Discussion of :

A bacterial clone synthesizing proinsulin

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Biochemistry

A bacterial clone synthesizing proinsulin

(rat preproinsulin/cDNA cloning/solid-phase radioimmunoassay/DNA sequence/fused proteins)

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Contributed by Walter Gilbert, June 9, 1978

ABSTRACT **We have cloned double-stranded cDNA copies of a rat preproinsulin messenger RNA in *Escherichia coli* χ1776, using the unique *Pst* endonuclease site of plasmid pBR322 that lies in the region encoding amino acids 181–182 of penicillinase. This site was reconstructed by inserting the cDNA with an oligo(dG)-oligo(dC) joining procedure. One of the clones expresses a fused protein bearing both insulin and penicillinase antigenic determinants. The DNA sequence of this plasmid shows that the insulin region is read in phase; a stretch of six glycine residues connects the alanine at position 182 of penicillinase to the fourth amino acid, glutamine, of rat proinsulin.**

Can the structural information for the production of a highe cell protein be inserted into a plasmid in such a way as to b expressed in a transformed bacterium? To attack this problem we used as a model rat insulin, an interesting protein that ca be identified by immunological and biological means.

Although mature insulin contains two chains, A and B, it i the product of a single longer polypeptide chain. The hormon is initially synthesized as a preproinsulin structure (1, 2). A hydrophobic leader sequence of 23 amino acids at the amin terminus of the nascent chain is cleaved off, presumably as th polypeptide chain moves through the endoplasmic reticulum (3–4), producing a proinsulin molecule. The proinsulin chair

minA1, supE42, Δ40[*gal-uvrB*], λ⁻, *minB2, rfb-2, nalA25, oms-2, thyA57, metC65, oms-1,* Δ29[*bioH-asd*], *cycB2, cycA1, hsdR2*) was provided by R. Curtiss.

DNA and Enzymes. pBR322 DNA, a gift from A. Poteete, was used to transform *E. coli* HB101. Plasmid DNA was purified according to the procedure of Clewell (16). Avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase), *E. coli* DNA polymerase I, and terminal transferase were gifts from T. Papas, M. Goldberg, and J. Wilson, respectively. Restriction enzymes were purchased from Be-

Abstract

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Outline:

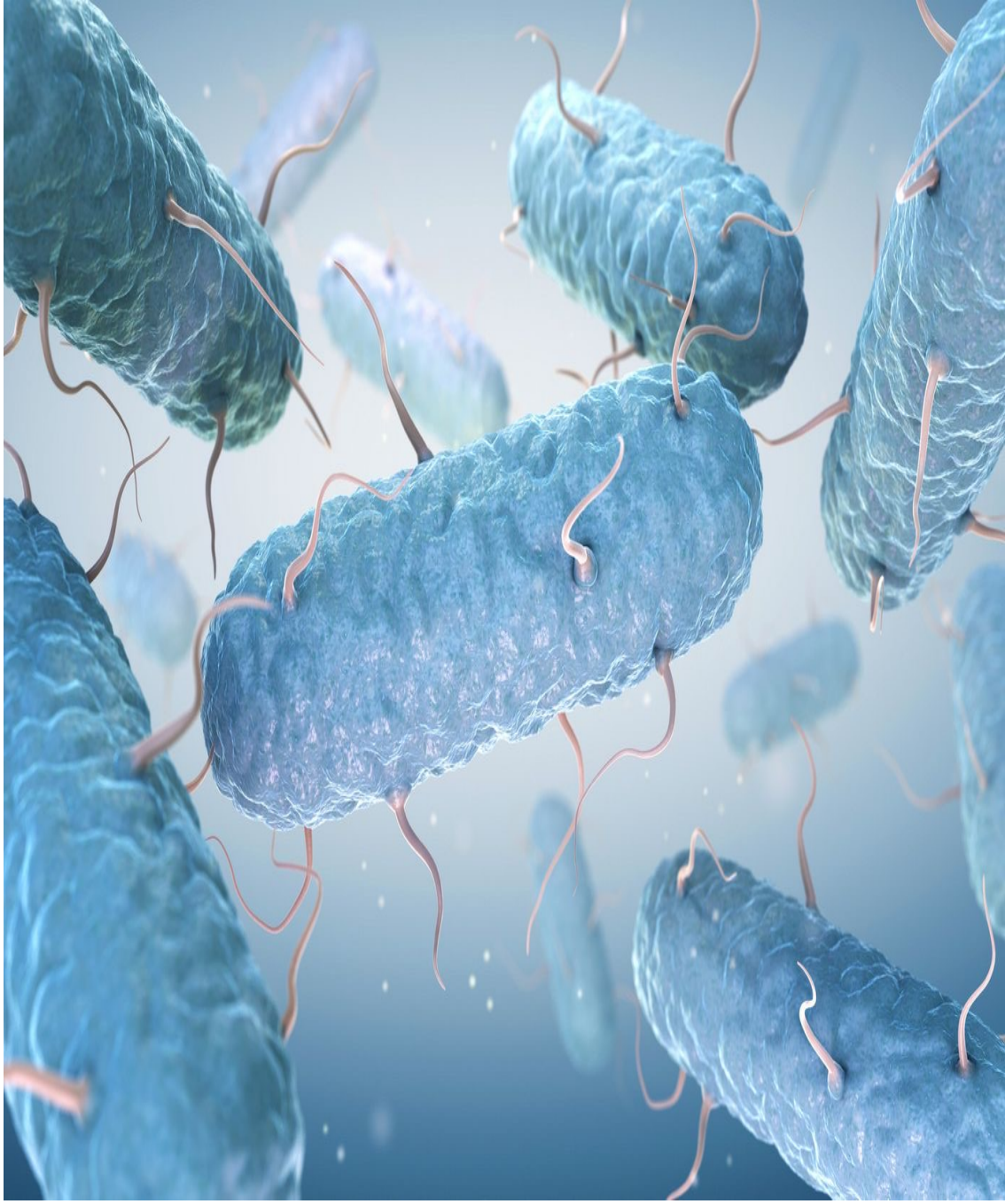
- Insulin.
- E.coli.
- pBR322 vector.
- Aim of the study.
- Methods and Material.
- Results.
- Discussion.

Insulin:

- Produced By pancreatic cells.
- Helps in maintain sugar level in body.
- Lacking Insulin Resulting many problems such as being a diabetic patient.

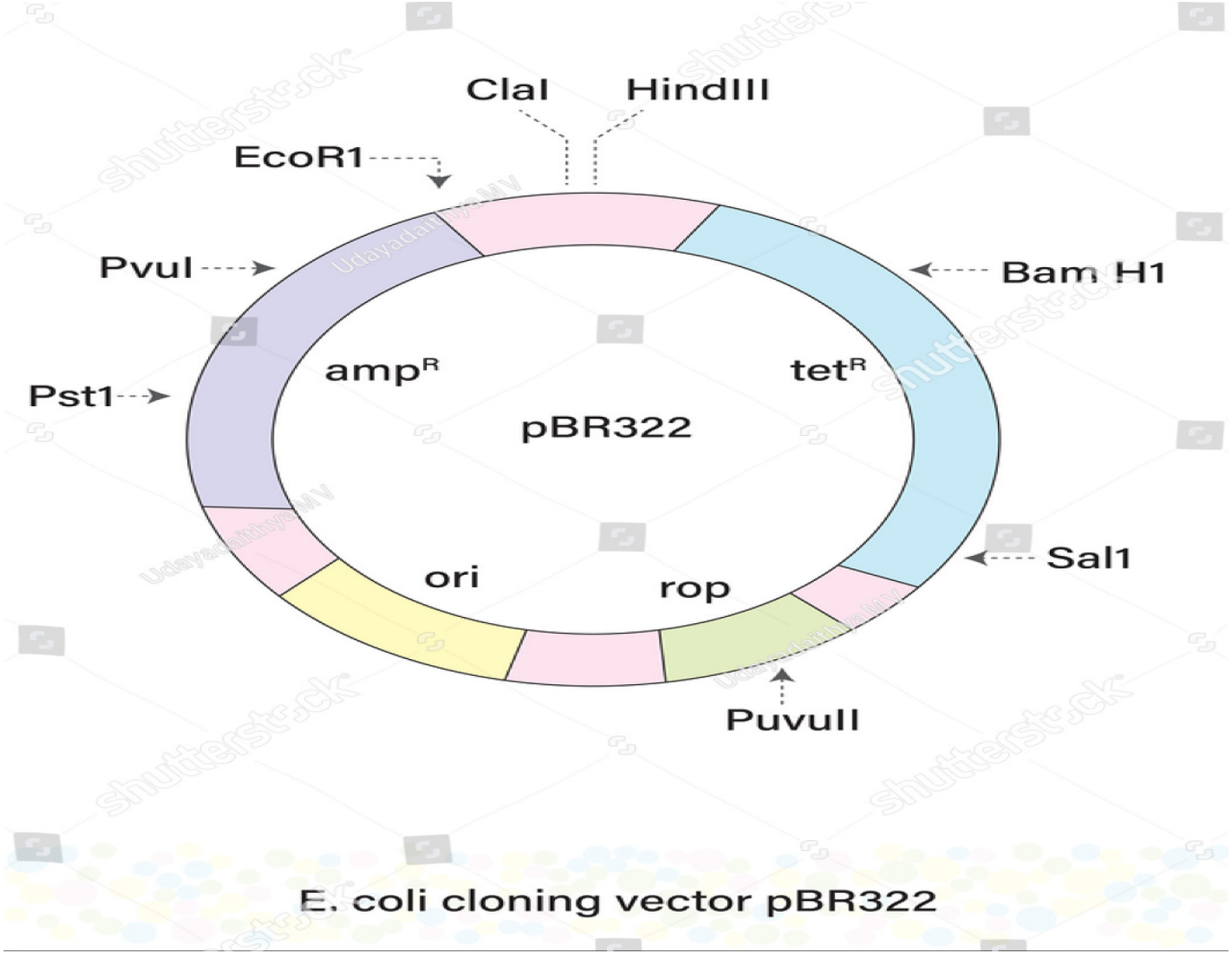
E.*coli*:

- E.coli consider as normal flora microorganism.
- Can found in human and animals.
- Large numbers of E.coli strains are harmless.
- Well known microorganism.



https://images.app.goo.gl/pz9T9zMrb2E9oqgYA

pBR322 Vector:



E. coli cloning vector pBR322

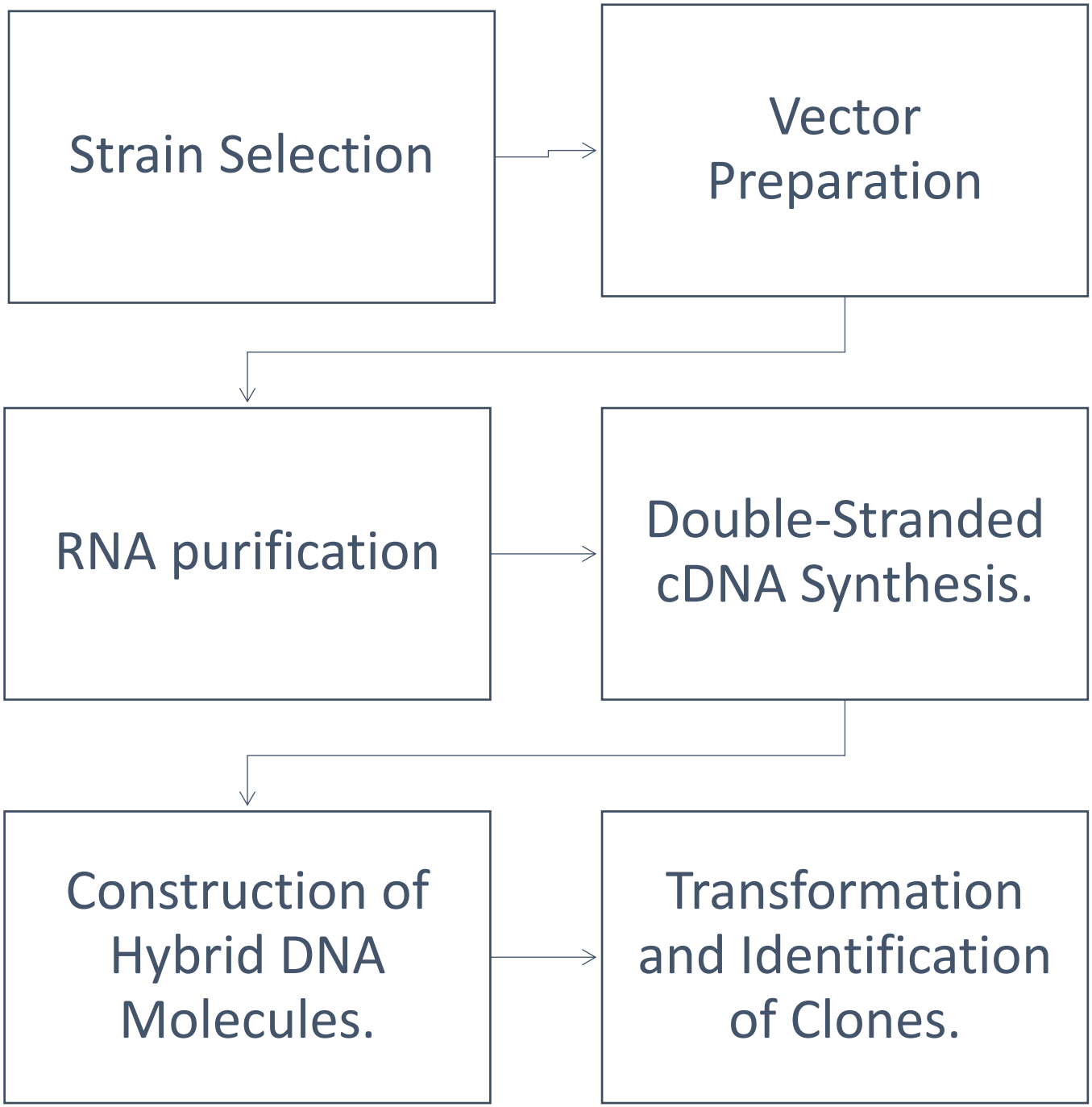
Aim of the study:

Can the structural information for the production of a higher cell protein be inserted into a plasmid in such a way as to be expressed in a transformed bacterium? To attack this problem, we used as a model rat insulin, an interesting protein that can be identified by immunological and biological means.

Materials and Methods:

Ullrich *et al.* (9) have cloned double-stranded cDNA copies of rat preproinsulin mRNA isolated from pancreatic islets and determined sequences covering much of those two genes. We have made double-stranded cDNA copies of mRNA from a rat insulinoma (10) and cloned these in the *Pst* (*Providencia stuartii* endonuclease) site of pBR322 (11), which lies within the penicillinase gene.

The *Escherichia coli* penicillinase is a periplasmic protein, the gene for which was recently sequenced (12). Penicillinase is synthesized as a preprotein with a 23 amino acid leader sequence (12, 13), which presumably serves as a signal to direct the secretion of the protein to the periplasmic space, and is removed as the protein traverses the membrane. Insertion of the structural information for insulin into the penicillinase gene should cause expression of the insulin sequence as a fusion product transported outside the cell.


Strain Selection
Vector Preparation
RNA purification
Double-Stranded cDNA Synthesis.
Construction of Hybrid DNA Molecules.
Transformation and Identification of Clones.

Results:

Table 1. Hybrid-arrested translation and immunoprecipitation of the cell-free products

Source of arresting DNA	Radioactivity, cpm/20 μl			
	Acid insoluble	Immunoprecipitable		% Immunoprecipitable*
		− Insulin	+ Insulin	
Control I (−DNA, −RNA)†	2,570			
Control II (−DNA, +RNA)‡	35,700	12,300	310	36.2
pBR322	28,800	7,850	245	29.0
Clone 3	15,100	3,630	264	26.9
Clone 13	19,600	5,190	350	28.4
Clone 15	18,600	4,850	252	28.7
Clone 16	29,200	8,830	247	32.2
Clone 17	24,000	6,700	316	30.0
Clone 18	15,900	3,690	251	25.8
Clone 19	8,650	587	277	5.0
Clone 20	15,100	4,070	231	30.6
Clone 21	21,100	5,170	223	26.7

Results:

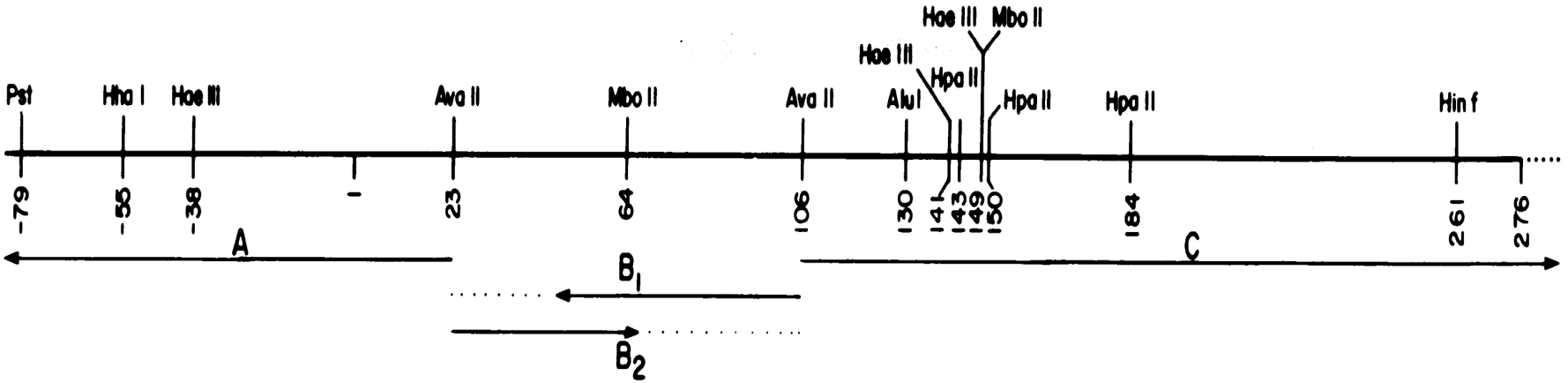


FIG. 1. Restriction map of the insertion in clone pI19. Each restriction site is identified by a number indicating the 5′-terminal nucleotide generated by cleavage at the message strand. Nucleotides are numbered beginning with the first base of the sequence encoding proinsulin. Nucleotides in the 5′ direction from position 1 in the message strand are identified by negative numbers, beginning with −1. Arrows indicate the sequenced fragments; those pointing to the left indicate sequences derived from the antimessage strand, and those pointing to the right indicate sequences derived from the message strand. The uniquely labeled restriction fragments were generated as follows: Following excision with *Pst*, DNA of the insertion was digested with *Ava* II and end labeled. Fragments A and C purified from a polyacrylamide gel were sequenced directly because the *Pst* ends do not label significantly. Fragment B was strand separated on a polyacrylamide gel and sequenced in both directions. The exact number of C·G pairs in the right-hand tail before the *Pst* site could not be counted.

Results:

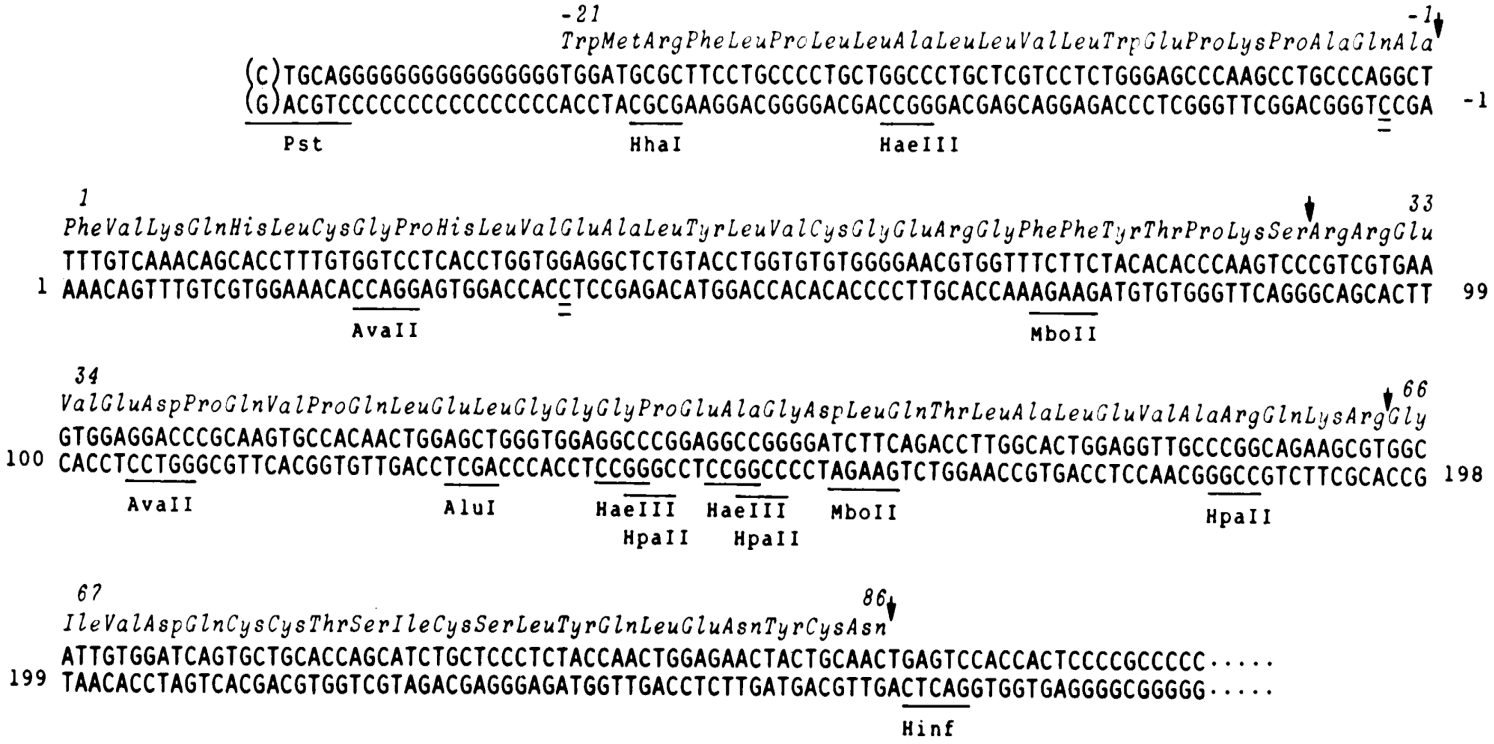


FIG. 2. DNA sequence of the insertion in clone pI19. Nucleotides are numbered using the convention described in Fig. 1. Accordingly, amino acids are numbered beginning with the first amino acid of proinsulin, while the last amino acid of the leader sequence (pre region) is numbered as −1. Restriction endonuclease cleavage sites experimentally verified are underlined and identified. The arrows indicate, in order, the ends of the leader sequence and the peptides B, C, and A. Two nucleotides indicated by double underlining are uncertain.

Results:

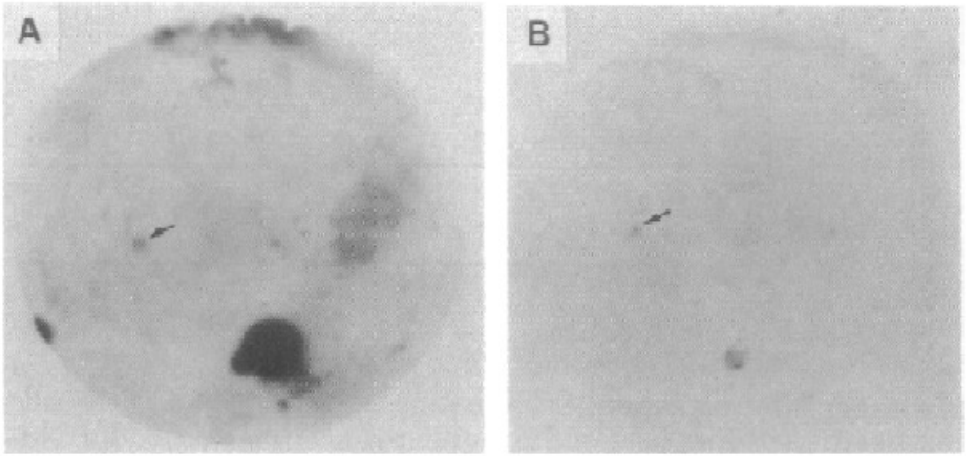


FIG. 3. Initial detection of penicillinase-insulin hybrid polypeptides in an insulin cDNA clone. Cells from colonies of the 48 insulin cDNA clones and from control colonies, χ1776 and χ1776-pBR322, were applied to an agarose/lysozyme/EDTA plate. Positive controls, 5 ng of insulin and 5 ng of penicillinase, each in 1 μl of wash buffer, also were spotted on plate. Antigen was adsorbed to an IgG-coated polyvinyl disk during a 1-hr incubation at 4°. Immobilized antigen was labeled by setting the plastic disk on a solution containing radioiodinated anti-insulin IgG. The autoradiographs are of disks precoated with anti-insulin IgG (*A*) or anti-pencillinase IgG (*B*), exposed on Kodak X-Omat R film using a Du Pont Cronex Lightning Plus intensifying screen for 12 hr at −70°. The arrows indicate the signal generated by clone pI47. The large exposed area in the lower right of (*A*) is the positive control for insulin detection.

Results:

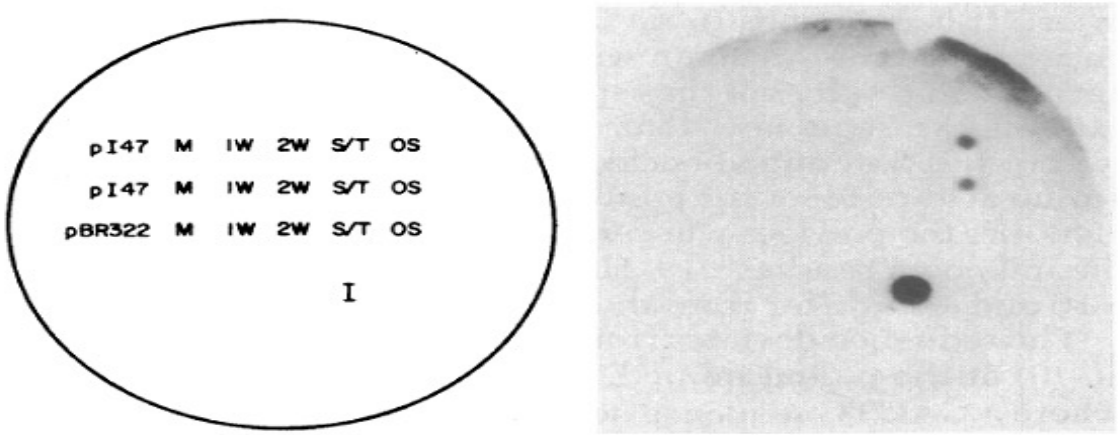


FIG. 4. Release of insulin antigen from χ1776-pI47 cells by osmotic shock. One liter of χ1776-pI47 cells growing at 37° in M9 medium supplemented with 1 g of tryptone, 0.5 g of yeast extract, and 0.5% glucose was harvested at a density of 5×10^7 cells per ml and washed two times in 10 ml of cold 10 mM Tris·HCl, pH 8/30 mM NaCl. The cells were then osmotically shocked (34) in the following manner: The final wash pellet was resuspended in 10 ml of 20% sucrose per 30 mM Tris·HCl, pH 8, at room temperature, made 1 mM in EDTA, shaken at room temperature for 10 min, centrifuged out, resuspended in 10 ml of cold distilled water, shaken in an ice bath for 10 min, and again pelleted. The resulting supernatant was termed the "water wash." As a control, 1 liter of χ1776-pBR322 was grown and treated in a similar manner. Aliquots (1 μl) of each fraction to be assayed for the presence of insulin antigen were applied to the surface of a 1.5% agar plate. (*A*) Positions of each fraction on the plate. M, medium; 1W, first wash supernatant; 2W, second wash supernatant; S/T, sucrose/Tris supernatant; OS, distilled water wash; I, insulin. (*B*) Autoradiograph showing results of a two-site radioimmunoassay of these fractions. Antigen was adsorbed to a polyvinyl disk and labeled by using anti-insulin IgG. The labeled areas correspond to the water washes and the positive control (1 ng insulin). A spectrophotometric assay for β-galactosidase (23) indicated that no more than 4% of cells lyse during this procedure.

Results: