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CHILD HEALTH THE GENOME PROJECT AND PHENYLKETONURIA*

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Child health and life expectancy improved together in many societies in the 20th century. As the incidence of diseases with major extrinsic causes declined, the heritability of disease as a whole increased. Accordingly, the genetic (intrinsic) causes of disease have become important. The genome project is an international venture out of which will come new knowledge about the biological basis of health and disease, and technologies to apply that knowledge in medicine and other disciplines. Phenylketonuria (PKU) at one time was seen only as a rare inborn error of metabolism causing severe mental retardation. Yet, the gene frequency is about 1% in Caucasian and Oriental populations, higher still in some populations with particular histories that have enhanced gene frequency. These genetic facts make PKU a paradigm in human genetics. This genetic disease, for which it was thought there was nothing to be done, has yielded to inquiries at clinical, metabolic, protein (enzyme) and DNA (PAH gene) levels. Results have shown that, early diagnosis (by population screening) and treatment (by low phenylalanine diet) largely prevents mental retardation. DNA analysis reveals particular associations between PKU mutations, RFLP haplotypes and populations; these associations are relevant for counselling. PKU illustrates well how medical science, molecular biology and gene mapping can improve knowledge and contribute to child health. *Key words: heritability, genomics, phenylalanine hydroxylase, PAH gene, phenylketonuria.*

When social conditions and the quality of life improve in human society, a consequence is increased heritability of the diseases that continue to affect individuals and families. Increased heritability, in this sense, means increased relevance of genetic causes of disease among all causes. Another consequence

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Many of the themes referred to here are documented in Development Brain Dysfunction Vol: 6 (Parts 1-3), 1993 an issue of that journal carrying an excellent article on PKU in Turkey and addressing all major themes.

of improved quality of life is decline in the perinatal mortality rate, improved neonatal survival rates and increased life expectancy. The result will be a change in population structure. The former relative excess of persons of pre-reproductive age will decline and there will be a relative increase in older-aged subjects. It could be said that improvements in child health care will increase the need for and relevance of knowledge relating to geriatric illnesses and the biological problems of senescence.

A necessary consequence of increased longevity, in the absence of any other change in population dynamics, will be population growth. This is one of the major problems facing the human species in some parts of the world now and on planet earth as a whole in the future. The most humane option to curtail population growth is the "fifth freedom"; it is the option to control reproduction in the context of personal choice.

These events, as I have described them, are some of the results of increased knowledge relating to health and disease and the applications of that knowledge. It has been said that science is an attack on ignorance and that what is not known is more important in science than what is known. Society tolerates scientists and their activities on the understanding that the facts will be converted into innovations, technologies and other applications to benefit society and individuals. To put it another way, the legacies of science are hypotheses (arrays of concepts), databases (arrays of facts), and innovations (arrays of technologies). In their particular way, the health sciences reflect this broad view. The lectures gave two illustrations: 1. The disappearance of mental retardation associated with phenylketonuria; and 2. The Human Genome Project.

A Place for Molecular Genetics and Biology in Medicine: The Medical Research Council of Canada (MRC) recently published its strategic plan. The document was written following a national consultation with scientists and the public. It contains some interesting phrases; here are two:

"Our potential for understanding the genetic bases for health and disease has never been greater. The prospective diagnostic and therapeutic benefits of molecular biology, including the evolution of stunningly creative frontiers of biotechnology, seem practically boundless. In it self, the multinational Human Genome Project, in which MRC is an active partner, promises to give us a virtual atlas of the human gene family, whose orderly expression is central to life and health".

"The boundaries of disciplines overlap, are redrawn as common, erode, disappear. The system is pluralistic in other ways. Research takes place in a wide variety of settings, from "dry labs" (office areas), to "centers of excellence" that may exist only electronically. The range of specialists, contributing expertise to

health issues, now includes lawyers and philosophers, engineers and physicists, clergy and social scientists, even historians".

The evolution of our knowledge about PKU and its applications in the past half decade in Quebec, for example (and in Turkey now), well illustrate these points of view. As for the Genome Project, which has been a cause for concern among many scientists and citizens, *The Economist* had a much more sanguine view (The Economist, Oct. 24, 1992, p. 96-98). The writer of the article entitled "The end of the beginning" expresses the opinion that the Genome Project is a search for data which in due course will be converted into tools for science, including the health sciences. Two interesting comments appear in the article.

"An entirely new field is being born, one that takes as its study not individual genes, but sets of them: genomics as opposed to genetics".

"When the genome project's nominal goal is reached, and a complete human genome is sequenced, nobody will notice. They will be too busy ..... finding out what it does and how it does it.

It is worth noting that a new journal with the title *Genomics* was established only 6 years ago and is now overflowing with articles every month. In the case of knowledge about PKU, it has advanced more rapidly since the mid-'80s, when tools were made available to study mutant genotypes and their expression in PKU, than in the previous 50 years following discovery of the disease in 1934.

Phenylketonuria: Multiple Beginnings and Endings: Phenylketonuria (PKU) is a classical Mendelian disorder; by this we mean that its principal cause is mutation in a single gene. On the other hand, PKU is a classical multifactorial disease; by this we mean that it has an equally important nutritional component of cause. The mental retardation associated with the Mendelian disorder is dependent on exposure to an essential nutrient: L-phenylalanine in the protein component of the diet. This fact permits treatment of the Mendelian disorder and the prevention of mental retardation by restriction of dietary phenylalanine. In brief, mutation and experience are each necessary and sufficient causes of the mental retardation in PKU[1].

PKU illustrates three important strategies in the study of genetic diseases. The first identifies, by segregation analysis, that a particular phenotype (disease) behaves as a Mendelian trait; in the second, one can work inward from the clinical disease to dissect phenotype at the levels of metabolite, biochemical reaction and polypeptide. With molecular biological techniques, one can get to the gene from the polypeptide through the corresponding cDNA. In the third approach, called reverse genetics by some people and positional clone (or expression cloning) by others, one works outward from the gene to explain the various levels of phenotype. The history of PKU illustrates all of these strategies.

Historical Perspectives: Asbjorn Følling discovered PKU in 1934. The story is well known. It is worth remembering, however, that this man, carefully trained in chemistry and nutrition and who had read articles by Garrod, used his knowledge and training to study the new disease. He recognized: a chemical signal for the disease; its origin in a disturbance of phenylalanine metabolism; the relationship between dietary phenylalanine and dishomeostasis; an association between the disorder and mental retardation; occurrence of the disorder in siblings; and a segregation pattern compatible with autosomal recessive inheritance. Dr. Følling's letter describing the new disorder brightened the final days of Garrod's life. Garrod was experiencing the loneliness of the long distance runner, and his concepts were not being used by colleagues in medicine. The fact that someone else now recognized their relevance was warmly recognized by Garrod.

Følling also identified the pathway affected by the abnormality in phenylketonuria. Others came to investigate that and other aspects of the disease. Judah Quastel and Lionel Penrose contributed to that knowledge and gave the name by which the disease is now known. George Jervis, among others, identified the enzyme defect and demonstrated a deficiency of phenylalanine hydroxylating activity in patients' livers. Seymour Kaufman, who has devoted more than a quarter century to delineating the precise defect in phenylalanine hydroxylase activity, opened up our knowledge of the hydroxylating reaction, thereby setting the scene for the discovery of other Mendelian disorders at other loci affecting components controlling the synthesis and recycling of the tetrahydrobiopterin cofactor.

Lionel Penrose was a contributor to many of these developments. As a geneticist whose particular interest was hereditary forms of mental retardation, he developed many important questions relevant to our analysis of PKU as a biological problem. It was Penrose who affirmed the necessary and sufficient conditions in the pathogenesis of the disease. He predicted that PKU would be treatable[2]. He also asked a fundamental question: If the disease has such an effect on the homozygote, and it is autosomal recessive, why is the gene so frequent in human populations? (He recognized that its frequency was about 1%). He also asked why this gene was not randomly distributed in human populations. (He noted a predilection for Celtic-derived populations). These fundamental questions[2] could not be answered until the era of molecular genetics had arrived. In the mean time, Penrose opened the door to proposals for the early diagnosis and treatment of PKU.

Screening and Treatment: Simple technological innovations made mass screening of the newborn infant to detect a chemical signal (hyperphenylalaninemia) a feasible social option. Robert Guthrie and colleagues were responsible for the most widely used test-a bacterial inhibition assay. It can be said that newborn screening for PKU and allied disorders has become the most widely applied genetic technology in human history. Society sees it as beneficial.

The rationale for newborn screening, in this case, is to achieve early diagnosis and an opportunity for intervention with a treatment that will prevent incipient disease from becoming established. The rationale was translated into practice when it became known that, by the use of a semi-synthetic diet low in phenylalanine, the metabolic phenotype could be controlled and near-normal homeostasis restored. The credit for this important development goes primarily to Louis Woolf, Horst Bickel, Marvin Armstrong and their colleagues. Thereafter it was shown that initiation of the diet within the first month of life was compatible with normal or near-normal intellectual development in the affected patient. Two important new developments in our understanding of PKU followed:

It soon became apparent that neonatal hyperphenylalaninemia is not always the equivalent of PKU. Some forms of hyperphenylalaninemia associated with deficient phenylalanine hydroxylase activity have only a mild metabolic phenotype-sometimes called non-PKU hyperphenylalaninemia. We have tended not to treat these persons. Other forms of hyperphenylalaninemia are associated with mutations at loci encoding the enzyme responsible for synthesis of tetrahydrobiopterin and the enzyme associated with recycling of dihydrobiopterin. Once the tetrahydrobiopterin-deficient forms of hyperphenylalaninemia were recognized, one could never counsel the patient and family with confidence until they were ruled out. Accordingly, national programs must now have access to the diagnostic tests to identify disorders of tetrahydrobiopterin synthesis and regeneration.

Another problem surfaced; it was maternal hyperphenylalaninemia. Half the patients with hyperphenylalaninemia are female. If the phenylalanine concentration is not in the normal range when a female conceives and carries a fetus, the product of that conception is at high risk of impaired brain development, microcephaly and other congenital malformations. The major legacy of successful PKU-prevention programs in the first generation is the need for effective programs to prevent the consequences of maternal hyperphenylalaninemia in the second. Most national programs now have registers of patients with hyperphenylalaninemia and prospective programs for the management of women at risk.

Phenylalanine Hydroxylase Genotypes: A new era of knowledge began when Savio Woo and colleagues cloned the PAH gene-first in rats, then in humans. It was shown that mutation at a single locus was sufficient to explain classical PKU; that one polypeptide was sufficient to form an effective homopolymer with phenylalanine hydroxylating activity in the presence of tetrahydrobiopterin; and that mutations causing PKU mapped to the PAH locus on chromosome 12q22-24.1.

The PAH gene encompasses more than 90kb of nucleotide sequences has a promotor region recently characterized, contains 13 exons, and has a suite of

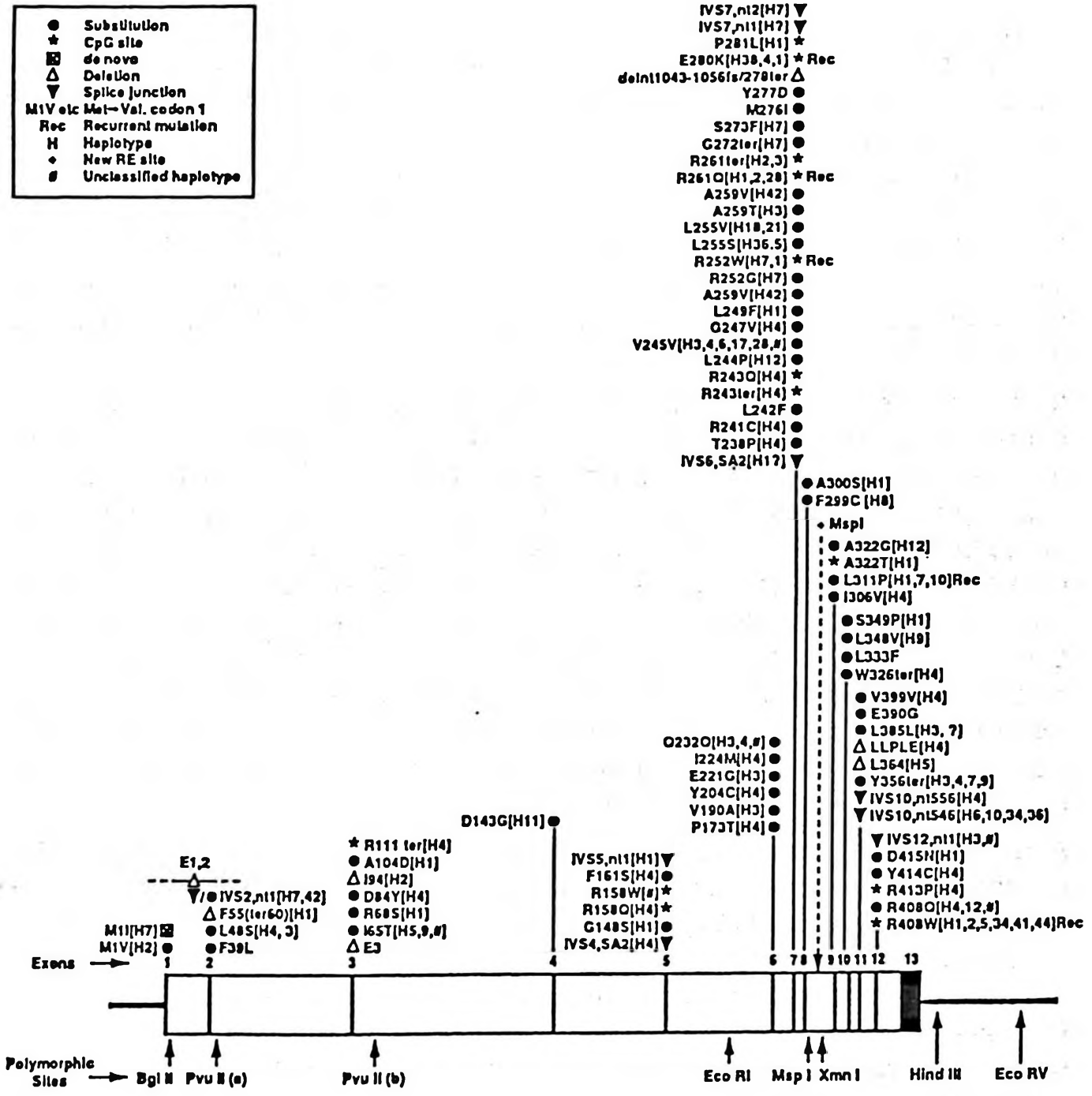

Fig. 1: Distribution of exons along 90 kb of genomic DNA encoding the human PAH gene in the chromosome region 12q.22-q24.1. Details about the 5' promoter region, not shown in the figure, are now available. Polymorphic restriction sites used in RFLP haplotypes are shown below the gene. Exons are numbered in Arabic numerals above the gene. Alleles are named by the following conventions. For missense mutations, the first letter and number following indicate the normal amino acid residue and codon number, respectively; the letter following is the replacement residue. For nonsense mutations, the suffix 'ter' (termination) is used after the codon affected. For deletion mutations, the prefix symbol, Δ, or the letters del (deletion), indicate the residue or exon affected. For splice-junction mutations, the intron affected (e.g. IVS2), the nucleotide involved (e.g. nt1,nt546) or region of the intron (e.g. SA2 for 3' end of junction) are named. Other symbols are explained on the figure.

polymorphic markers-7 of which are diallelic and one (in the Hind III region at the 3' end of the gene) is multiallelic. These markers provide informative haplotypes. Analysis of the gene with the corresponding probes and primers for PCR amplification of segments within the gene has identified a great many mutations associated with the phenylketonuria phenotype (Fig. 1); over 100 are now known. The mutations are non-randomly distributed in human populations and have both inclusive and exclusive associations with the RFLP haplotypes. Accordingly, it is possible to analyze the PAH locus and to study their histories and the corresponding histories of the populations with which they associate. The PAH locus has thus become very powerful for the study of human population genetics.

The molecular information has been put to work in other ways. For example, expression analysis of constructs has identified that some PKU mutations generate null phenotypes of unstable proteins or non-functional homopolymers. Others affect the specific activity of the enzyme, and these are of particular interest because they will identify residues essential for enzyme activity. There is correspondence between the severity of the mutational effect on enzyme activity, the degree of hyperphenylalaninemia in the absence of treatment, and the requirement for restriction of phenylalanine in the diet[3]. These broad correlations have predictive value for management of the patient.

Mutation analysis shows that some mutations are public in their distribution, meaning that they are prevalent in particular geographic regions and populations, e.g. R408W, IVS12,ntI, R261Q and IVS10,nt546 in Europe. Careful analysis of the association between mutation, haplotype and population is beginning to reveal information about the origns of these mutations in human history. Exon 7 is an important region in the gene, and mutation in this exon is a prevalent cause of PKU not because this exon is hypermutable, but probably because it is critical for the function of PAH. A number of mutations affect CpG dinucleotides, a hypermutable sequence in the human genome. Deletions or duplications as causes of mutation, are not unusually frequent in the PAH gene. Recombination within the gene may explain the occurrence of particular mutations on a different haplotype (e.g. R408W on haplotypes 1 and 2). But R408W also occurs on haplotype 44 in Orientals; here, recurrent mutation at a CpG dinucleotide is the likely explanation. Mean while, by means of the relevant PKU mutations, it may be possible to date the origin of recombinant European chromosomes. The findings are important and we are reminded that Penrose posed the corresponding questions long ago.

Histories of Genes: Histories of Populations: One of the hypotheses posed by Penrose was that PKU had a Celtic origin in Europeans. (Penrose did not know about the exceptionally high prevalence of PKU in Turkey nor about its occurrence in Orientals at frequencies resembling those in Europeans). In this regard, our

own analysis of PKU mutations in Quebec populations is relevant. Quebec represents a region founded by Europeans in the New World, first by French settlers in the 17th and early 18th centuries, then by British settlers between the mid-18th and-20th centuries, and finally by non-French Europeans and others in the late 20th century. These various founding families and their descendants have clustered in different geographic regions of the province. Accordingly, the molecular analysis of contemporary PKU chromosomes is likely to reveal stratification-and it does. At three regional centers caring for PKU patients identified by the provincial newborn screening program, we found three sets of PKU patients with three different sets of mutations. PKU, at these different centers, is three different sets of diseases according to its cause (mutation). These findings may have implications for the refinement of treatment.

We have found something else. We noticed that some mutations are actually shared by the three populations with their distinctive historical origins. At first this was puzzling, until we recognized that one of the shared mutations (IVS12nt1) had a broad European origin compatible with the origins of all varieties of founders. Another shared mutation, R408W on haplotype 1, could be traced to ancestors (Irish or Scottish) of Celtic origin in each family. Accordingly, we hypothesized that this particular mutation, very common in Quebec, was in fact the "Celtic chromosome" proposed by Penrose. Subsequent studies (Eisensmith, personal communication, 1993) show that R408W on haplotype 1 is the major PKU mutation in contemporary European populations with strong Celtic ancestry. Studies such as this indicate how founder effects and subsequent genetic drift explain the origin of PKU in populations whose histories are compatible with expansion in relative genetic isolation. I suspect that analysis of PKU mutations in Turkish populations will yield analogous findings and will have the corresponding relevance for the management of the disease.

New Beginnings: Evidence is accumulating that eraly diagnosis and treatment, with the best possible methods now available, does not accomplish all that we would desire in PKU. Abnormalities are identifiable by magnetic resonance imaging in the brain of treated PKU patients. These abnormalities are fewer, the better the treatment is. They imply that we must refine treatment, be more rigorous with it, and probably impose more burdens on families. It is not surpising, then that among affected families there is a gradually increasing demand for prenatal diagnosis. This demand is already apparent in Turkey. Until recently it was not possible to contemplate prenatal diagnosis for PKU. Genotype analysis now makes it possible. Identification of heterozygotes at risk in families is technically difficult at the phenotype level for a variety of reasons. It is feasible (some say easy) by analysis of haplotypes in informative families. Prenatal diagnosis, indirectly by haplotype association or directly by mutation analysis, is increasingly accessible and relevant in some societies.

Envoie: We return then to themes raised by Garrod, the person who highlighted inherited susceptibility to disease; by Følling who showed that PKU was a significant cause of mental retardation; and by Penrose, who asked basic questions about the origin and significant of PKU mutations in human populations. (We may yet discover that PKU mutations conferred a selective advantage on heterozygotes at some time in human history). This knowledge was not reason for despair; ways were discovered to achieve universal screening and early diagnosis of individuals harboring PKU mutations and to treat them before brain development was irreversibly impaired. It is fair to say that PKU, the disease, has disappeared from societies where the technologies are available; the associated genes continue to be transmitted at unchanging frequencies. Meanwhile, other problems attached to the disease continue, notably the issue of maternal hyperphenylalaninemia and the challenge to improve treatment of the phenotype. Physicist John Ziman said that "reliable knowledge" will serve as a basis for action, can lead to correct prediction and is relevant to choices of action that matter. All that we know about PKU fits the definition of "reliable knowledge".

I return to the theme of molecular biology in medicine and the relevance of the Genome Project, to close with some basic points of view. The basic question in medicine remains: why does this patient have this disease now? It is the question that distinguishes between the patient with the disease and the incidence of the disease; it also distinguishes the patient with the disease from the disease the patient has. Many of us work with a medical model which recognizes that manifestations of disease (the substrates for diagnosis and treatment) have their origin in a deviant cellular process (pathogenesis of the disease) which, in turn, originates in a cause which is both intrinsic (biological) and extrinsic (experience). To understand pathogenesis and cause is to have access to prevention. Medical education puts primary emphasis on the manifestation of disease. Interest in prevention is to some extent counter-intuitive, because if all diseases could be prevented there would be no need to practice medicine as we know it.

The history of PKU is one of the great illustrations of this theme. I said at the beginning that if we could improve social and environmental conditions, and thus the quality of life, we would increase the heritability of disease. Extrinsic causes can change almost overnight; intrinsic causes (mutations) change at a glacial pace. It follows that to know the human genome and its mutations will be increasingly relevant to an understanding of the biological basis of health and disease. This will have profound implications for medical education and the way we design and practice health care.

One can think about medicine in three frameworks of time and organism. When we meet with the patient, we recognize an individual in the present moment (now). But the individual belongs to a family which has a recent history and may

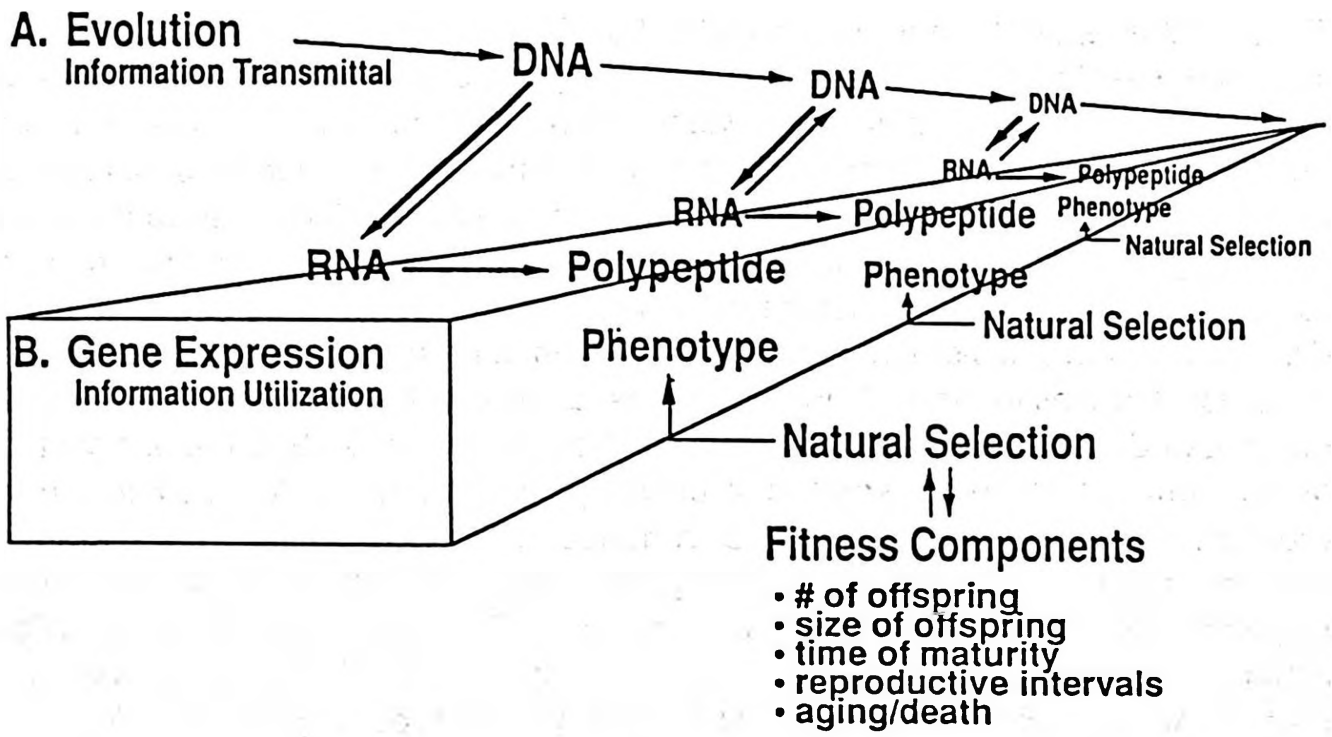

Fig. 2: Two biological paradigms: evolution, or information transmittal; and gene expression, or information utilization. The central dogma of biology links one to the other. Selection acts on phenotype to select an object (the corresponding gene). Fitness components which are the substance of 'life histories' are the products of natural selection.

have a future one if the person is or becomes a parent. Finally, the family belongs to a species (Homosapiens) whose demic expansion and evolution through its genetic footprints passed on to contemporary human populations. Accordingly, we are interested in two paradigms of genetics (Fig. 2). In one we are considering transmittal of information; this is the paradigm of evolution. The study of PKU contributes to the documentation of human evolution. It also describes an important gene that has been conserved and has evolved through mammalian and earlier species. The gene is a marker for history. In the other paradigm, we are concerned with utilization of information: how genotype becomes phenotype. The association between mutant genotypes and the corresponding phenotypes in PKU is but one of a hundred ways to illustrate how change in information can explain disease. The link between the two paradigms (transmittal and utilization) is of course the central dogma of biology; DNA makes RNA makes protein.

It all becomes manifest in what some call "life histories", which are the fundamental factors underlying the biology of individuals and populations. As practitioners of health care, we are contributing to the life histories of the human species. Accordingly, we are modifying the evolution of our own species. These are major responsibilities to consider in our roles as members of our species, as members of families, as individuals, whether we are patients or well persons, and as citizens no matter where we live on planet Earth.

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