1
Early Recombinant Protein Therapeutics

Pierre De Meyts[1,2,3]

[1] *Department of Cell Signalling, de Duve Institute, Catholic University of Louvain, Avenue Hippocrate 75, 1200, Brussels, Belgium*
[2] *De Meyts R&D Consulting, Avenue Reine Astrid 42, 1950, Kraainem, Belgium*
[3] *Global Research External Affairs, Novo Nordisk A/S, 2760, Måløv, Denmark*

1.1
Introduction

The successful purification of pancreatic insulin by Frederick Banting, Charles Best, and James Collip in the laboratory of John McLeod at the University of Toronto in the summer of 1921 [1–3], as reviewed in the magistral book of Bliss [4], ushered in the era of protein therapeutics. Banting and McLeod received the Nobel Prize in Physiology or Medicine in 1923. The discovery of insulin was truly a miracle for patients with Type 1 diabetes, for whom the only alternative to a quick death from ketoacidosis was the slow death by starvation on the low-calorie diet prescribed by Allen of the Rockefeller Institute [5–7]. Insulin went into immediate industrial production (from bovine or porcine pancreata) from the Connaught laboratories of the University of Toronto and, under license from the University of Toronto by Eli Lilly and Co. in the United States, by the Danish companies Nordisk Insulin Laboratorium and Novo (who merged in 1989 as Novo Nordisk), and by the German company Hoechst (now Sanofi), all of which remain the major players in the insulin business today.

Insulin also turned out to be a blessing for scientists interested in protein structure. It was the first protein to be sequenced [8, 9], earning Fred Sanger his first Nobel Prize in 1958. It was the first protein to be assembled by total peptide synthesis [10–14]. It was the first peptide hormone to have its minute blood levels measured by radioimmunoassay [15], earning Rosalyn Yalow the Nobel Prize in Physiology or Medicine in 1977. The structure of insulin was solved by X-ray crystallography in 1969 by Nobel Laureate Dorothy Hodgkin and her team in Oxford [16], providing a rationale for detailed structure–activity relationships studies [17] and, later, for protein engineering of insulin and insulin analogs.

Protein Therapeutics, First Edition. Edited by Tristan Vaughan, Jane Osbourn, and Bahija Jallal.

For about 60 years after the discovery of insulin, major therapeutic advances were in formulation to improve pharmacokinetic and pharmacodynamic (PK/PD) properties and provide longer acting insulins, and in purification (reviewed in Ref. [18]). The introduction of the first insulin injection pen in 1985 (Novopen) made insulin treatment somewhat more bearable for diabetic patients.

In 1982, insulin once again was at the forefront of a therapeutic revolution by becoming the first DNA recombinant protein therapeutics on the market (human insulin, Humulin by Eli Lilly), kickstarting the biotechnology era. By 2008, 130 protein therapeutics (of which 95 were produced by genetic engineering) had been approved by the US Food and Drug Administration (FDA) [19], and in 2016, 206 [20]. These comprise hormones, interferons, interleukins, hematopoietic growth factors, tumor necrosis factors, blood clotting factors, thrombolytic drugs, enzymes, monoclonal antibodies, and vaccines.

In this introductory chapter, I will attempt to retrace the pioneering early steps in the development of recombinant protein therapeutics.

1.2 The Birth of Genetic Engineering

The term "molecular biology" is said to have been coined in 1938 by Warren Weaver, head of the Division of Natural Sciences at the Rockefeller Foundation. While the field developed since the 1930s, it got a major impetus in 1953 with the discovery of the double-helical structure of DNA by Watson and Crick [21, 22], who deduced from it the mechanism of genetic self-duplication. For this achievement, Watson and Crick were awarded the Nobel Prize in Physiology or Medicine in 1962.

While studying the structure, function, and replication of the genes of simian virus 40 (SV40), Paul Berg of Stanford University had the idea in the early 1970s that viral DNA could be used to transduce inserted nonviral genes into mammalian cells. He and his colleagues succeeded in developing a general way to join two DNAs together *in vitro,* a process called DNA recombination. This involved synthesizing cohesive ends onto the end of DNA segments and covalently joining them *in vitro* using DNA ligase. They succeeded in inserting both lambda phage genes and the galactose operon of *Escherichia coli* into circular SV40 DNA [23]. Recombinant DNA technology was born. Paul Berg received the Nobel Prize in Chemistry in 1980 for this work, shared with Walter Gilbert and Fred Sanger (his second Nobel Prize) for their work on sequencing DNA methods. Interestingly, this landmark paper does not report successful expression of the recombinant DNA into mammalian cells (in part due to biohazard concerns), but Berg and colleagues later developed suitable vectors for this purpose (Figure 1.1) [24].

Berg's very basic goal was to insert bacterial, viral, and simple eukaryotic genes into mammalian cells, in order to understand better how human genes are organized and function. What triggered the rise and explosive growth of the biotechnological industry was a different postulate: how to transduce mammalian genes encoding proteins of therapeutic importance into bacteria or simple eukaryotes (and eventually mammalian cells). The key experiments toward this end were done

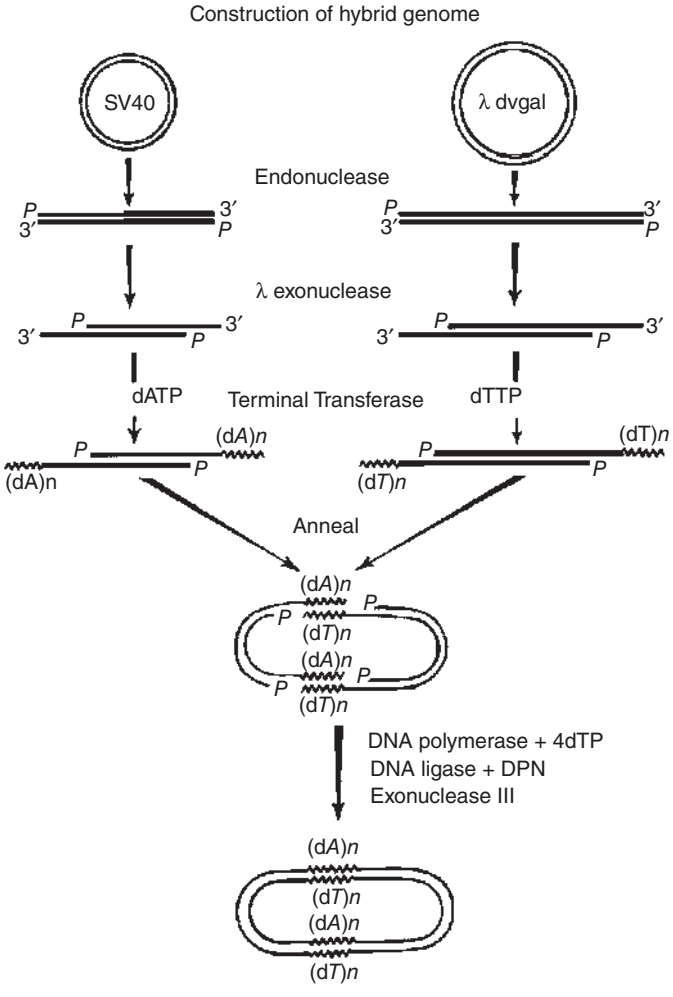

Figure 1.1 Paul Berg's construction of hybrid genome. (From his speech: The Nobel Prize in Chemistry 1980. Nobelprize.org. Nobel Media AB 2014. Web. 12 Jan 2017. http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1980/ [24].)

by a team of scientists that included Stanlry N. Cohen from Stanford University, as well as Herbert Boyer and Howard Goodman from the University of California, San Francisco (UCSF), who were to become major players in the nascent biotechnology industry [25, 26].

They transferred and expressed frog ribosomal DNA into bacterial cells using a constructed plasmid vector, pSC101. This plasmid contained a single site for the restriction enzyme EcoRI and a gene for tetracycline resistance. EcoRI was used to cut the frog DNA into small fragments, which were combined with the plasmid that had also been cleaved with EcoRI. The aligned sticky ends of the DNA fragments were joined using DNA ligase. The plasmids were then transferred into *E. coli* and plated onto a growth medium containing tetracycline. The

cells that incorporated the plasmid carrying the tetracycline resistance gene grew and formed colonies of bacteria, some of which carried the frog ribosomal RNA gene. The colonies were shown to express frog ribosomal DNA.

This led to the very first biotechnology patent granted to Cohen and Boyer in 1980, to the Wolf Prize in Medicine in 1981 and the National Medal of Science in 1988 to Cohen, and to the Lasker Award in 1980 and National Medal of Science in 1990 to Boyer.

Berg Boyer and Cohen with other leading molecular biologists raised concern in 1974 about the potential biohazards of recombinant DNA [27], and organized the influential Asilomar Conference in February 1975, which resulted in stringent National Institutes of Health (NIH) guidelines for recombinant DNA work in June 1976; however, these were later relaxed when the technology became more familiar.

Herbert Boyer made a bold move in April 1976 by founding the biotechnology company Genentech in South San Francisco with a young venture capitalist Robert A. Swanson. Genentech to this day (acquired by Roche, Switzerland, in 2009) remains one of the most successful biotech companies. Since 1976, more than 2000 such companies have been founded, and major pharmaceutical companies have subsequently strived to become more biotechnologically oriented.

1.3 Recombinant Human Insulin

1.3.1 The Race to Clone the Human Insulin Gene and to Produce Human Recombinant Insulin

The race to produce the first recombinant protein therapeutics has been vividly described in the excellent book of Hall [28]. By 1976, Eli Lilly and Co. became concerned that the supply of animal pancreata would become insufficient at some point in the future to meet the needs of diabetic patients worldwide. This was supported by a report of the National Diabetes Advisory Board published 2 years later [29]. Lilly was aware of the promise of genetic engineering and had established an in-house group of scientists to evaluate its possibilities.

Forty years ago, in May 1976, Lilly convened one of its periodic academia–industry symposia (the 16th since 1922), focused this time on the theme of making insulin by genetic engineering. Leading molecular biologists and insulin researchers from all over the United States were invited: Donald F. Steiner (the discoverer of proinsulin [30]) and Shu Jin Chan from the University of Chicago, Grady Saunders and Peter Lomedico from University of Texas, William J. Rutter, Raymond Pictet, and Howard M. Goodman from UCSF, and William (Bill) Chick, Argiris Efstratiadis, and Walter Gilbert (who would later become a co-winner of the 1980 Nobel Prize in Chemistry) from Harvard University.

Bill Chick presented his results with establishing an insulin-producing cell line from an irradiated rat insulinoma, which became an obvious tool for attempting to isolate the rat insulin gene (rat and mice have two, in fact). The race was on [28].

The following year, Axel Ullrich and colleagues in the UCSF team led by Bill Rutter and Howard Goodman reported the construction of recombinant bacterial plasmids containing complementary DNAs (cDNAs) for the coding sequence or rat preproinsulin I and the A sequence or rat preproinsulin II [31]. In 1980, Graeme Bell in the Rutter–Goodman team determined the sequence of the human insulin gene [32]. The same year, Talmadge and colleagues in the Gilbert team showed that bacteria (*E. coli*) can process mature rat preproinsulin to proinsulin [33].

However, in the end the race to produce recombinant human insulin was won by a team that scooped all others by choosing a radically different approach from those trying to use cloned genes: they chose to use completely synthetic DNA.

Herbert Boyer missed the Lilly May 1976 meeting because he was busy founding Genentech in April, and forming an alliance with a team at the City of Hope National Medical Center in Duarte, California, in order to produce recombinant human insulin, and ultimately sell it [28].

The City of Hope team included Arthur (Art) D. Riggs, a geneticist and molecular biologist whose major interests were how genes are turned on and off, X-chromosome inactivation, and DNA methylation, basically founding the now popular field of epigenetics. Riggs had recruited in 1975 a highly talented Japanese organic chemist Keiichi Itakura (then in Ottawa), who was at the forefront of the difficult art of making synthetic DNA.

Riggs and Itakura had realized the potential power of combining chemical DNA synthesis technology and recombinant DNA technology, and Herbert Boyer had independently come to the same conclusion. The project got strong support from Rachmiel Levine, the Medical Director of the City of Hope, considered by many to be the father of modern diabetes research. Riggs and Itakura chose to first try the synthetic approach on a simpler peptide hormone, somatostatin, with only 14 amino acids. The project was rejected for funding by the NIH as unrealistic, but with Boyer's collaboration and Genentech's support, the effort to make somatostatin in *E. coli* was completed successfully in 1977 [34]. This represented the first synthesis of a functional polypeptide product from a gene of chemically synthesized origin. Then, in 1978, the City of Hope–Genentech team reported the successful chemical synthesis of separated genes for the A chain and B chain of human insulin [35], and in 1979 their successful expression in *E. coli* [36]. Synthetic genes for human insulin A and B chains were cloned separately in plasmid pBR322. The cloned synthetic genes were then fused to an *E. coli* β-galactosidase gene to provide efficient transcription and translation and a stable precursor protein. The insulin peptides were cleaved from β-galactosidase, detected by radioimmunoassay, and purified. Complete purification of the A chain and partial purification of the B chain were achieved. These products were mixed, reduced, and reoxidized. The presence of insulin was detected by radioimmunoassay.

This triumph launched the biotechnology industry. It also endeared Genentech with investors [28]. Some of the key players behind this historical milestone are shown in Figure 1.2. In this figure are David Goeddel, one of the first scientists hired by Genentech and who later became Genentech's research director, and Roberto Crea, who soon after the insulin project set up the DNA chemistry lab at Genentech.

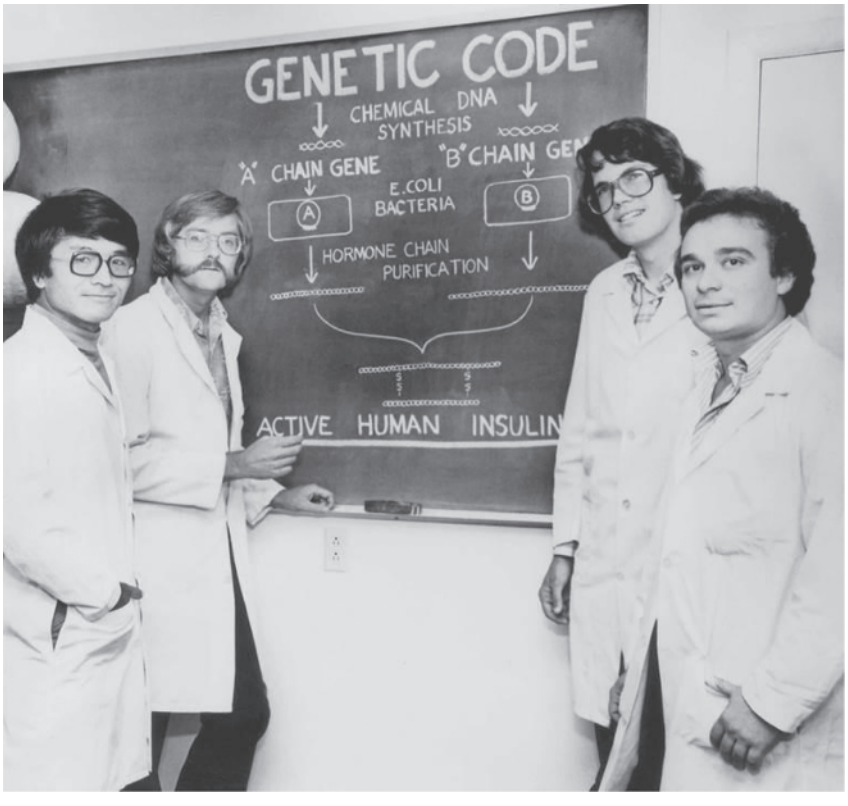

Figure 1.2 From left to right: Keiichi Itakura, Art Riggs, David Goeddel, and Roberto Crea. (Picture courtesy of City of Hope, used with permission of the scientists pictured.)

The process (Figure 1.3) was licensed by Genentech to Eli Lilly and Co. [38]. Crystals of the recombinant insulin are shown in Figure 1.4. In 1981, my group showed that the recombinant insulin had the same affinity for the insulin receptor as native insulin [39]. On October 29, 1982, recombinant human insulin was approved by the FDA and went on the market under the name Humulin. The two-chain recombination process was later replaced by the expression in *E. coli* of a gene for human proinsulin, which after expression and purification was then enzymatically converted to insulin [40]. More details about the precise processes can be found elsewhere [18, 41].

Retrospectively, aside from preventing an eventual shortage of animal insulins, it is not clear whether recombinant human insulin presented a significant therapeutic advantage over the highly purified mono-component porcine insulins that Novo had on the market since 1973 [42]. However, it paved the way for the entire biotechnology industry and cleared the hurdles for FDA approval of a recombinant protein therapeutic. Moreover, it also opened up the possibility to prepare recombinant insulin analogs with improved PK/PD properties [43].

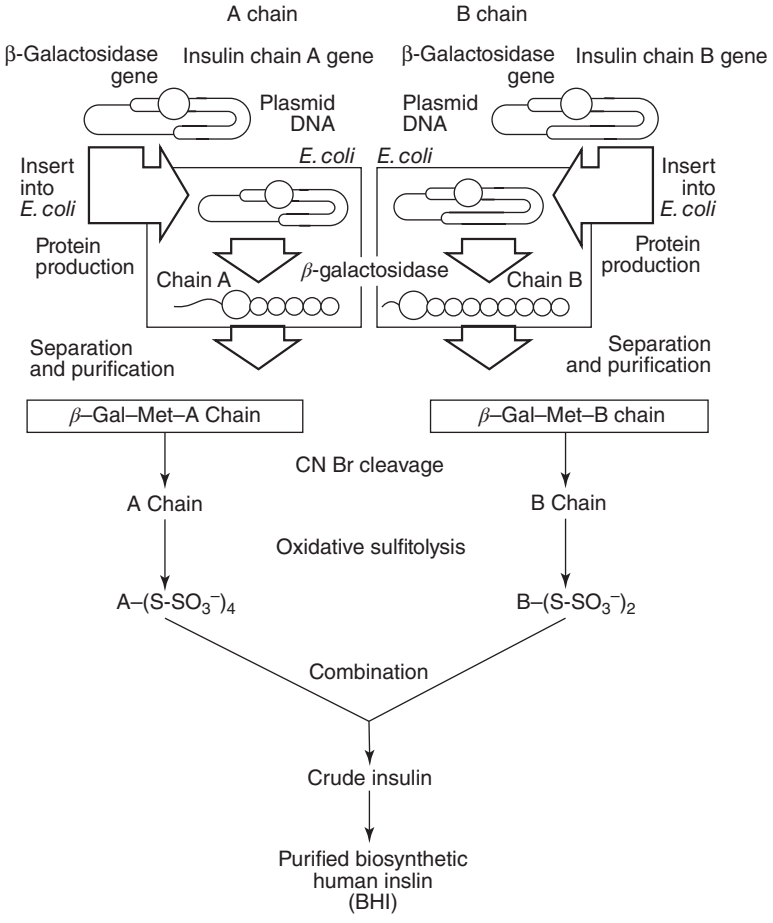

Figure 1.3 Production of insulin from separate genes encoding the A and B chains of human insulin. (From Ref. [37], used with permission. © E.P. Kroeff.)

1.3.2
Novo's Counter Strategy: Semisynthetic Human Insulin

The research management at the Danish company Novo, one of the main competitors to Eli Lilly, headed at the time by Jørgen Schlichtkrull, the father of highly purified mono-component porcine insulins [42], had been cautious of the whole DNA recombinant approach. Some of their chemists even referred to the bacterial insulin as "sewer insulin"! The prospect of a shortage of animal pancreata, which had motivated Lilly in the first place, did not appear to be an issue in a country that has 4.5 times more pigs than humans. However, when it became clear that Lilly was going soon to be on the market with a recombinant human insulin, it was too late to jump on the genetic engineering bandwagon. A quick strategy was needed to counter the risk of losing European market share to Humulin.

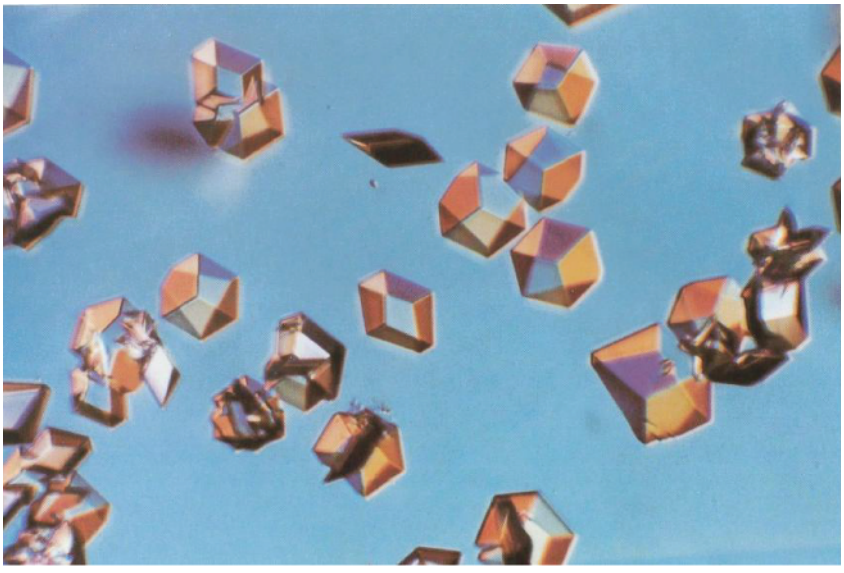

Figure 1.4 Zinc crystals of recombinant human insulin produced from the separate chains recombination process. (From Ref. [38], used with permission © Raven Press 1981.)

It had been known for some time that peptide bond formation can be enzymatically catalyzed by the reverse reaction of proteases (for review see Ref. [44]). Ken Inouye in Japan pioneered the enzymatic semi-synthesis of insulin analogs from desoctapeptide insulin [45]. Dietrich Brandenburg and Axel Wollmer were pioneers in insulin chemistry at the German Wool Research Institute in Aachen, where the total insulin synthesis had been previously accomplished by director Zahn in 1965 [11]. In 1979, Brandenburg and Wollmer organized the second International Insulin Symposium in Aachen, which was attended by the top insulin researchers in the world [46]. Jan Markussen, the leading insulin chemist from Novo was there. So was I.

Hans Gattner from Aachen presented how to make human insulin from porcine insulin by enzymatic semi-synthesis [47]. The only amino acid difference between the two is a Thr at the C-terminal position 30 of the B chain in human insulin replacing an Ala in porcine insulin. The semi-synthesis consisted in removing the Ala with carboxypeptidase from porcine insulin (extracted from pancreas) and using trypsin to replace with a Thr in a two-step process. A similar approach was published independently in 1979 by Morihara *et al.* [48]. Neither scientist patented their method.

Upon return to Denmark, Markussen applied for a patent to make a one-step trypsin transpeptidation of porcine insulin to human insulin [49]. The Aachen group published their similar one-step procedure in 1981 [50]. This process was easily scalable for industrial production, and Novo had its semisynthetic human insulin (Novolin) approved in Europe and on the market in early 1982, beating Eli

Lilly to the finish line by several months for their human insulin. The progress in insulin semi-synthesis is reviewed in Ref. [51].

Retrospectively, one could argue that this whole process of humanizing porcine insulin was a rather futile exercise, aside from the marketing strategy. The marketing of the time was focused on "Why use an insulin that is not your own"? In fact, the B30 amino acid has no influence on the biological activity of insulin, and modern insulin analogs prepared today in yeast lack entirely this B30 residue (the single-chain precursor can be cleaved at Lys B29 by the processing enzyme). Indeed, modern insulin analogs could therefore be considered both porcine and human.

1.3.3 Yeast Recombinant Insulin

In order to fill the expertise gap in DNA recombinant methods, Lars Thim, a young Novo protein chemist, and Niels P. Fiil, a seasoned microbiologist from the University of Copenhagen who had joined Novo in 1980, made contact in 1982 with Zymogenetics, a young biotech company based in Seattle, founded in 1981 by two scientists from the University of Washington, Earl W. Davie and Benjamin D. Hall, and by the late Michael Smith from the University of British Columbia, who became a Nobel Laureate in Chemistry in 1995. Zymogenetics was acquired by Novo in 1988, became independent again in 2000, and was acquired by Bristol Myers Squibb in 2010.

In collaboration with Zymogenetics scientists, Thim established a method for the secretion and processing of dibasic insulin precursors in the yeast *Saccharomyces cerevisiae* [52]. Proinsulin in the pancreas is processed to insulin by a trypsin/carboxypeptidase B-like enzyme system [53]. *S. cerevisiae* possesses a similar enzymatic system (*KEX2*) [54] within the secretory compartments to process the pheromone mating factor α1 (MFα1) gene product into a peptide with 13 amino acid residues [55]. The construct used for yeast expression consisted of a double-stranded human preproinsulin cDNA cloned from human pancreas, fused to MFα1, and inserted within the triose phosphate isomerase gene. Together, these fragments provide sequences that ensure a high rate of transcription for the insulin precursor-encoding genes and also provide presequences that direct the insulin precursors into the yeast secretory pathway and lead to the secretion of the expression products into the growth medium [52]. The entire expression unit was inserted into the plasmid CPOT [52] (Figure 1.5).

The purified secretion product was converted to insulin by trypsin and carboxypeptidase B [52].

Novo and Nordisk merged in 1989 as Novo Nordisk, ending 66 years of competition. The Novo Nordisk yeast DNA recombinant insulin was approved and on the market in 1991 under the name Novolin.

And what about Hoechst (today Sanofi), the other major player in the insulin field? They had also missed the nascent genetic engineering revolution. In order to catch up, they made a deal in 1981 with Howard M. Goodman who had by then

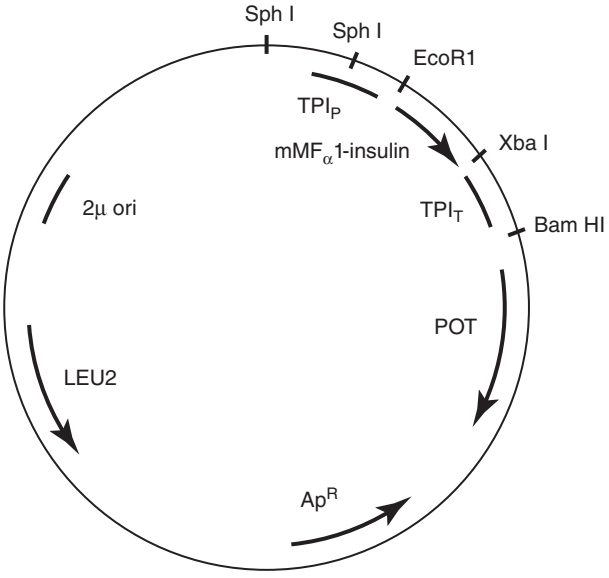

Figure 1.5 Yeast expression plasmid for dibasic insulin precursors. (From Ref. [52], used with permission © PNAS 1986.)

moved to Massachusetts General Hospital in Boston (part of Harvard Medical School), giving him a US$ 70 million unrestricted research grant over 10 years, with the understanding that he would train some Hoechst scientists and that Hoechst would get licensing rights from discoveries. However, there was a level of general uncertainty within German public opinion around genetically modified organisms, and coupled with efficient opposition from Green parties, Hoechst's *E. coli*-based DNA recombinant insulin (Insuman) was significantly delayed and did not start production and achieve approval until 1998 [56].

1.4 Recombinant Human Growth Hormone

Up to 1985, children with growth hormone deficiency (dwarfism or small for gestational age) were treated with the growth hormone (somatropin) extracted from the pituitary glands of human cadavers. This led to over 160 cases of the prion-caused Creutzfeldt–Jakob disease, which has a mean incubation time of 12 years but may not be obvious until 30 years after contamination. This gave some urgency to ensure the availability of DNA recombinant growth hormone. Growth hormone is a much larger peptide than insulin (191 compared to 51 amino acids), so it was not possible to manufacture it via the complete DNA synthesis route that worked so well for insulin. However, unlike insulin, it is a linear peptide that does not require complex processing of a pro form to a two-chain form, so it did prove amenable to direct expression in *E. coli.*

Growth hormone became a focus of research in Howard Goodman's lab at UCSF in the late 1970s, where Axel Ullrich was still struggling to express a cloned

insulin gene in bacteria [28]. A German post-doctoral scientist, Peter Seeburg, together with John Shine from Australia (and Joseph Martial from Belgium, and John D. Baxter from the United States from a separate department), determined the sequence of the rat growth hormone gene in 1977 and demonstrated that it could be expressed in bacteria [57, 58]. The cDNA sequence of the human growth hormone RNA was subsequently determined [59], and the gene (GH1) was shown to be part of a cluster of five genes [60]. In August 1978, Howard Goodman reported the successful expression of the human growth hormone gene in *E. coli* at a Benson Symposium in Copenhagen, with Peter Seeburg, Joseph Martial, John Shine, Axel Ullrich, and John D. Baxter as coauthors [59].

In 1978, Peter Seeburg and Axel Ullrich moved to Genentech after a difference of opinion with Goodman [28]. In October 1979, a Genentech team led by David Goeddel and Peter Seeburg, and in collaboration with Itakura and colleagues at the City of Hope, reported in *Nature* the successful expression in *E. coli* of a hybrid native and synthetic DNA sequence coding for human growth hormone ([61], Figure 1.6). The path to the recombinant human growth hormone has been reviewed by both Seeburg and by the UCSF team [63, 64].

The recombinant growth hormone was approved by the FDA in 1985 as Protropin, and later as Nutropin. The research was funded by the Swedish company

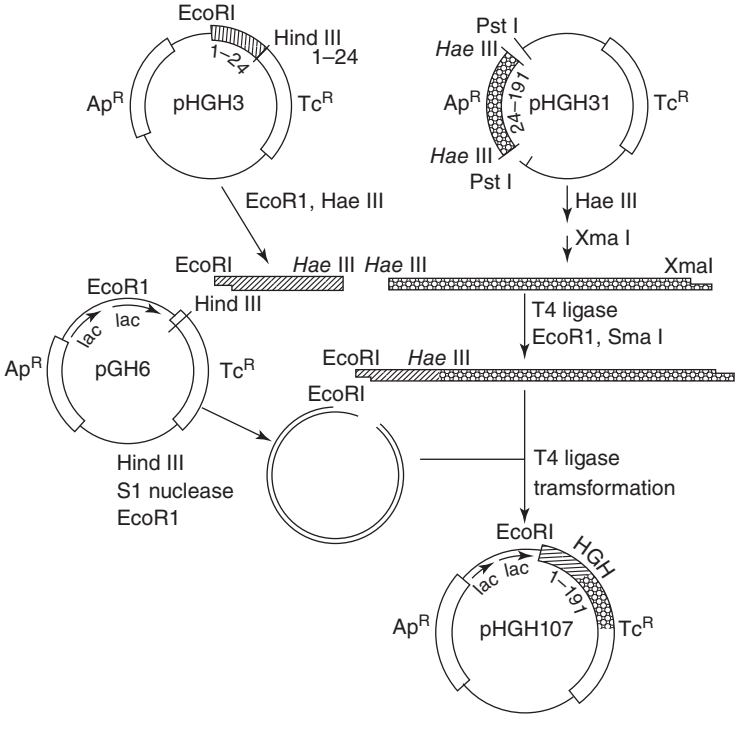

Figure 1.6 Assembly of the plasmid containing the human growth hormone gene. For detailed explanation, see Ref. [61]. (From Ref. [62], used with permission, © G. Miozzari and Walters Kluwer.)

AB Kabi, and both Genentech and Kabi (later acquired by Pharmacia followed by multiple mergers) received co-marketing rights.

Human growth hormone became the first major product directly marketed by Genentech. This success story became somewhat overshadowed when it became apparent that Peter Seeburg had taken to Genentech aliquots from his human growth hormone clones from UCSF without permission from the lab chief or the University, and may have used them for the process to make the recombinant growth hormone [28]. By then Seeburg was Director of Molecular Neuroscience at the Max Planck Institute in Germany. He was accused of making up one of the plasmids described in the 1979 *Nature* paper to hide the supposed theft, which he acknowledged (calling it a "technical inaccuracy") in the trial for patent infringement that ensued between UCSF and Genentech [65]. David Goeddel and the other coauthors have strongly denied Seeburg's allegations that the experiments were not conducted exactly as described in the *Nature* paper (see Ref. [66] for both Goeddel's and Seeburg's comments).

In any case, in 1999 Genentech settled for a $200 million payment to UCSF, of which $85 million went to Seeburg, Shine, Goodman, Baxter and Martial [65].

In 1985, Nordisk Insulin Laboratorium in Denmark (who had been selling human pituitary-extracted growth hormone since 1973 under the name Nanonorm) came up with its own version of bacterial recombinant growth hormone, under the name Norditropin, approved in Europe and Japan in 1988 and by the FDA in 1995 (by then Nordisk had merged with Novo).

1.5 Recombinant Human Interferons

In parallel to the race to produce recombinant human insulin with the involvement of Genentech as narrated above, another race at the end of the 1970s was in place for the production of recombinant human interferon, with heavy involvement of another biotech start-up, Biogen, and some of the same key players as in the insulin and growth hormone sagas. The narrative that follows is based on the lively and candid account of Weissmann [67] as well as other sources.

Interferon, a substance that protected cells from viral infection, was discovered and named in 1957 [68] by Alick Isaacs and Jean Lindenmann at the National Institute of Medical Research in London. This was the first of a class of molecules known as cytokines.

Interferons are proteins with antiviral activity that are produced from cells in response to a variety of stimuli [69]. They turned out to be a highly complex class of multifunctional proteins encoded by over 20 different genes in animals including humans (for contemporary reviews see Refs [70,71]).

In the summer of 1977 at a Gordon Conference, Charles Weissmann, then director of the Institute for Molecular Biology at the University of Zurich, and Peter Lenguyel, Professor of Molecular Biophysics and Biochemistry at Yale University [72, 73], decided to collaborate to clone mouse interferon, for which Lenguyel had a sensitive assay [67, 73]. Weissman and Lenguyel were both born in Hungary 2

years apart and became friends while working together in Nobel Laureate Severo Ochoa's lab at New York University [73]. Cloning interferon was a huge challenge because at the time no sequence information was available, and it was not appreciated that it consisted of multiple proteins. Moreover, this very potent substance was produced in very tiny amounts by induced cells, such as leukocytes or fibroblasts.

In 1977, and again in 1978, Charles Weissmann was approached by two venture capitalists from Inco, a company that had invested in the early days of Genentech [67], with a proposal to create a similar biotech venture in Europe, to be called Biogen. In 1978, they organized the "First European Microbiology Conference" in Geneva, with a roster of prominent European molecular biologists as prospective members of Biogen's Scientific Board, plus the future Nobel Laureate Walter Gilbert of Harvard University (already mentioned in the insulin saga) and Phillip Allen Sharp, then Director of the Massachusetts Institute of Technology (MIT) Center for Cancer Research (who would share in 1993 the Nobel Prize in Physiology or Medicine with Richard J. Roberts for the discovery of mRNA splicing). Biogen was thus founded.

Weissmann was commissioned by the Scientific Board to clone human IFN cDNA, a move promoted by the prospect of interferon being not only an antiviral agent but also potentially an antitumor agent [67, 71]. Weissmann made a partnership with Kari Cantell, a virologist from the Central Public Health Laboratory in Helsinki who was able to produce about a gram of crude IFN per year from leukocytes from 90 000 blood donors from the Finnish Red Cross [67, 74].

In 1979, Walter Gilbert became the CEO of Biogen, and the Board attracted support from Schering Plough, with an investment that allowed the creation of a Biogen laboratory in Geneva. At Christmas the same year, the Weissmann team had established conclusive evidence that they had cloned and expressed human leukocyte interferon (later known as IFNα) in *E. coli.* Weissmann announced the results in a seminar at MIT on January 15, 1980, and then at a highly publicized press conference [75], which raised questions about the then rather untested relationships between university labs and commercial companies [67]. The article was published in *Nature* on March 27, 1980 [76]. Six months later, David Goeddel and colleagues at Genentech, in collaboration with the group of Stanley Pestka at the Roche Institute of Molecular Biology (a leading interferon researcher), also reported the production of leukocyte human interferon in *E. coli,* but this time in sufficient amounts to demonstrate *in vivo* antiviral activity in monkeys [77]. In September 1980, Tadatsugu Taniguchi, a former postdoc of Weissmann now back in Japan, reported the cloning and expression of the human fibroblast interferon gene (later known as IFNβ) in *E. coli* [78]. He is now a member of the National Academy of Sciences, USA. In 1982, Goeddel and colleagues at Genentech reported the cloning and expression of IFNγ in *E. coli* [79].

From then on, the research, both academic and commercial, on interferons progressed at a fast pace (see Refs [67, 70, 71]).

Recombinant human IFN α2b interferon from Schering-Plough (licensed from Biogen) and from Hoffmann-La Roche (licensed from Genentech) was approved

by the US FDA in 1986 for the treatment of hairy cell leukemia. The further evolution of Biogen has been well documented [67]. The company went public in 1983 and merged with the San Diego-based IDEC pharmaceuticals. It went through major reorganizations, moving to Cambridge, MA, USA, and divesting the Geneva, Zurich, and Ghent labs. Walter Gilbert was replaced as CEO in December 1984 and Charles Weissmann resigned in 1988. Biogen is today a multi-billion dollar company. A dozen different interferon preparations from several companies have now been approved by the FDA for various indications including cancers, multiple sclerosis, and chronic hepatitis C infection.

1.6 Recombinant Human Erythropoietin

Erythropoietin (EPO) is a glycoprotein cytokine/hormone/growth factor principally produced in the kidney and controls red blood cell production in the bone marrow. For a good overview, see the excellent review of Jelkmann from the University of Lübeck [79]. EPO was the first of the hematopoietic growth factors – a group that includes thrombopoietin, granulocyte-colony stimulating factor (GCSF), and granulocyte-macrophage colony-stimulating factor (GMCSF) – to be characterized.

Its existence was first suggested 110 years ago by Paul Carnot, Professor of Medicine at the Sorbonne in Paris, France, and his coworker Clotilde Deflandre [80, 81]. Although the experimental data are now believed to be an artifact [79], the hypothesis they generated regarding the existence of a circulating hemopoietic substance proved to be correct and influential. This hemopoietic substance was named EPO by Bonsdorff and Jalavisto at the Institute of Physiology of the University of Helsinki in 1948 [82]. It took another 30 years to finally be able to isolate and purify it. This was done by the group of Eugene Goldwasser in the Department of Biochemistry at the University of Chicago [83], starting from 2550 l of urine from Japanese patients with aplastic anemia collected by Takaji Miyake from Kumamoto University, generating 8 mg of protein. Goldwasser (who died in 2010 at age 88) has written a nice personal account of the struggle to purify, clone, and express EPO [84]. He and the University of Chicago did not patent EPO, and neither made a cent from what became a blockbuster drug [85].

Enter Amgen. Initially called Applied Molecular Genetics, the biotech company Amgen (in Thousand Oaks, California) was founded on April 8, 1980, by the venture capitalist William K. Bowers and associates, and went public in 1983. The first CEO was one of the co-founders, George B. Rathman, a scientist–businessman. They partnered with Goldwasser's group and obtained the purified EPO. Amgen scientists Fu-Kuen Lin from Taiwan and colleagues managed to clone the EPO gene and express it in Chinese hamster ovary (CHO) cells [86]. Amgen had naturally patented EPO. The article came out in the *Proceedings of the National Academy of Sciences* (PNAS) in October 1985 [86].

However, the Amgen/Goldwasser teams were scooped with respect to publication by Takaji Miyake (now at Wright University in Dayton, Ohio), who had made

an alliance with a competing biotech company, Genetics Institute Inc. in Boston, founded in 1980 by two leading molecular biologists from Harvard, Thomas Maniatis and Marc Ptashne. Miyake provided Genetics Institute with the purified EPO he had made with Goldwasser, and ended up as last author on the paper that came out in *Nature* on February 28, 1985 [87], reporting the cloning and expression of human EPO in COS cells, 9 months ahead of the Amgen article. The project was supported by Chugai Pharmaceuticals in Japan. But Amgen had patented first. This resulted in protracted patent litigation, ultimately won by Amgen. Amgen's EPO was approved by the FDA on June 1, 1989, and produced under the name Epogen. Shortly afterward, Amgen (then associated with Japan's Kirin Breweries) cloned and produced GCSF [88], which was approved by the FDA in February 1991 as Neupogen. The Genetics Institute was subsequently (1992–1996) absorbed by Wyeth (then American Home Products).

1.7
Recombinant Tissue – Type Plasminogen Activator

Tissue-type plasminogen activator (t-PA) is a serine protease normally present on endothelial cells, which is therapeutically used for thrombolysis, the pharmacological dissolution of a blood clot by the IV infusion of plasminogen activators that activate the fibrinolytic system (Figure 1.7, see Refs [89, 90] for reviews). Thrombolysis by t-PA has become the staple of the treatment of acute myocardial infarction [90].

t-PA was originally purified and characterized in 1981 by Désiré Collen from the Katholieke Universiteit Leuven in Belgium from human melanoma cells in culture [91]. t-PA was cloned and successfully expressed, in collaboration between Collen and Diane Pennica and colleagues at Genentech, in both *E. coli* [92] and mammalian CHO cells [93]. This project stimulated another important

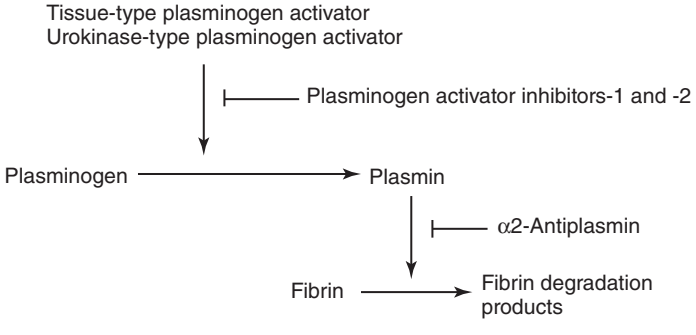

Figure 1.7 The fibrinolytic system. The proenzyme plasminogen is activated to the active enzyme plasmin by tissue-type or urokinase-type plasminogen activator. Plasmin degrades fibrin into soluble fibrin degradation products. Inhibition of the fibrinolytic system may occur through plasminogen activators, by plasminogen activator inhibitors, or more directly via plasmin, mainly by α2-antiplasmin. (Adapted from Ref. [90].)

biotechnological development, that of large-scale tissue-culture fermentation and purification procedures [94], critical for recombinant proteins that require specific biological processes such as glycosylation, which cannot be obtained in unicellular organisms like *E. coli* or yeast.

Genentech's CHO-produced t-PA (Activase or alteplase), the first of the early recombinant protein therapeutics not produced in unicellular organisms, was approved by the FDA in 1987 for acute myocardial infarction and later for acute ischemic stroke and pulmonary embolism.

Désiré Collen received several major scientific awards for his work on t-PA, including the Belgian Francqui Prize in 1984, the Louis-Jeantet Prize for Medicine in 1985, and the Interbrew-Baillet Latour Prize in 2005.

1.8 Recombinant Hepatitis B Virus (HBV) Vaccine

The very first FDA-approved recombinant vaccine for human use was developed by Chiron Corporation, a US West Coast biotech company (based in Emeryville, CA) founded in 1981 by three academic scientists from the University of California, William (Bill) J. Rutter (chairman), already mentioned above in the insulin saga, Edward Penhoet, and Pablo DT Valenzuela. It went public in 1983. They were active in the areas of biopharmaceuticals, vaccines, and blood tests. Based on their research [94–96], they managed to express hepatitis B surface and core antigens in *E. coli* [96]. This became the basis of a vaccine licensed to Merck and Co. and approved by the FDA in July 1986 as Recombivax.

Chiron was acquired by Novartis AG from Switzerland in April 2006.

1.9 Postscript

In this introductory chapter, I have not attempted to provide an encyclopedic description of the processes involved in producing the first DNA recombinant therapeutic proteins, nor of their biological properties and all therapeutic applications that developed after the initial FDA approval. I have focused on the period 1972–1985, which saw the transition from basic molecular biology to industrial biotechnology, trying to give a feeling for the challenges of the time, especially in terms of the sometimes delicate relationships between academic and industry-sponsored research, and the fierce competition that developed between the leading groups involved, which was in fact a major contributor to success. This melting pot in the end broke down barriers between academia and industry, making it much easier to transit between the two. I should also make it clear that I have in no way attempted to give a complete picture of the scientific accomplishments of the scientific protagonists involved in this story, most of whom went on to brilliant careers in both academia and biotech, and became some of the most influential scientists of the last decades.

Acknowledgments

I would like to dedicate this essay to the memory of Ronald E. Chance of Eli Lilly and Co., who sadly passed away in 2015. Ron was the driving force (with Bruce Frank and colleagues) in bringing recombinant human insulin to the market, and transforming the perspectives of the whole pharmaceutical industry in the process. I fondly remember our collaboration in the early days preceding the launch of Humulin, and many years of friendly interactions afterward.

I also would like to acknowledge many enlightening interactions over the years with many of the scientists cited in this chapter: in particular, Arthur Riggs, Keiichi Itakura, and Rachmiel Levine during the 5 years I spent at City of Hope, and Jan Markussen and colleagues during my 20 years at the Hagedorn Research Institute at Novo Nordisk in Denmark. Axel Ullrich kindly served on the advisory board of the Hagedorn Research Institute for several years. I also had a nice collaboration with Jim Wells at Genentech on growth hormone. I am grateful to Arthur Riggs, Keiichi Itakura, and Yoko Fujita-Yamaguchi for a critical reading of the manuscript.

References

1 Banting, F.G. and Best, C.H. (1922) The internal secretion of the pancreas. *J. Lab. Clin. Med.*, **7**, 251–256.
2 Banting, F.G., Best, C.H., Collip, J.B., Campbell, W.R., and Fletcher, A.A. (1922) Pancreatic extracts in the treatment of diabetes mellitus: preliminary report. *Can. Med. Assoc. J.*, **12**, 141–146.
3 Banting, F.G., Campbell, W.R., and Fletcher, A.A. (1923) Further experience with insulin in the treatment of diabetes mellitus. *BMJ*, **1**, 8–12.
4 Bliss, M. (2007) *The Discovery of Insulin. 25th Anniversary Edition*, The University of Chicago Press.
5 Allen, F. (1915) Prolonged fasting in diabetes. *Am. J. Med. Sci.*, **150**, 480–485.
6 Allen, F., Stillman, E., and Fitz, R. (1919) *Total Dietary Regulation in the Treatment of Diabetes*, The Rockefeller Institute for Medical Research, New York.
7 Mazur, A. (2011) Why were "starvation diets" promoted for diabetes in the pre-insulin period. *Nutr. J.*, **10**, 23.
8 Ryle, A.P., Sanger, F., Smith, L.F., and Kitai, R. (1955) The disulphide bonds of insulin. *Biochem. J*, **60**, 541–546.
9 Nicol, D.S. and Smith, L.F. (1960) Amino-acid sequence of human insulin. *Nature*, **187**, 483–485.
10 Katsoyannis, P.G. (1964) The synthesis of the insulin chains and their combination to biologically active material. *Diabetes*, **13**, 339–348.
11 Zahn, H., Brinkhoff, O., Meienhofer, J., Pfeiffer, E.F., Ditschuneit, H., and Gloxhuber, C. (1965) Combining of synthetic insulin chains into biologically active preparations (in German). *Z. Naturforsch. B*, **20**, 666–670.
12 Kung, Y.T., Huang, W.T., Chen, C.C., and Ke, L.T. (1966) Total synthesis of crystalline insulin. *Sci. Sin.*, **15**, 544–561.
13 McElhiny, V.K. (1966) Total synthesis of insulin in Red China. *Science*, **153**, 281–283.
14 Sieber, P., Kamber, B., Hartmann, A., Johl, A., Riniker, B., and Rittel, W. (1974) Totalsynthese von Humaninsulin unter gezielter Bildung der Disulfidbindungen. *Helv. Chim. Acta*, **57**, 2617–2621.
15 Yalow, R.S. and Berson, S.A. (1960) Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.*, **39**, 1157–1175.

16 Adams, M.J., Blundell, T.L., Dodson, E.J., Dodson, G.G., Vijayan, M., Baker, E.N., Harding, M.M., Hodgkin, D.C., Rimmer, B., and Sheat, S. (1969) Structure of rhombohedral 2 zinc insulin crystals. *Nature*, **224**, 491–495.

17 Blundell, T., Dodson, G., Hodgkin, D., and Mercola, D. (1972) Insulin: the structure in the crystal and its reflection in chemistry and biology. *Adv. Prot. Chem.*, **26**, 279–402.

18 Owens, D.R., Vora, J.P., and Dolben, J. (1991) Human insulin and beyond: semi-synthesis and recombinant DNA technology reviewed, in *Biotechnology of Insulin Therapy. Frontiers in Pharmacology and Therapeutics* (ed. J.C. Pickup), Blackwell Scientific Publications, Oxford, London, Edinburgh, Boston, MA, Melbourne, Paris, Berlin, Vienna, pp. 24–41.

19 Leader, B., Baca, Q.J., and Golan, D.E. (2008) Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discovery*, **7**, 21–39.

20 Baca, Q.J., Leader, B., and Golan, D.E. (2016) Protein therapeutics, in *Principles of Pharmacology. The Pathophysiologic Basis of Drug Therapy*, 4th edn (eds D.E. Golan, E.J. Armstrong, and A.W. Armstrong), Lippincott Williams and Wilkins, Philadelphia, PA, pp. 955–978.

21 Watson, J.D. and Crick, F.H.C. (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*, **171**, 737–738.

22 Watson, J.D. and Crick, F.H.C. (1953) Genetical implications of the structure of deoxyribonucleic acid. *Nature*, **171**, 964–967.

23 Jackson, D.A., Symons, R.H., and Berg, P. (1972) Biochemical method for inserting new genetic information into DNA of simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2904–2909.

24 Berg, P. (1980) Dissections and Reconstructions of Genes and Chromosomes, Nobel lecture, www.nobelprize.org (accessed 17 May 2016).

25 Cohen, S.N., Chang, A.C., Boyer, H.W., and Helling, R.B. (1973) Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3240–3244.

26 Morrow, J.F., Cohen, S.F., Chang, H.C., Boyer, H.W., Goodman, H.M., and Helling, R.B. (1974) Replication and transcription of eukaryotic DNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1743–1747.

27 Berg, P., Baltimore, D., Boyer, H.W., Cohen, S.N., Davis, R.W., Hogness, D.S., Nathans, D., Rohn, R., Watson, J.D., Weissman, S., and Zinder, N.D. (1974) Potential biohazards of recombinant DNA molecules. *Science*, **185**, 303.

28 Hall, S.S. (1987) *Invisible Frontiers: The Race to Synthesize a Human Gene*, The Atlantic Monthly Press, New York, p. 334.

29 Health Trust Digital Library (1978) *Report of the National Diabetes Advisory Board. A Study of Insulin Supply and Demand*. Publication No 78-1588, , US Dept 06, Health, Education and Welfare, Washington, DC.

30 Steiner, D.F., Cunningham, D., Spigelman, L., and Aten, B. (1967) Insulin biosynthesis: evidence for a precursor. *Science*, **157**, 697–700.

31 Ullrich, A., Shine, J., Chigwin, J., Pictet, R., Tischer, E., Rutter, W.J., and Goodman, H.M. (1977) Rat insulin genes: construction of plasmids containing the coding sequences. *Science*, **196**, 1313–1319.

32 Bell, G.I., Pictet, R.L., Rutter, W.J., Cordell, B., Tischer, E., and Goodman, H.M. (1980) Sequence of the human insulin gene. *Nature*, **284**, 26–32.

33 Talmadge, K., Kaufman, J., and Gilbert, W. (1980) Bacteria mature preproinsulin to proinsulin. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 3988–3992.

34 Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heynecker, H.L., Bolivar, F., and Boyer, H.W. (1977) Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science*, **198**, 1056–1063.

35 Crea, R., Krasjewski, A., Hirose, T., and Itakura, K. (1978) Chemical synthesis of genes for human insulin. *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5765–5769.

36 Goeddel, D.V., Kleid, D.G., Bolivar, F., Heynecker, H.L., Yansura, D.G., Crea, R., Hirose, T., Krasjewski, A., Itakura, K., and Riggs, A.D. (1979) Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 106–110.

37 Chance, R.E., Kroeff, E.P., Hoffmann, J.A., and Frank, B.H. (1981) Chemical, physical and biologic properties of biosynthetic human insulin. *Diabetes Care*, **4**, 147–154.

38 Chance, R.E., Kroeff, E.P., and Hoffman, J.A. (1981) Chemical, physical and biological properties of recombinant human insulin, in *Insulins, Growth Hormone and Recombinant DNA Technology* (ed. J.L. Gueriguian), Raven Press, New York, pp. 71–86.

39 Keefer, L.M., Piron, M.A., and De Meyts, P. (1981) Human insulin prepared by recombinant DNA techniques and native human insulin interact identically with insulin receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 1391–1395.

40 Frank, B.H., Pettee, J.M., Zimmermann, R.E., and Burck, P.J. (1982) The production of human proinsulin and its transformation to human insulin and C-peptide, in *Peptides: Synthesis-Structure-Function*, Proceedings of the Seventh American Peptide Symposium, (eds D.H. Rich and E. Gross), Pierce Chemical Company, Rockford, IL, pp. 729–738.

41 Gueriguian, J.L. (1981) *Insulins, Growth Hormone and Recombinant DNA Technology*, Raven Press, New York.

42 Schlichtkrull, J., Brange, J., Christiansen, A.H., Hallund, O., Heding, L.G., Jørgensen, K.H., Munkgaard Rasmussen, S., Sørensen, E., and Vølund, A. (1974) Monocomponent insulin and its clinical implications. *Horm. Metab. Res. (Suppl. Ser.)*, **5**, 134–143.

43 Brange, J., Ribel, U., Dodson, G., Hansen, M.T., Havelund, S., Melberg, S.G., Norris, F., Norris, K., Snel, L., Sørensen, A.R., and Voigt, H.O. (1988) Monomeric insulins obtained by protein engineering and their medical implications. *Nature*, **333**, 679–682.

44 Chaiken, I.M. (1981) Semisynthetic peptides and proteins. *CRC Crit. Rev. Biochem.*, **11**, 255–301.

45 Inouye, K., Watanabe, K., Morihara, K., Tochino, Y., Kanaya, T., Emura, J., and Sakakibara, S. (1979) Enzyme-assisted semi-synthesis of human insulin. *J. Am. Chem. Soc.*, **101**, 751–752.

46 Brandenburg, D. and Wollmer, A. (1980) *Proceedings 2nd International Insulin Symposium*, Walter De Gruyter, Aachen.

47 Gattner, H.G., Danho, W., and Naithani, V.K. (1980) Enzyme-catalysed semisynthesis with insulin derivatives, in *Proceedings of the 2nd International Insulin Symposium* (eds D. Brandenburg and A. Wollmer), Walter De Gruyter, Aachen, pp. 117–123.

48 Morihara, K., Oka, T., and Tsusuki, H. (1979) Semi-synthesis of human insulin by trypsin-catalysed replacement of Ala-B30 by Thr in porcine insulin. *Nature*, **280**, 412–413.

49 Markussen, J. (1980) Process for preparing insulin esters. UK Patent Application GB 2069502 A.

50 Jonczyk, A. and Gattner, H.G. (1981) A new semi-synthesis of human insulin: trypsin transpeptidation of porcine insulin with L-threonine tert-butyl ester. *Hoppe Seyler's Z. Physiol. Chem.*, **362**, 1591–1598.

51 Morihara, K. (1990) Enzymatic semisynthesis of insulin: an update. *J. Mol. Recogn.*, **3**, 181–186.

52 Thim, L., Hansen, M.T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G., and Fiil, N.P. (1986) Secretion and processing of insulin precursors in yeast. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6766–6770.

53 Steiner, D.F., Docherty, K., and Carroll, R. (1984) Golgi/granule processing of peptide hormone and neuropeptide precursors: a minireview. *J. Cell. Biochem.*, **24**, 121–130.

54 Thorner, J. (1985) Pheromone-processing proteases of the yeast *Saccharomyces cerevisiae*. *Nature*, **314**, 384–385.

55 Stötzler, D., Kiltz, H.H., and Duntze, W. (1976) Isolation of four related peptides exhibiting alpha factor activity from *Saccharomyces cerevisiae*. *Eur. J. Biochem.*, **69**, 397–400.

56 Peerenboom, E. (1998) Hoechst production of recombinant human insulin finally begins after 14-year battle. *Nat. Biotechnol.*, **16**, 409.

57 Seeburg, P.H., Shine, J., Martial, J.A., Baxter, J.D., and Goodman, H.M. (1977) Nucleotide sequence and amplification in bacteria of structural genes for rat growth hormone. *Nature*, **270**, 486–494.

58 Seeburg, P.H., Shine, J., Martial, J.A., Ivarie, R.D., Morris, J.A., Ullrich, A., Baxter, J.D., and Goodman, H.M. (1978) Synthesis of growth hormone by bacteria. *Nature*, **276**, 795–798.

59 Goodman, H.M., Seeburg, P., Martial, J.A., Shine, J., Ullrich, A., and Baxter, J.D. (1979) Structure and expression in bacteria of growth hormone genes, in *Specific Eukaryotic Genes* (eds J. Engberg, H. Knurow, and V. Leick), Munskgaard, Copenhagen, pp. 179–190.

60 Barsh, G.S., Seeburg, P.H., and Gelinas, R.E. (1983) The human growth hormone gene family: structure and evolution of the chromosomal locus. *Nucleic Acids Res.*, **11**, 3939–3958.

61 Goeddel, D.V., Heynecker, H.L., Hozumi, T., Aretzen, R., Itakura, K., Yauzara, D.G., Ross, M.J., Miozzari, G., Crea, R., and Seeburg, P.H. (1979) Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature*, **281**, 544–548.

62 Miozzari, G.F. (1981) Strategies for obtaining expression of peptide hormones in *E. coli*, in *Insulins, Growth Hormone and Recombinant DNA Technology* (ed. J.L. Gueriguian), Raven Press, New York, pp. 13–48.

63 Seeburg, P.H. (1986) Human growth hormone: from clone to clinic. *Cold Spring Harbor Symp. Quant. Biol.*, **51** (Part 1), 669–677.

64 Martial, J.A., Hallevell, R.A., Baxter, J.D., and Goodman, H.M. (1992) Human growth hormone:- complementary DNA cloning and expression in bacteria. *Biotechnology*, **24**, 293–298.

65 Dalton, R. and Schiermeyer, Q. (1999) Genentech pays 200 million dollars over growth hormone "theft". *Nature*, **402**, 335.

66 Henner, D., Goeddel, D.V., Heynecker, H., Itakura, K., Yausura, D., Ross, M., Miozzari, G., and Seeburg, P.H. (1999) UC-genentech trial. *Science New Series*, **284**, 1455–1456.

67 Weissmann, C. (2001) Recombinant interferon – the 20th anniversary, in *Recombinant Protein Drugs: Milestones in Drug Therapy* (eds P. Buckel, M.J. Parnham, and J. Bruinvels), Series, Birkhaûser Verlag, Basel, Boston, MA Berlin, pp. 3–41.

68 Isaacs, A. and Lindenmann, J. (1957) Virus interference. 1. The interferon. *Proc. R. Soc. London, Ser. B*, **147**, 258–267.

69 Pestka, S., Langer, J.A., Zoon, K.C., and Samuel, C.E. (1987) Interferons and their actions. *Annu. Rev. Biochem.*, **56**, 727–777.

70 Pestka, S., Krause, C.D., and Walter, M.R. (2004) Interferons, interferon-like cytokines, and their receptors. *Immunol. Rev.*, **202**, 8–32.

71 Dunn, G.P., Koebel, C.M., and Schreiber, R.D. (2006) Interferons, immunity and cancer immunoediting. *Nat. Rev. Immunol.*, **6**, 836–848.

72 Lenguyel, P. (1982) Biochemistry of interferons and their actions. *Annu. Rev. Biochem.*, **31**, 251–282.

73 Lenguyel, P. (2014) Wanderings in biochemistry. *J. Biol. Chem.*, **289**, 19254–19258.

74 Cantell, K. (1998) *The Story of Interferon. The Ups and Downs in the Life of a Scientist*, World Scientific, London.

75 Wade, N. (1980) Cloning gold rush turns basic biology into big business. *Science*, **207**, 507.

76 Nagata, S., Taira, H., Hall, A., Johnsrud, L., Streuli, M., Ecsodi, J., Boll, W., Cantell, K., and Weissmann, C. (1980) Synthesis in *E. coli* of a polypeptide with human leukocyte interferon activity. *Nature*, **284**, 316–320.

77 Goeddel, D.V., Yelverton, E., Ullrich, A., Heynecker, H.L., Miozzari, E., Holmes, W., Seeburg, P.H., Dull, T., May, L., Stebbing, L., Crea, R., Maeda, S., McCandliss, R., Sloma, A., Tator, J.M., Gross, M., Fanilletti, P.L., and Pestka, S. (1980) Human leukocyte interferon produced by *E. coli* is biologically active. *Nature*, **287**, 411–416.

78 Taniguchi, T., Guarente, L., Roberts, T.M., Kimelman, D., Douhan, J. 3rd, and Ptashne, M. (1980) Expression of the human fibroblast interferon gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 5230–5233.

79 Jelkmann, W. (2007) Erythropoietin after a century of research: younger than ever. *Eur. J. Haematol.*, **78**, 183–205.

80 Carnot, P. and Deflandre, C. (1906) Sur l'activité hémopoietique du sérum au cours de la régénération du sang. *C. R Acad. Sci. Paris*, **143**, 384–386.

81 Carnot, P. and Deflandre, C. (1906) Sur l'activité hémopoiétique des différents organes au courrs de la régénération du sang. *C. R. Acad. Sci. Paris*, **143**, 432–435.

82 Bonsdorff, E. and Jalavisto, E. (1948) A humoral mechanism in anoxic erythrocytosis. *Acta Physiol. Scand.*, **16**, 150–170.

83 Miyake, T., Kung, C.K., and Goldwasser, E. (1977) Purification of human erythropoietin. *J. Biol. Chem.*, **252**, 558–5564.

84 Goldwasser, E. (1996) Erythropoietin: a somewhat personal history. *Perspect. Biol. Med.*, **40**, 18–31.

85 Goozner, M. (2004) *The 800 Million Dollars Pill. The Truth Behind the Cost of New Drugs*, University of California Press.

86 Lin, F.K., Suggs, S., Lin, C.H., Browne, J.K., Smalling, R., Egrie, J.C., Chen, K.K., Fox, G.M., Martin, F., Stabinski, Z., Badrawy, S.M., Lai, P.H., and Goldwasser, E. (1985) Cloning and expression of the human erythropoietin gene. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7580–7584.

87 Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, J.D., Kaufman, R.J., Mufson, A., Seehra, J., Jones, S.S., Hewick, R., Fritsch, E.F., Kawakita, M., Shimizu, T., and Miyake, T. (1985) Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature*, **313**, 806–810.

88 Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P.H., Zsebo, K.M., Murdock, D.C., Chazin, V.R., Bruszevski, J., Lu, H., Chen, K.K., Barendt, J., Platzer, E., Moore, M.A.S., Mertelsmann, K., and Welte, K. (1986) Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemia myeloid cells. *Science*, **232**, 61–65.

89 Collen, D. and Lijnen, H.R. (1991) Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood*, **78**, 3114–3124.

90 Collen, D. and Lijnen, H.R. (2001) Tissue-type plasminogen activator: helping patients with acute myocardial infarction, in *Recombinant Protein Drugs: Milestones in Drug Therapy* (eds P. Buckel, M.J. Parnham, and J. Bruinvels), series, Birkhaüser Verlag, Basel, Boston, MA, Berlin, pp. 107–126.

91 Rijken, D.C. and Collen, D. (1981) Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. *J. Biol. Chem.*, **256**, 7035–7041.

92 Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennett, W.F., Yilverton, E., Seeburg, P.H., Heynecker, H.L., Goeddel, D.V., and Collen, D. (1983) Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature*, **301**, 214–221.

93 Collen, D., Stassen, J.M., Marafino, B.J., Builder, S., De Cock, F., Ogez, J., Tajiri, D., Pennica, D., Bennett, W.F., Salwa, J., and Hoyng, C.F. (1984) Biological properties of human tissue-type plasminogen activator obtained by expression of recombinant DNA in mammalian cells. *J. Pharmacol. Exp. Ther.*, **231**, 146–152.

94 Valenzuela, P., Gray, P., and Quiroga, M. (1979) Nucleotide sequence of the gene coding for the major protein of hepatitis -B virus surface antigen. *Nature*, **280**, 815–819.

95 Edman, J.C., Hallewell, R.A., Valenzuela, P., Goodman, H.M., and Rutter, W.J. (1981) Synthesis of hepatitis B surface and core antigens in *Escherichia coli*. *Nature*, **291**, 505–506.

96 Standring, D.N. and Rutter, W.J. (1986) The molecular analysis of hepatitis B virus. *Progr. Liver Dis.*, **8**, 311–333.