PRODUCTION AND PROCESSING OF RECOMBINANT HUMAN THERAPEUTIC PROTEINS IN *E. COLI*

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INTRODUCTION

Large scale production of human therapeutic proteins which are required in substantial quantities, and which cannot be produced in abundant quantities from traditional sources, has been facilitated by the use of recombinant DNA (r-DNA) technology. This technology has been used to clone genes either in simplified forms or genes have been engineered depending upon situations. Therapeutic proteins have been isolated from non-human sources for use in human ailments e.g. bovine and/or porcine inusulin for treating human diabetes. Use of non-human source proteins for therapy in human runs the risk of developing unwanted immunological responses. Consequently, the need for producing and using identical human proteins for human therapy assumes significance, and in this direction the contributions of r-DNA technology are invaluable.

RECOMBINANT DNA PROTEINS CURRENTLY IN HUMAN USE

The first r-DNA derived product approved by Food and Drug Administration (FDA), USA in 1982 was human insulin produced in E.Coli. Thereafter, several human proteins produced by r-DNA technology have been approved by FDA, USA and/or other Regulatory Authorities of different countries. The following table indicates the main r-DNA proteins which are already in therapeutic use (1-4).

RECOMBINANT GENE PRODUCTS UNDER COMMERCIAL DEVELOPMENT

A large number of human proteins are also in the making; several of these are in advanced stages of development. Some of these proteins are mentioned below (Table-2).

TABLE-1

(r-DNA Proteins currently in human therapeutic use)

Sl.No.	PROTEINS	USE	r-DNA sources
1.	Insulin*	Diabetes	E.coli & Yeast
2.	Human Growth Hormone	Dwarfism	Mouse Mammary Cells, E.coli
3.	Interferons*	Viral disease, Cancers & AIDS	E.coli
4.	Interleukins	Various cancers	E.coli
5.	Tissue Plasminogen activators	Thrombolysis	Chinese Hamster Overy Cell Line
6.	Erythropoietin	Anaemia	Chinese Hamster Overy Cell line
7.	Hepatitis-B* surface antigen	Vaccine against Hepatitis-B	Yeast
8.	Streptokinase*	Thrombolysis	E.coli
9.	Epideramal Growth Factor	Wound healing including burns	E.coli

* Items approved for marketing in India.

Recombinant proteins are usually made in microorganisms like E.coli and yeasts. The advanced knowledge concerning genetics and physiology of E.coli has accounted for the preferential use of E.coli as a host for gene expression. Additional advantages include rapid generation of biomass due to high rates of cell growth and availability of low-cost culture conditions. Important drawbacks include its inability to exert certain post-translational modifications (e.g. disulfide bond formation, glycosylation and acetylation) which usually lead to wrong protein folding. In such situations, use of animal cell lines is preferred, as otherwise the biological activity of the proteins gets substantially modified.

There is no estimate on the number of different proteins present in human body. However, from the size of the human genome, assuming a level of gene expression of around 5%, it is estimated that the number of human proteins could be as many as 100,000 to 150,000. The number of proteins which have been identified, and

on which preliminary or in depth knowledge has been gained is less than 300; much less numbers have been cloned and expressed. The area has thus substantial future potential and several years will elapse before worthwhile harnessing of the r-DNA technology for the production of a sizeable number of authentic human proteins shall be meaningful and significant. In this paper, efforts have been made to give a glimpse on the progress made in engineering human therapeutic proteins in E.coli and the processing strategies of such proteins for gainful exploitations for commercial use.

TABLE-2

Sl.No.	PROTEINS	MAIN USE	r-DNA SOURCES	Ref.
1.	Atrial Natriuretic Factor (ANF)	Hypertension and Kidney disease	Yeast	5
2.	Antithrombin III	Anticoagulant	CHO cell line	6
3.	Blood Factor VIII	Haemophilia	CHO cell line BHK-21 cell line	7 8
4.	Blood Factor IX	Haemophilia-B	CHO cell lines	9
5.	Epidermal Growth Factor (EGF)	Wounded healing including burns	E.coli	10
6.	Human recombinant antibodies	Infections, Various Cancers	CHO Mouse myeloma	11 12
7.	Hepatitis-B surface antigen	Vaccine against Hepatitis-B	C-127 cell line CHO cell line	13 14
8.	Plasminogen activator(including chimerics and modifications	Thrombolysis	E.coli CHO Spodoptera frugiperda cell lines Modified protein	15 16 17 18
9.	Superoxide dismutase	Cardiac treatment and Organ transplants	E.coli	19
10.	Tumour Necrosis Factor (TNF)	Antitumour and Antiviral Therapy	E.coli	20
11.	Urokinase	Thrombolysis	E.coli	21

EXPRESSION OF FOREIGN PROTEINS IN E.COLI

Any DNA sequence can be enriched by a number of methods. The cleavage sites on DNA sequences can be mapped by restriction endonucleases and the nucleotide sequences, coding as well as non-coding, can be identified. Suitable expression vectors are thereafter required for the expression of gene products.

The benefits from obtaining production of heterologous proteins in E.coli are enormous, as much as known about the growing conditions of the organism in large commercial fermenters. Moreover the metabolic as well as the reproductive rates of E.coli are much faster. E.coli duplicate in about 20 minutes (called generation time) and this is about 20,000 times faster than man, and this implies much faster productivity. Taking advantge of this faster reproductive rates, Genentech Labs Inc. (USA) cloned in 1977 Somatostatin in E.coli and had obtained 5 mg of the protein from 100 gms. of the bacterium in a 8 litr. fermenter in a couple of days!To produce these quantities from the lamb brains, nearly 500,000 lambs would have to be sacrificed. This would adequately illustrate the power of recombinant DNA technology as a tool.

In order to express the heterologous (eukaryotic) DNA sequences into proteins in E.coli, it is necessary to have a thorough understanding of the signals that determine the efficiency of transcription and those that determine the efficiency of translation of the messenger RNA (m RNA). The currently held theory of the mechanism of gene expression in prokaryotes (including E.coli) and eukaryotes are briefly described below. Schematically the steps are shown in Fig.1. The Operon Model originally proposed by Monod and Jacob (22) is based on the control of the metabolism of lactose. The model is known as "negative control" model. In this system, the lactose operon contains three structural genes designated as "Z", "Y" and "A"; these code for three enzymes which are involved in lactose metabolism. Beta-galactocidase catabolises the formation of galactose and glucose from lactose; lactose permease facilitates entry of lactose into the bacterial cell; and thiogalactoside trans-acetylase helps transfer of acetyl groups to galactosides. These three structural genes are transcribed as a single polycistronic m RNA which codes for all the three proteins. The basic feature of this model is the coordinated expression of several genes, as in this case of "Z", "Y" and "A" genes. Transcription is effected by DNA dependent RNA-polymerase that binds to DNA at the promoter site. The beginning of transcription is "upstream" of the structural polycistron at a specific initiation site. The transcript contains the Shine-Dalgarno (S-D) sequence at the 5 end which is complementary to ribosomal RNA, thus facilitating m RNA - ribosome complex formation. Protein synthesis or translation then proceeds. In prokaryotic system

transcription and translation are united, and towards the downstream of the structural polycistron (in figure 1, the "A" gene), there is a signal for termination of transcription where the complex of the DNA-RNA polymerase is unstable. The transcription of the structural polycistron is controlled by a regulatory gene which is called the "i" gene in the lactose operon which codes for a repressor protein that binds specifically to a site of the DNA sequence adjacent to the promoter called **operator sequence**, a 22 base pair DNA sequence which is located 6 base pairs downstream of the lac Pribnow box. The transcriptional start site of the promoter is at the beginning of the lac operator. Thus, the lac repressor prevents the RNA plymerase from binding to the promoter through physical hinderance. As a result, the transcription is inhibited and thus the synthesis of the enzymes coded in the structural genes is prevented. The inducer binds to the repressor and alters the three dimensional structure of the repressor preventing its binding to the operator; therefore the RNA polymerase is able to transcribe the genes for lactose metabolism.

Besides, the above negative control system, there are other control systems such as the **"positive control"** system as well as a process called **"attenuation"**. In the positive control system, the inducer would bind to the protein which would stimulate transcription. In attenuation control, the system operates on genes responsible for the synthesis of certain amino acids. For more details on the control of genes in prokaryotes, several recent reviews may be consulted (23-28).

EXPRESSION OF EUKARYOTIC GENES

In eukaryotes the operation of genes are quite different and indeed much less is known about eukaryotic expressions compared to the prokaryotes. DNA is maintained with histone and other proteins as a complex structure in cells known as chromatins. Chromatins undergo significant alterations during transcription and such changes are associated with the control of genes. The lactose repressor type of molecules as in **Fig. 1** have not yet been discovered in eukaryotes. Evidence has however been obtained of the existence of steroid hormone receptors which are involved in the control of gene expression.

The problems associated with expressing eukaryotic genes in E.coli have been studied exhaustively and significant amount of information is available. Thus no polycistronic m RNA have been found yet and each structural cistron is transcribed separately with its own promoter and transcription initiation as well as termination sites. The RNA - polymerase is more complex and exists for r RNA, t RNA and m RNA synthesis. The promoter sequence are different from prokaryotes. Once the RNA polymerase is attached to the cistron, the synthesis of RNA begins.

Attachment of RNA is influenced by non-coding, Cis-acting DNA sequences which are believed to be responsible for dragging the RNA-polymerase to the coding region (29). After the onset of transcription, the RNA molecule is capped by 7 - methylguanosine attached by a 5' - 3' phosphate linkage to the end of m RNA. The transcription is then terminated. Before translation, RNA undergoes significant processing. Processing involves besides capping, trimming with ribonuclease or addition of the "poly A tail" to the 3 - end and removal of introns. Introns have to be removed from the m RNA or else protein synthesis can not occur. Introns are removed by the action of splicing enzymes and thereafter they are very precisely ligated with specific ligases to bring back the exons together in the correct reading frame (30). After the protein is produced, this is subject to post-translation modifications for its maturation like glycosylation, addition of prosthetic groups, specific peptide cleavage, formation of multi subunit structure etc., which are specific to the types of cells involved in the protein synthesis.

Prokaryotes do not have the machinery to process introns and therefore genes containing intron will not be expressed in E.coli. In addition specific maturation operations of eukaryotic proteins cannot also be carried out by prokaryotes. These clearly indicate the current limitation of the prokaryotic expression systems for the expression of certain complex eukaryotic proteins.

Expression in prokaryotes of eukaryotic genes containing introns is overcome by isolating the m RNA, and undertaking on it in-vitro, the reactions of reverse transcriptase, DNA polymerase and S1 nuclease, and followed by in-vivo gene cloning. The enzyme reverse transcriptase produces a single strand of DNA complementary to m RNA. The DNA polymerase will then synthesise the second strand and the S1 nuclease will thereafter cut the unwanted loop of DNA created in the previous reaction. Now the double stranded c DNA without the introns can be used as the coding sequence in prokaryotes. The c DNA approach has certain limitations like difficulties in obtaining the starting m RNA in sufficiently pure form and problems encountered in obtaining the proper c DNA sequence synthesised by reverse transcriptase. It is often necessary to ligate additional DNA sequence on to the c DNA to make the gene suitable for the expression vector.

If the amino acid sequence of the required protein is known then it is possible to construct the DNA molecule with the required nucleotide sequence. In this process the constructed DNA can be tailored to a vector and the codons preferred by the prokaryotes could also be inserted. This system has been used to produce alpha interferon of 166 amino acids wherein a DNA sequence of 514 base pairs were used (31).

IMPORTANCE OF PROMOTER SEQUENCE

Promoter sequence plays a major role in transcription. For maximising the expression of a homologous or heterologous gene a strong promoter is required. In some cases where the gene product is cytostatic or cytotoxic to the host organism, a controlable (regulated) promoter is necessary against a non controlable (constitutive) promoter. The strength of a promoter is determined by the frequency with which the DNA polymerase starts the transcription cycle leading to a corresponding increase in the level of m RNA. Thus, the obvious choice is to use the promoters whose natural gene product in E.coli is abundant. Some of the common E.coli promoters used for expression are **Plac-UV5, Ptrp, Plpp and Prrn.** The **Plac-UV5** is a regulated promoter, though one of the weakest described here. The **Ptrp** is about 4 times stronger than **Plac-UV5,** but less easy to regulate. The **Plpp** is a very strong but constitutive promoter. However, the **Plpp** promoter has been tamed into a regulated promoter by placing **lac operator** site downstream of the promoter (32). The **Prrn** directs the transcription of the ribosomal RNA genes and is one of the most powerful promoters of E.coli. The use of the ribosomal RNA promoter for gene expression is promising provided it can be repressed or induced effectively. Lack of sufficient knowledge of the mechanism of regulation of these rrn - promoters reduces their application in developing inducible expression systems based on these promoters.

To amalgamate the useful features of various promoters, some hybrid promoters have been constructed in the laboratory. An excellent example of this approache is **tac promoter,** a hybrid of **trp** and **lac-UV5** promoters, joined at position -20 with respect to the transcription initiation site. The **tac promoter** is an inducible promoter and is about 10 times as efficient as the **lac-UV5** promoters.

Some bacteriophage promoters have also been found very useful for development of expression vectors. Important ones are bacteriophage λ PR and λ PL promoters, the rightward and the leftward promoters of coliphage lambda. The promoters are regulated by a repressor coded by the phage cI gene. Temperature sensitive mutants (cI 857) of the cI gene have been isolated. The repressor binds to these distinct regions of about 17 nucleotides long separated by spacers of 3 to 7 bases. At 30 C the repressor is able to block transcription from these promoters, but at 40 C the repressor destabilises leading to depression of the promoters. The efficiency of PL promoter is about the same as that of the hybrid promoter **tac.**

All the promoters described above are recognized by E.coli RNA polymerase. An interesting addition to the arsenal of molecular biologists has been promoters from bacteriophage T7 (33).

The sequences needed for **T7** of E.coli promoter activity are quite different; so vectors have been constructed where the target DNA will be transcribed very actively by **T7** RNA polymerase but poorly if at all, by E.coli RNA polymerase. Such vectors allow toxic genes to be cloned under the control of the **T7** promoter leading to the production of large amounts of specific RNAs and proteins both in vitro and in vivo.

The nucleotide sequence of certain promoters in E.coli are indicated in Table-3.

TABLE-3

(The base sequence of characterised promoters)

Particulars	DNA dependent RNA polymerase binding site (-35 sequence)		"Pribnow Box" m RNA initiation site (10 sequence)
E.coli Consensus sequence	TTGACA		TATAAT
ptrp	TTGACA	ATTAATCATCGAACTAG (17 bp)	TAACTA ... Trans.*
plac UV5	TTTACA	CTTTATGCTTCCGGCTCG (18 bp)	TATAAT ... Trans.
ptac	TTGACA	ATTAATCATCGGCTCG (16 bp)	TATAAT ... Trans.
pl	TTGACA	TAAATACCACTGGCGGT (17 bp)	GATACT ... Trans.
tc	TTGACA	GCTTATCATCGATAAGC (17 bp)	TTTAAT ... Trans.
Eukaryotic Consensus	CCAAT		ATA

*Trans. = Transcription

The commonly used promoters are the **lac** or **trp** or **tac** which are placed upstream of the coding sequence. Several **lac** promoter based vectors have been constructed with the first few nucleotides of Z gene coding for beta galactosidase and the coding sequence to be expressed is inserted downstream of the beta galactosidase N-terminal coding sequence to give a fusion protein.

TRANSLATION

The m RNA in E.coli would translate efficiently and that the initiation shall be brisk if it contains a start codon (AUG or GUG alongwith an optimal Shine - Dalgarno (S-D) sequence upstream of the initiation codon. Identified S-D sequences vary in length from 3 to 9 bases and the polyuridine sequence 5 UAAGGAGGU3 is one full length S-D sequence. The S-D sequence or at least 4 nucleotides of the sequence AGGAGGU should be positioned seven to nine nucleotides upstream of the initiation codon. The S-D sequence should not be set apart and these should not overlap with the nucleotide sequence recognised by a RNA binding protein. Besides, there is a preferential choice of placement of the initiation codon for better translation. In general for efficient translation the initiation codon AUG alongwith 3 to 9 bases lying between 3 to 12 bases upstream of S-D sequence is preferred. The S-D bases are complimentary to the 3 end of the 16 S r RNA molecule and are involved in the initiation of translation by forming a complex between m RNA and 30 S subunit of the ribosome. Other features that govern the translation are the m RNA stability and its secondary structure. The amount of gene product synthesized in the cell is determined not only by the strength of promoter but also by the efficiency of initiation of translation of m RNA and its half life.

GENE FUSION STRATEGIES

Eukaryotic proteins are usually produced in E.coli as fusion proteins. In designing the recombinant polycistron, the N-terminal coding sequence of an E.coli gene alongwith the control region is ligated to the desired coding sequence. The polycistron integrated into the appropriate plasmid when introduced and cloned in E.coli, will be recognised by the E.coli RNA - polymerase as native and translation will be effected. The protein shall be chymeric, the N-terminal shall be prokaryotic while the C-terminal will be eukaryotic. The N-terminal part is recognised as "SELF" by the cell and longer the N-terminal part is, more stable the heterologus protein shall be. The schematic representation of the process is shown in **Fig. 2.**

The strategy of designing a fusion protein is to ultimately remove the fusion part to get the desired protein.Cleavage of the fusion protein can be achieved at the junction by chemical methods or by enzymatic methods, and some of the cleavage peptide-sites that could be used in fusion protein strategy are indicated in Table 4 (32).

While choosing a cleavage peptide the factors to be considered are the stability and the effects of the native protein to the cleavage reaction, the specificity of the cleavage reaction as well as the ease of availability of the cleaving enzymes (when

required). Native proteins could be made stable by making them to form an intracellular inclusion body or by exporting the protein either into the periplasmic space or into the culture medium. Inclusion bodies are formed by designing the fusion of the native protein with a well expressed E.coli protein. For exporting native proteins, a fusion is made with an export leader sequence of prokariotic gene like beta lactamase, Omp A, alkaline phosphatase, etc. and the heterologous protein shall be transported either into the periplasmic space or would be exported to the outer cell wall or into the culture medium; the leader export sequence would usually be cleaved by enzymes present in the cell membrane.

TABLE-4

CHEMICAL AND ENZYMATIC METHODS THAT HAVE BEEN USED TO CLEAVE FUSION PROTEINS SITE - SPECIFICALLY

Sl.No.	CLEAVAGE METHOD	RECOGNITION SEQUENCE
	CHEMICAL	
1	Cyanogen bromide	- Met ↑
2	Formic acid	- Asp ↑ Pro -
3	Hydroxylamine	- Asn ↑ Gly -
	ENZYMATIC	
4	Collegenase	- Pro - Val ↑ Gly - Pro -
5	Enterokinase	- Asp - Asp - asp ↑ Lys -
6	Factor Xa	- Ile - Glu - Gly - Arg ↑
7	Thrombin	- Gly - Pro - Arg ↑
8	Trypsin	- Arg ↑
9	Clostripain	- Arg ↑
10	Ala 64 - subtilisin	- Gly - Ala - His - Arg ↑

Note : Arrow ↑ indicates cleaving site

Fusion proteins are also designed for enabling efficient isolation and purification wherein a part of the chimeric protein is used as the affinity handle or a part can have particular charge properties which would facilitate separation on ion-exchange resins. Using such properties fusion proteins have been purifi; a few examples are indicated in Table-5 (32).

TABLE-5

GENE FUSION SYSTEMS USED TO FACILITATE PROTEIN PURIFICATION

GENE PRODUCT	ORIGIN	SECRETION	LIGAND
β-Galactosidase	Escherichia coli	-	TPEG, APTG
Protein A	Staphylococcus aureus	+	IgG
CAT	Escherichia coli	+	Chloramphenicol
Poly (Arg)	Synthetic	-	Ion-exchange
Poly (Glu)	Synthetic	-	Ion-exchange
Z	Synthetic	+	IgG
Phos S	Escherichia coli	+	Hydrosylapatite
Cysteine	Synthetic	+	Thiol
Protein G	Streptococci	+	Albumin
MBP	Escherichia coli	+	Maltose
GST	Escherichia coli	-	Glutathione
Flag peptide	Synthetic	+	Specific IgG
Poly (His)	Synthetic	+	Zn, Cu

Numerous fusion genes in correct reading frame have been created based on lac. operon and using the N-terminal sequence of betagalactosidase to form the heterologous proteins. One of the earliest examples was the fusion of betagalactosidase to somatostatin in E.coli (34). Fusion peptide approach has been used for the expression of human insulin (35), thymosin (36), neoendorphin (37), betaendorphin (38), proinsulin (39), etc.

In order tó make commercial application of r DNA technology a success, the levels of expression of the required protein would have to be very high. Achievement of high levels of production of the designed protein is a result of great effort and investment. All said and done on r DNA technology, so far less than a dozen of products have become a commercial reality and relatively only

small numbers are in pipeline for exploitation, some of which are mentioned in the text earlier. Insulin, interferons and growth hormones have been over expressed (35,40). Factors influencing high levels of expression are selection of the codons, strength of promoters, secondary structures of m RNA in relation to ribosome binding site, efficiency of transcription, copy no. of plasmids, stability and physiology of host cell. Cassette vectors are now available with desired restriction sites and all the signals optimally spread (41), yet it can not be said with certainty that such cassettes would yield desired results. Best results are obtained by making several permutations and combinations. Once desired clone is obtained it has to be standardised for maintenance of growth conditions for optimal production of the desired proteins.

DOWNSTREAM PROCESSING

Engineered proteins in E.coli are mostly in the inclusion bodies and need to be solublised by various methods. In order to facilitate isolation several new techniques for the purfication of proteins have emerged. This was specifically necessary because the quantities of such recombinant proteins present either as inclusion bodies or in the periplasm of microorganisms or in the cell soup were in very small quantities compared to the conventional microbial metabolites as indicated below (Table 6).

TABLE-6

Typical Product Concentration

Products	Products in Broth	Products in Cells	Concentration
Ethanol (42)	Yes	-	50-120 g/l
Organic acids (42) Lactic, Citric etc)	Yes	-	40-100 g/l
Antibiotics (43)			
Penicillin	Yes	-	18-35 g/l
Tetracycline	Yes	-	20-30 g/l
Rifampicin	Yes	-	12-24 g/l
Gentamicin	Yes	-	1-2.5 g/l
Vitamins B-2 (42)	Yes	-	10-15 g/l
tPA (44)	Yes	Yes	55 mg/l
Human growth hormone (45)	-	Yes	20.5 g/l
Human Chorionic Gonadotropin (46)	-	Yes	1.1 mg/l

The concentration of bioproducts are usually in mg quantities or less per litre of the fermented broth and the products are either available in the supernatent or within the cells as inclusion bodies (47, 48). These would thus require special processing for recovery which are quite different from the conventional fermentation products. The future development of biotechnology is thus greatly linked with the development of efficient downstream processing systems and this is a great challenge to the engineers specially the chemical engineers, and the instrumentation specialists for enabling to produce gms or kilograms quantities of such high value-added products. This can happen only by concerted team-work of people of multidisciplinary background with the biologists and the chemical engineers working as the main players, alongwith close interaction with people of other disciplines.

The r DNA technology has led to a need for the development of reliable and efficient manufacturing processes. The first factor is to have an efficient host which may be a microorganism or a transformed cell line. Depending upon the host will the selection or design of the bioreactors be made. For growing E.coli or yeast the stirred tanks would usually be the best choice and several commercial variations are already available from leading companies. Usually the batch mode or fed batch mode are preferred for growths.

As has been indicated in Table-6, the concentration of recombinant protein after fermentation is very low. The process therefore assures more importance and requires more attention. As the products are not usually found in the supernatent but within the cells, the latter need to be handled very carefully to take out the targeted protein. Usually the cells are disrupted by treatment with alkali, detergents or enzymes, or are subjected to shear (liquid or solid shear) abrasion or osmotic shock. The cell-debri are separated subsequently by centrifugation or other mass separation techniques. The filtrate containing the product is subjected to nuclease treatment and proteins are then precipitated by salts or solvents alongwith pH alterations, wherever applicable. This enables partial purification only. The precipitate is redissolved, subjected to enzyme or chemical treatment for clearing the unwanted proteins if any and allowed to either precipitation or aqueous two phase extraction or chromatography followed by freeze drying depending upon the extent of purification sought. Injectable biologically active human proteins like insulin, erythropoietin, growth hormone, interleukins, interferons, hepatitis-B surface antigen etc. require intricate purification and processing; the detailed industrial procedures are however never published. It has been possible to purify many of these proteins by affinity chromatography using monoclonal antibodies as indicated below (Table-7) from the published literature.

TABLE-7

Protein substances purified using monoclonal antibody columns

Protein Substances	References
Tissue plasminogen activator	44
Urokinase	49
Alpha Interferon	50
Beta Interferon	51
Interleukin 2	52
Blood factor VIII c	53
Prolactin	54

Recombinant proteins passing through the rigorous steps of isolation and purification are often not in their active stage of folding; thereby they are lacking in the manifestation of their true biological activities although immunologically they are active. Intact immunological properties derived primarily from amino acid sequence of proteins are no indications of true biological activities; the latter are functions of proper folding of the proteins. Great care and manipulations are required to bring the proteins to their proper folding conformations. Renaturation of proteins were earlier believed to be spontaneous natural phenomena in appropriate physiological conditions (55-57). However, soon it was found that once denatured, it was not possible to renature most of them. Subsequently, with the discovery of **chaperons**, which are enzymes believed to be mediating the proper folding of proteins, evidences started gathering that proper folding of target proteins including renaturation of denatured ones is associated with certain other proteins, namely the chaperons which are not parts of the target proteins (58-64). More recently ribosomes, the protein synthesing machinery have been attributed to renaturation of inactivated proteins and such renaturation phenomena could be established on proteins obtained from different sources, using 70S ribosome from E.coli (65). This study indicated that unlike the involvement of ATP in protein folding by chaperons, ribosomes could independently direct refolding and renaturation. Taking clue from these results it should be possible to device appropriate columns containing immobilized ribosomes to use them for the refolding and renaturation of those inactivated proteins which are not amenable

to spontaneous natural refolding. This operation could be done at the final stage of processing of the recombinant proteins after effecting all the purification steps, so that the final protein products in appropriate physiological solutions are available for further processing into finished dosage forms.

CONCLUDING REMARKS

The decade of 1990s shall see the introduction of several life saving bioactive proteins to be used in in-vivo human therapy which shall be produced in unnatural hosts by r-DNA technology. E. coli shall contribute substantially as the 'tamed' micro organism in such production technologies. While the molecular biologists will contribute significantly towards the upstreams of the production technology by choosing proper host cells and appropriately modifying them, the microbiologists alongwith the chemical engineers and chemical technologists shall provide information on optimising process parameters and designing appropriate bioreactors, while the chemical engineers alongwith the instrumentation specialists, the protein chemists, the analytical chemists and others shall be instrumental to the final isolation, purification, packaging and product delivery to the medical profession and the consumers. It would not be possible to work in isolation by any one specialist group. It would be the right kind of people working together with proper equipment and instrumentation supports, all interwoven together into a complex and smooth fabric to have realisable benefits for the individual organizations as well as for the society.

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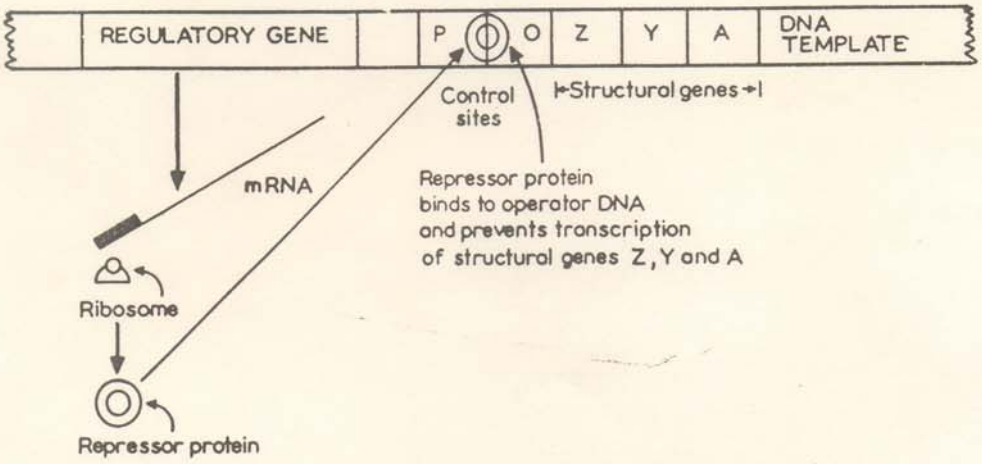
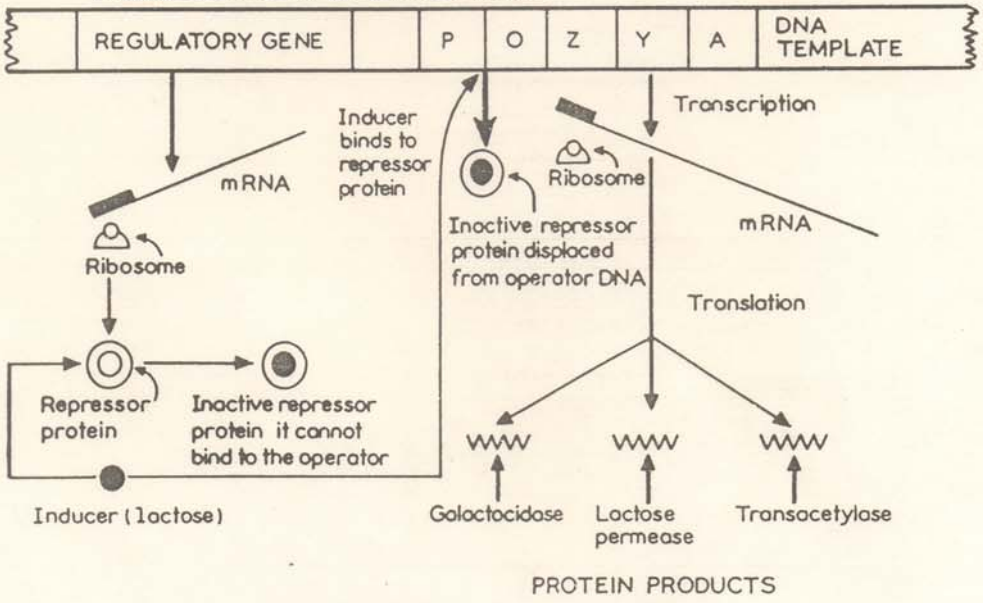
FIGURE. 1(a)

(LACTOSE OPERON MODEL OF PROTEIN SYNTHESIS IN E. coli)

REPRESSED STATE OF LACTOSE OPERON.



INDUCED STATE OF LACTOSE OPERON



PROTEIN PRODUCTS

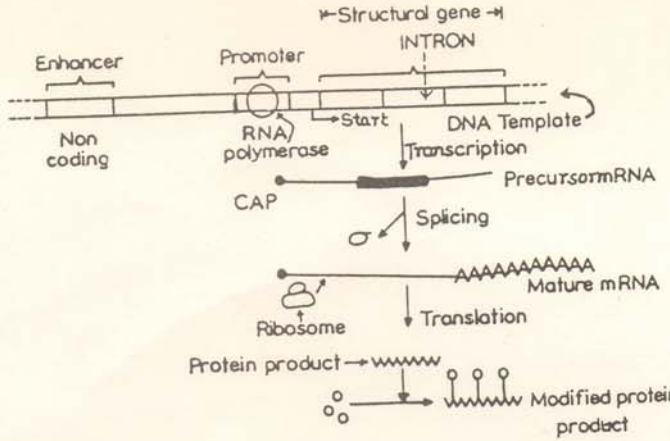


Figure 1(b) Outline of Mechanism of Protein Synthesis in Eukaryotes

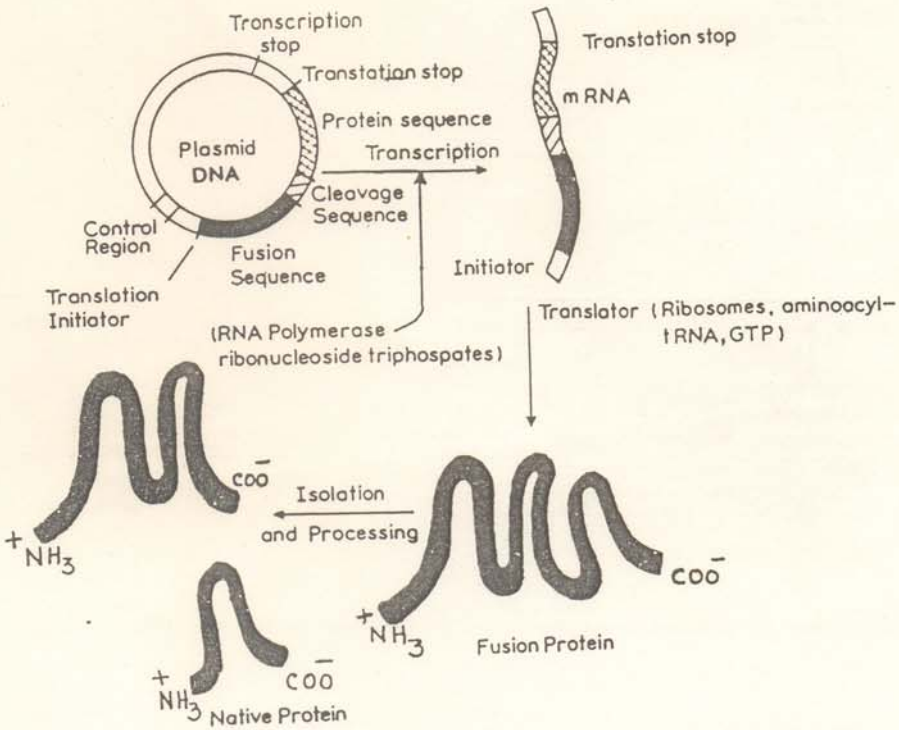


Figure 2 : Plasmid - directed expression of cleaveable fusion proteins