CAG UCG UGG CUU GGG CCC CUG CAG UUU

100

CUC AGC AGA GUC UUC ACC AAC AGC UUG GUG UUU GGC ACC UCG GAC CGU

110 120

GUC UAU GAG AAG CUG AAG GAC CUG GAG GAA GGC AUC UUG GCC CUG AUG

130 140

CGG GAG CUG GAA GAU GGC ACC CCC CGG GCU GGG CAG AUC CUC AAG CAG

150

ACC UAU GAC AAA UUU GAC ACA AAC AUG CGC AGU GAC GAC GCG CUG CUC

160 170

AAG AAC UAC GGU CUG CUC UCC UGC UUC CGG AAG GAC CUG CAU AAG ACG

180

GAG ACG UAC CUG AGG GUC AUG AAG UGC CGC CGC UUC GGG GAC GCC AGC

190

UGU GCC UUC UAG UUGCCAGC.... (poly A)

Table 2

BACTERIAL STRAINS AND PLASMIDS

Bacterial strains: E.coli K514 (Colson at al., 1965),

E.coli JM83 (Messing et al., 1981)

Plasmids	markers	specification	source reference
pAT153	Ap, Tc	pBR322 derivative lacking 803 bp fragment.	Twigg and Sherrat, 1980
pWS1.1	Su, Tc	R300B derivative (Barth, 1979) EcoRI-PvuII fragment has been replaced by 2066 bp fragment from pBR322 (hence Tc)	W.Shuch (ICI) unpublished
pUC8	Ap, B-galactosidase	see ref.	Viera and Messing, 1982
Bin19	Km, B-galactosidase	see ref.	Bevan, 1984

Table 3

PBGH 17.2 (10.256 kb)

PstI	BamHI	PvuII	BamHI+PstI
7.721	8.716	8.485	7.441
1.375	1.54	1.771	0.9
0.8			0.8
0.36			0.475
			0.36
			0.28

PBGH 229 (3.959 kb)

PstI	BamHI	BamHI+PstI
3.559	3.959	2.436
0.4		1.1123
		0.4

TABLE 4

	BamHI	BamHI+PstI	EcoRI+HindIII	PvuII	HaeII
PUC8	2 716	2 708 8	2 686 30	2 416 300	1 923 423 370
PUC8+280	2 988	2 708 280	2 686 302	2 416 303 269	1 923 439 370 256
PUC8+280+400 correct orientation	3 388	2 708 400 280	2 686 702	2 416 494 269 209	1 923 839 370 256
PUC8+280+400 incorrect orientation	3 388	2 708 400 280	2 686 702	2 416 397 306 269	1 923 839 370 256

pBGH 17·2

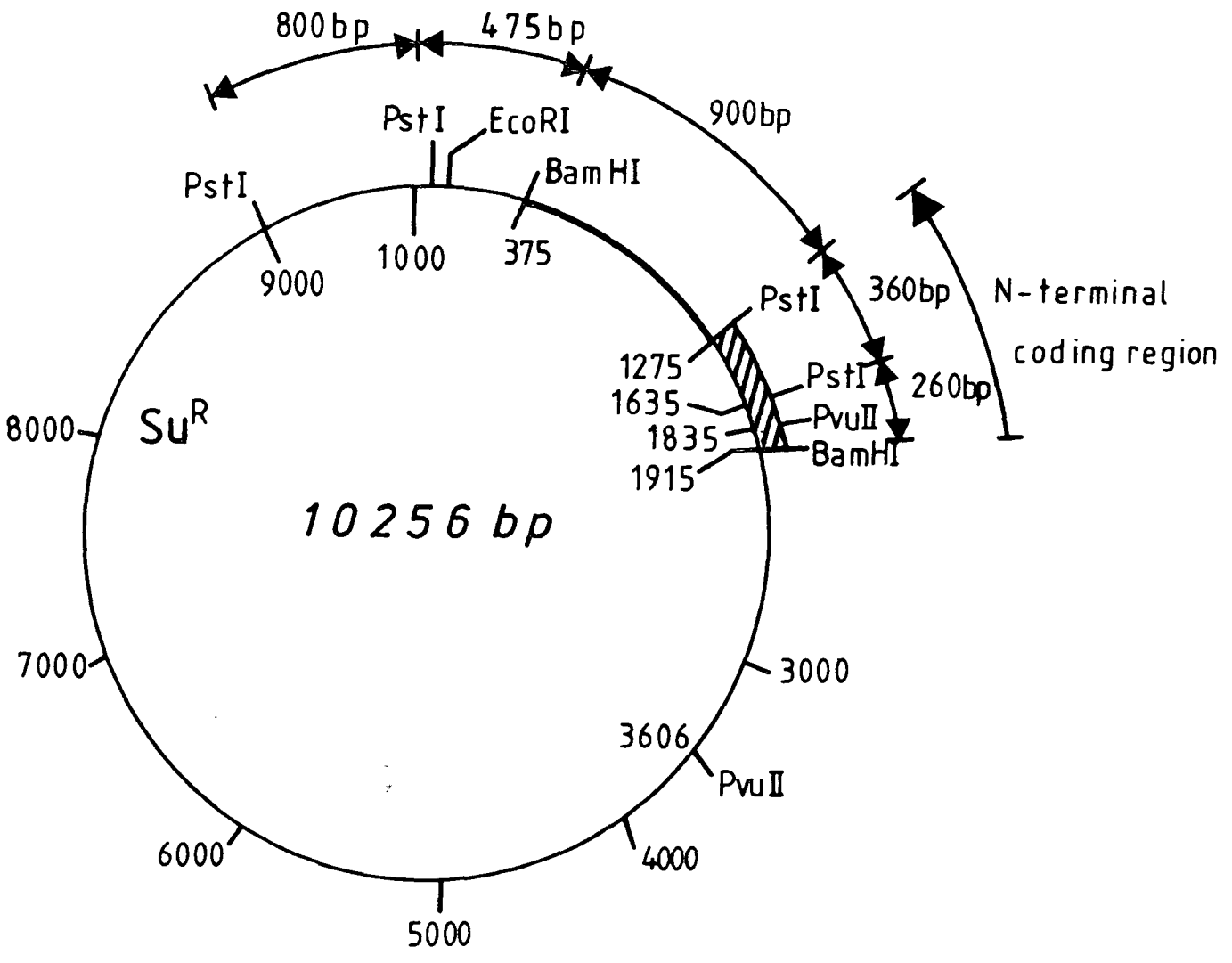
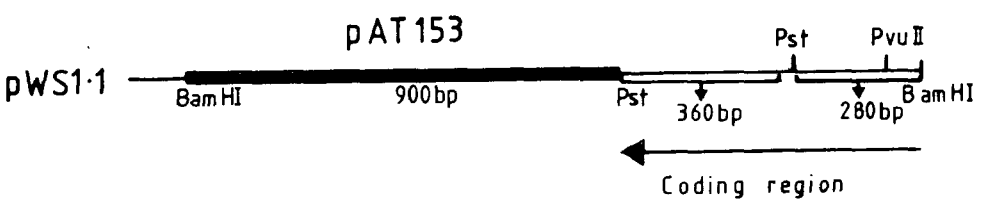


Fig. 1 *pBGH 17·2*



BGH 229

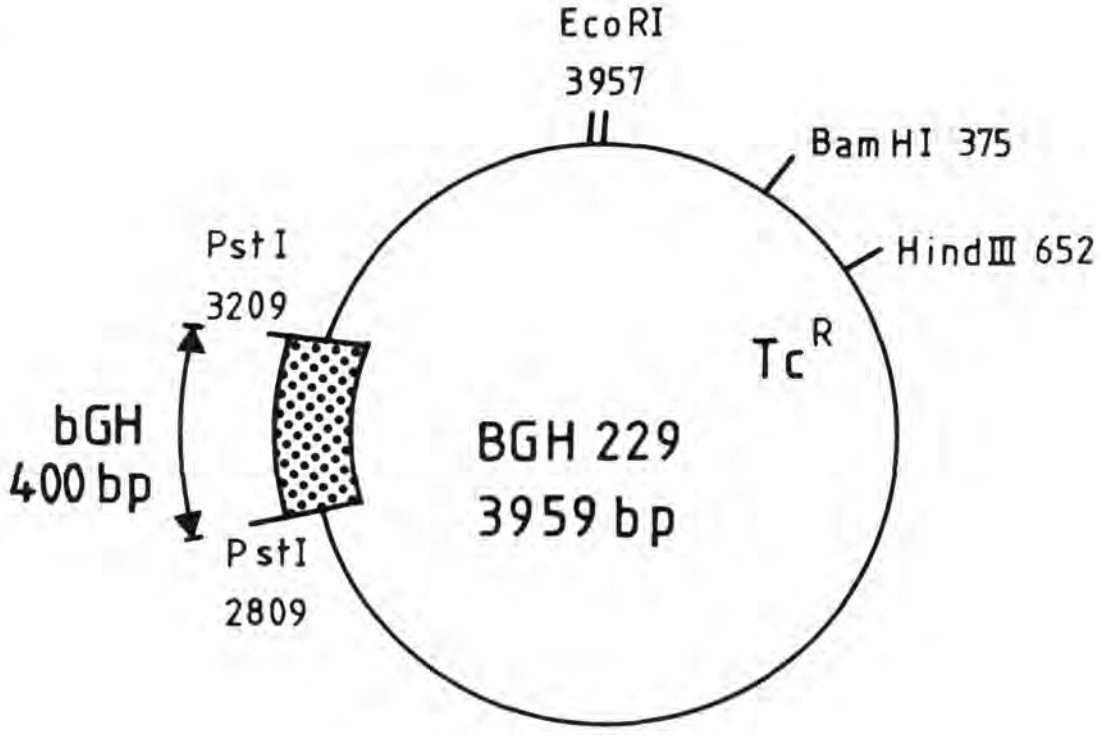

EcoRI
3957
Bam HI 375
Hind III 652
Pst I
3209
bGH
400 bp
PstI
2809
BGH 229
3959 bp
Tc R

Fig. 2

pUC 8 : Vector for bGH

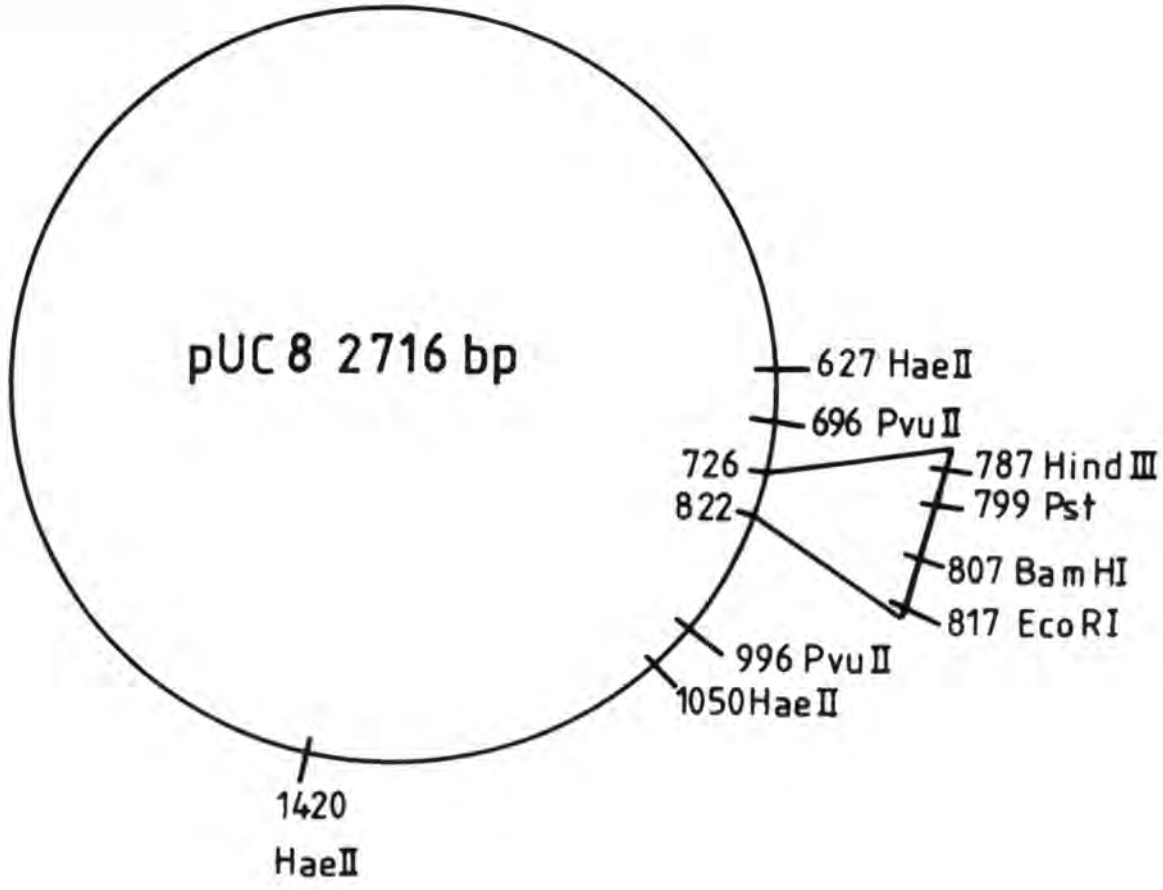


Fig. 3

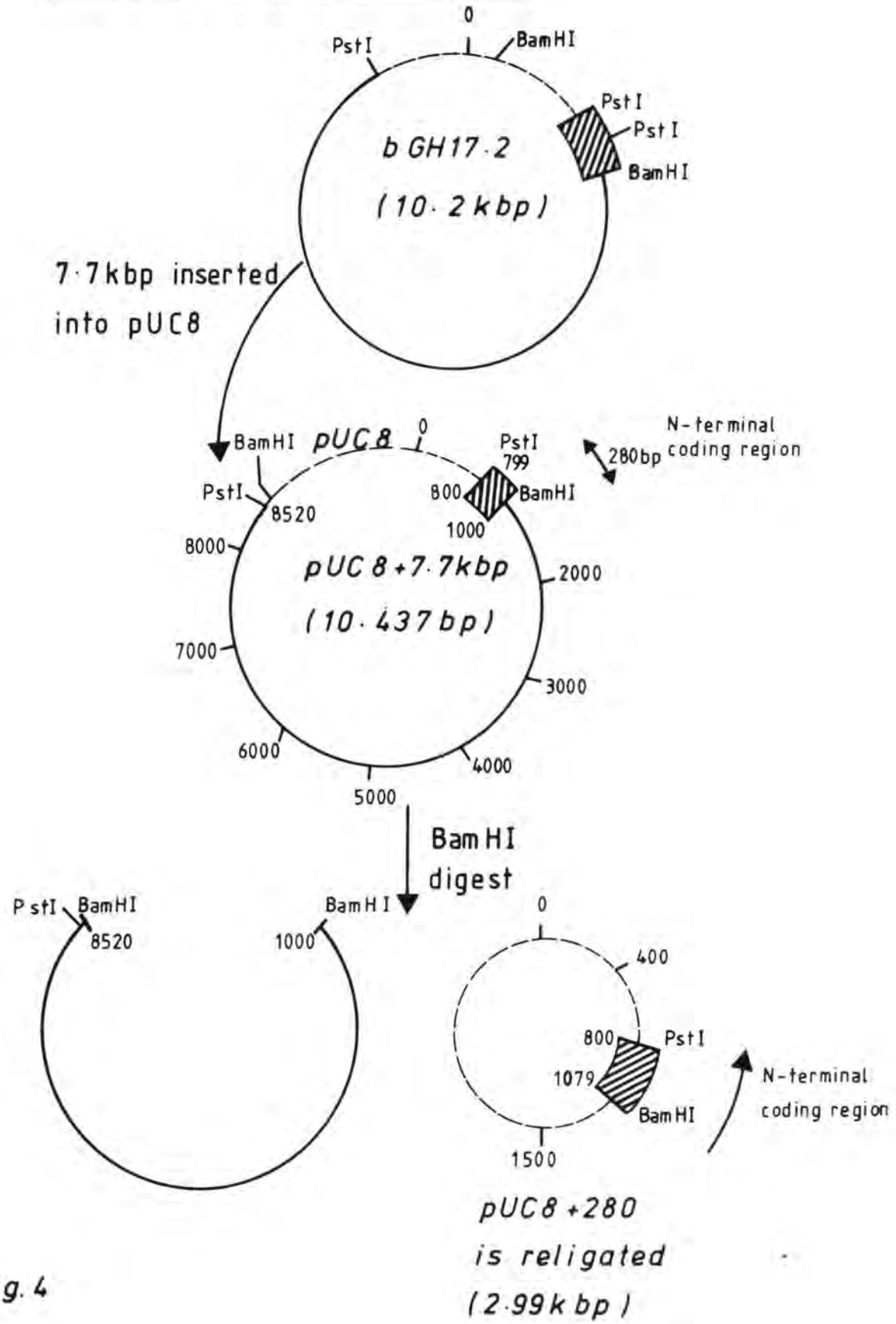

Insertion of 7·6kbp into pUC8
0
PstI
BamHI
PstI
PstI
BamHI
b GH17·2
(10·2kbp)
7·7kbp inserted
into pUC8
BamHI
pUC8
0
PstI
799
800
BamHI
1000
PstI
8520
8000
2000
pUC8+7·7kbp
(10·437bp)
7000
3000
6000
4000
5000
280bp
N-terminal
coding region
BamHI
digest
PstI
BamHI
8520
BamHI
1000
0
400
800
PstI
1079
BamHI
1500
N-terminal
coding region
pUC8+280
is religated
(2·99kbp)

Fig. 4

pUC8 with N-terminus of the bGH sequence inserted in the BamHI - PstI site.

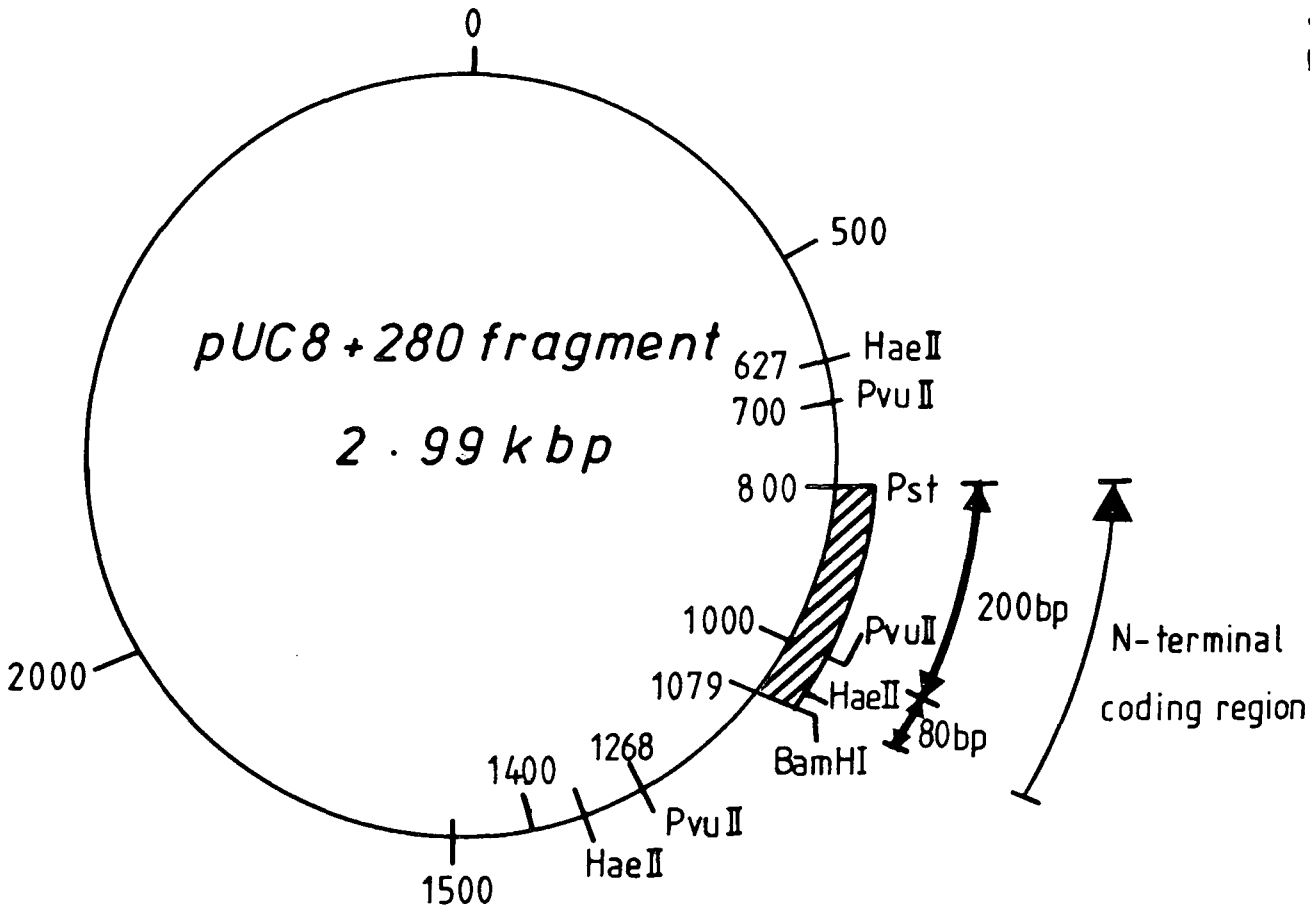


Fig.5

pUC8 carrying complete bGH (400 bp C-terminal fragment in the correct orientation.)

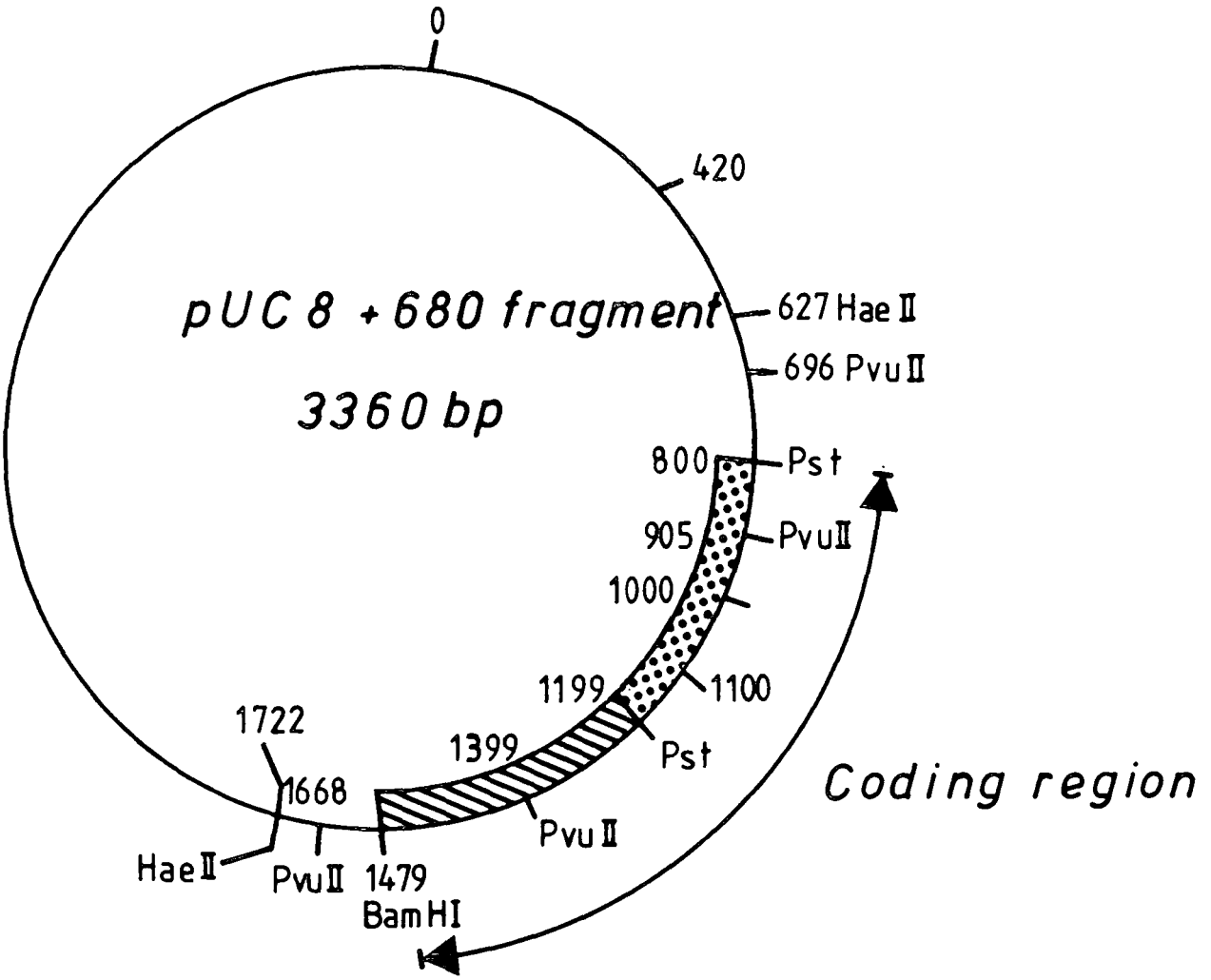


Fig. 6

pUC8+280 with the 400bp fragment in the wrong orientation

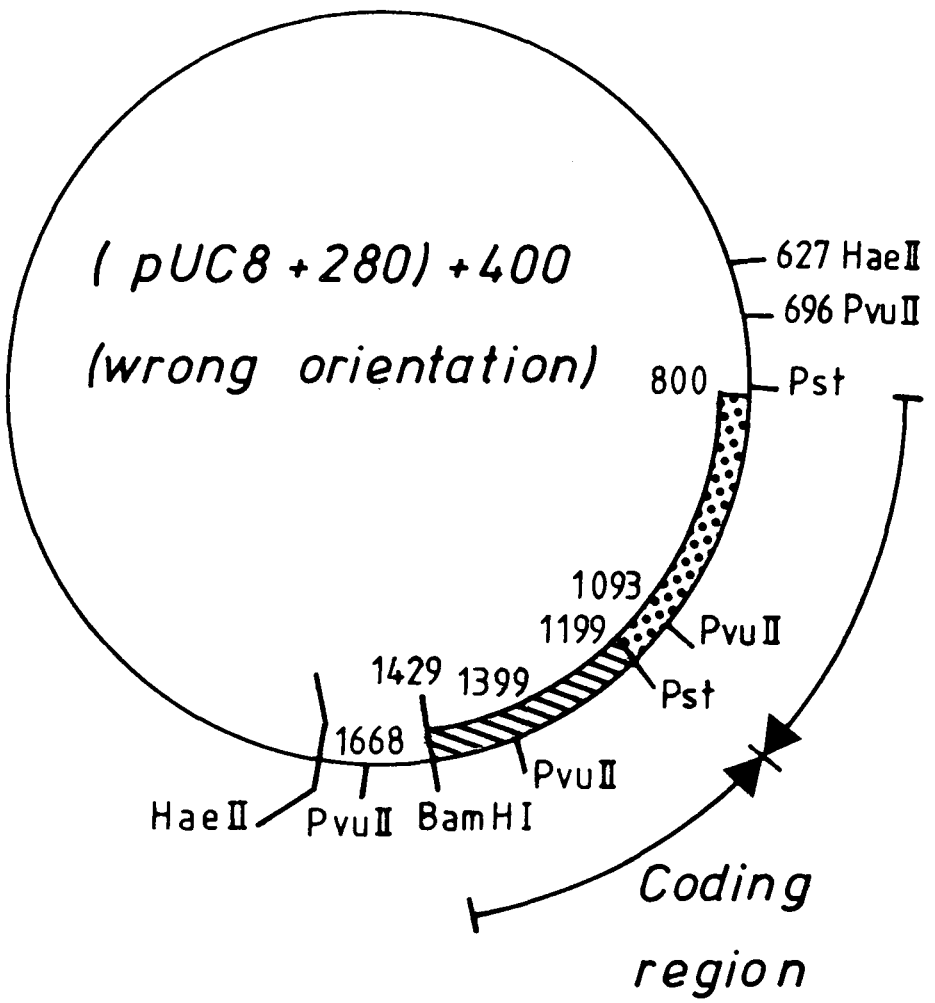


Fig.7

Proposed construction Bin19+bGH

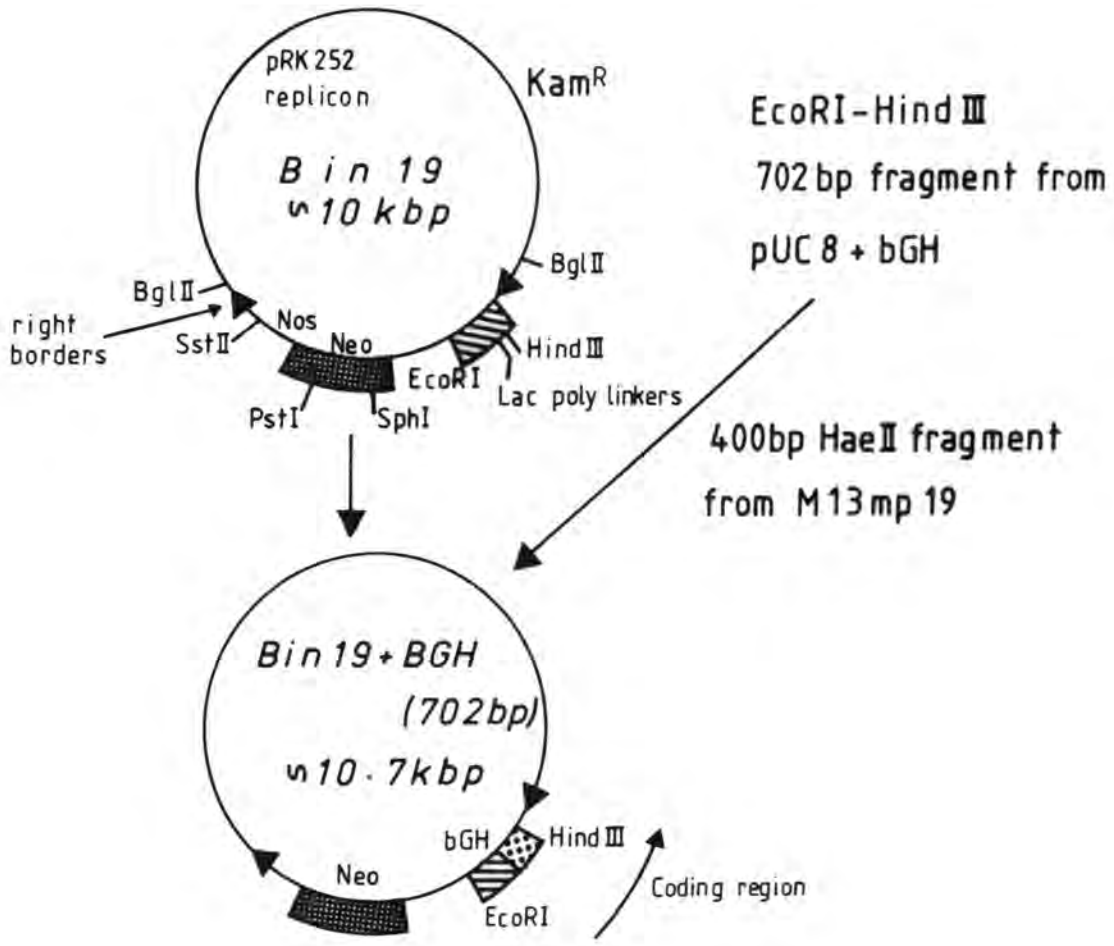


Fig. 8

Proposal construction of Bin19+bGH+Rubisco promoter

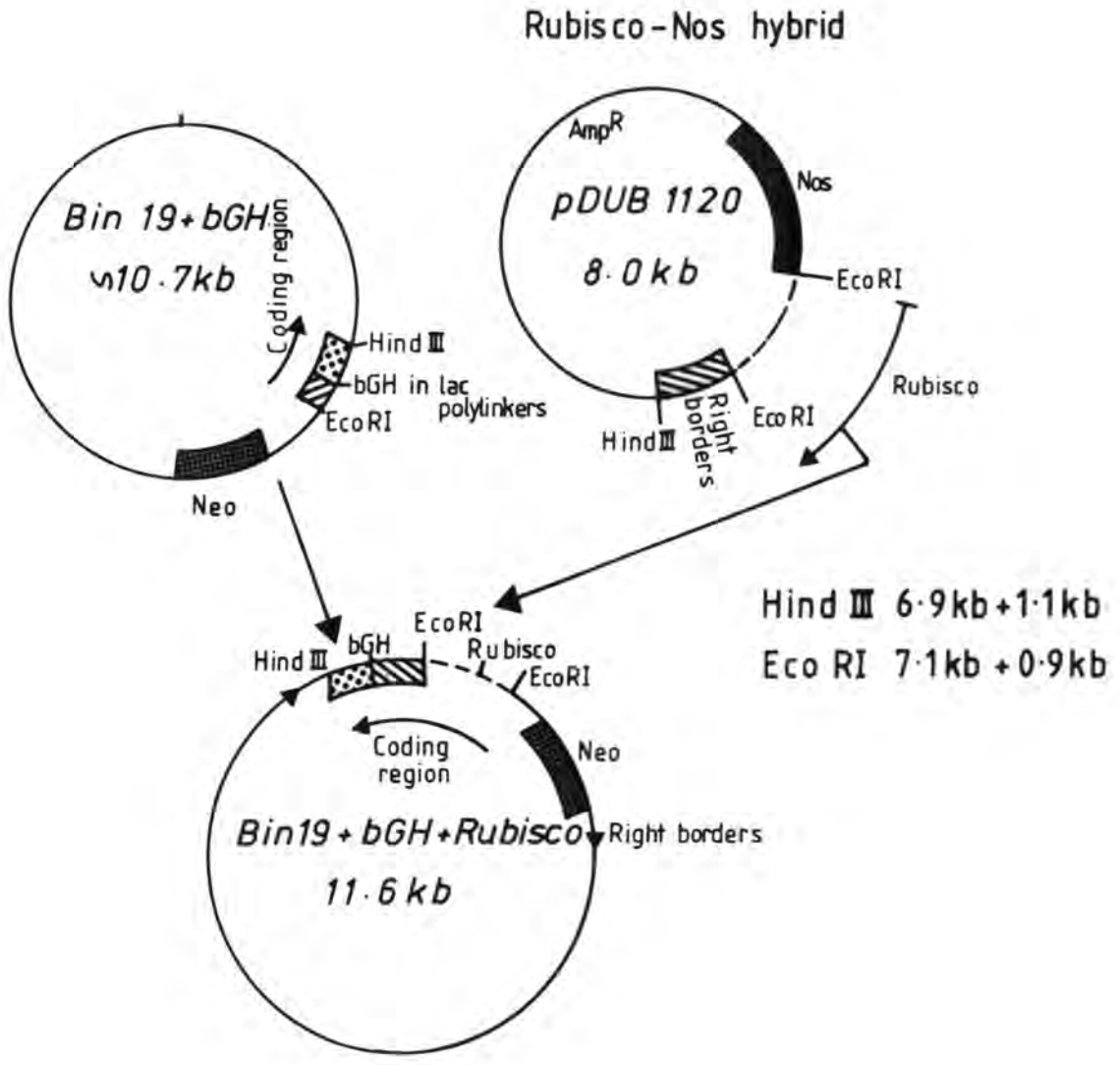
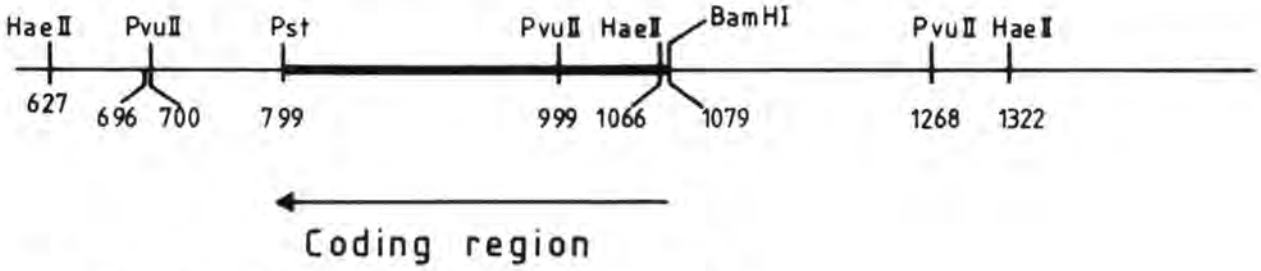


Fig. 9

pUC 8 + the first fragment of bGH



pUC 8 + bGH (in the correct orientation)

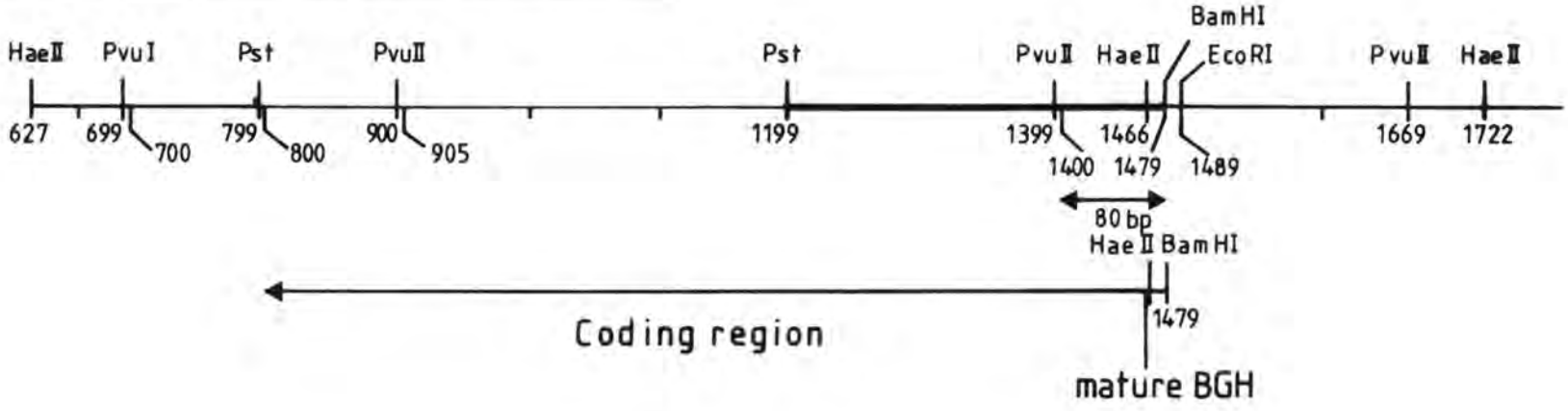


Fig. 10

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