Regulation of gene expression during ageing

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Deterioration or ageing of functions that occurs in all organisms after they attain reproductive ability is the sum total of the decline in the activities of various organs. The functions of various organs begin to deteriorate at different times of the life span and at different rates. So it is unlikely that a single gene is responsible for ageing of multicellular organisms. Studies on isoenzyme patterns of enzymes such as lactate dehydrogenase and alanine aminotransferase, show that the genes responsible for coding of different subunits of the enzymes are sequentially expressed during the life span. Also, the decrease in the levels of enzymes seen after adulthood is reversible and can be brought back to adult level by inducing their genes by steroid hormones which act on genes after binding to their receptors. Studies on transcription of several genes show that their expression declines after adulthood. This has been shown to be due to the decrease in the levels of *trans*-acting nuclear proteins that bind to specific *cis*-acting elements in the promoter regions of these genes. These proteins are inducible by steroid hormones. Hence the deterioration of functions that occurs after adulthood and leads to ageing can be delayed, and the adulthood period can be prolonged by manipulation of the expression of genes.

AGEING or deterioration of various functions occurs in all eukaryotic organisms after they attain reproductive maturity. The cause of this universal phenomenon is an intellectually challenging problem in biology. The questions that have confronted researchers in this field are: Why do functions of all organisms undergo deterioration after reproductive maturity is attained? Why do all members of a species have a more or less fixed life span? Why does a rat live for three years, an elephant for 70 years, and a human being for 100 years? At what age after attaining maturity does the process of ageing begin? Is there a single trigger, a master switch, which sets in motion the process of deterioration? If so, how is it switched on? Or, are there multiple switches that get switched on at different stages of the life span and set in motion the process of deterioration of various functions of the organism leading to ageing and death? Is this process programmed like the process of development?

Answers to these questions may help in designing experiments to postpone or prevent the onset of the ageing process. This would prolong the active and youthful period in humans from age 20–40 years to say 20–60 or more, and would greatly increase the active period and quality of life. The objective is to ensure better health for a longer period, and not merely prolongation of the years lived. Prolongation of the adult period is also expected to postpone or defer the onset of 'old age' diseases such as cardiovascular and cerebro-vascular diseases, cancer, arthritis, Alzheimer's disease, etc. which set in generally after age 50 when the persons are at the peak of their careers.

Earlier studies have shown: (i) decrease in the number of post-mitotic cells; (ii) accumulation of age pigment; (iii) decrease in levels of hormones; (iv) decrease in permeability of membranes; (v) increase in cross-linking and tensile strength of collagen; (vi) increase in free radicals; (vii) decrease in levels of enzymes, though some enzymes do not show any change, and a few others increase in level and (viii) decrease in antibody level and immunocompetence after the reproductive period. These changes being secondary in nature, do not explain the basic cause of ageing (reviewed by Kanungo[1]).

The fact that (i) all individuals of a species have a more or less fixed maximum life span, (ii) long-lived parents have long-lived progeny and short-lived parents have short-lived progeny, and (iii) identical twins have similar longevity, indicate that the cause(s) of ageing may lie at the level of genes. This is strengthened by the finding on the mutants of the free-living nematode, *Caenorhabditis elegans*. *C. elegans* has a life span of only about two weeks and has a fixed number of post-mitotic cells. It has several mutants which have different longevities. The longevity of *age*-1 mutant is 60% longer than that of the wild type[2]. Kenyon *et al.*[3] and Kenyon[4] reported that mutation in the *daf*-2 gene doubles the longevity. The extension of longevity also requires *daf*-16 gene. There are other *daf* genes which have regulatory effects on longevity. This indicates that the products of *daf*-2 and *daf*-16 genes accelerate the ageing process, and when they are mutated, the ageing process is deferred and, therefore, longevity is increased. Longevity in *C. elegans* and in other species may, therefore, be genetically regulated. The variability in the pattern of ageing among individuals within a species, especially in higher organisms, may not only be due to genetic differences but also due to the differences in their nutrition, and the types of stress such as tem-

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perature, radiation, pollution including free radicals, and psychological stress to which they are subjected to during their life span.

Another important observation is that the functions of different organs begin to deteriorate at different times, and at different rates. For example, in the mammals the activity of the thymus declines earlier than that of the skeletal muscle, and that of the latter declines earlier than that of the brain. This indicates that different genes are involved in the ageing of different organs, and there is no single or master gene that sets in motion the ageing process of the organism as a whole, because if such a gene were present, functions of all organs would begin to deteriorate at the same time.

On the basis of the above observations, Kanungo[1,5,6] put forward the 'gene regulation' theory of ageing, according to which ageing occurs due to the changes in the expression of genes after reproductive ability and other adulthood activities have been attained (Figure 1). The life span of an organism has three broad phases: development, adulthood and senescence. Developmental stage is programmed as the embryonic and post-embryonic events up to reproductive maturity are well timed, and occur in a specific sequence. These events take place due to sequential activation of several early phase genes (genes A–F) according to a set programme. Some genes of the late developmental period, genes E and F, switch on certain genes like G and H of the early reproductive phase. These genes, in turn, switch on sequentially other genes of the reproductive (adult) phase, and the organism attains reproductive ability. Continued reproduction and other stresses to which the organism is exposed during adulthood cause depletion of certain factors which are necessary for keeping essential genes active and maintaining adult functions. Also, continued reproduction and other stresses may cause accumulation of certain factors that may cause expression of some undesirable genes such as oncogenes leading to cancer. Thus the decline in functions that begins after attainment of reproductive ability is due to destabilization of the regulation or the homeostatic expression of genes required for maintenance of adulthood functions.

The various tenets of the 'gene regulation' theory are testable. For example, one can find out if (i) the expres-

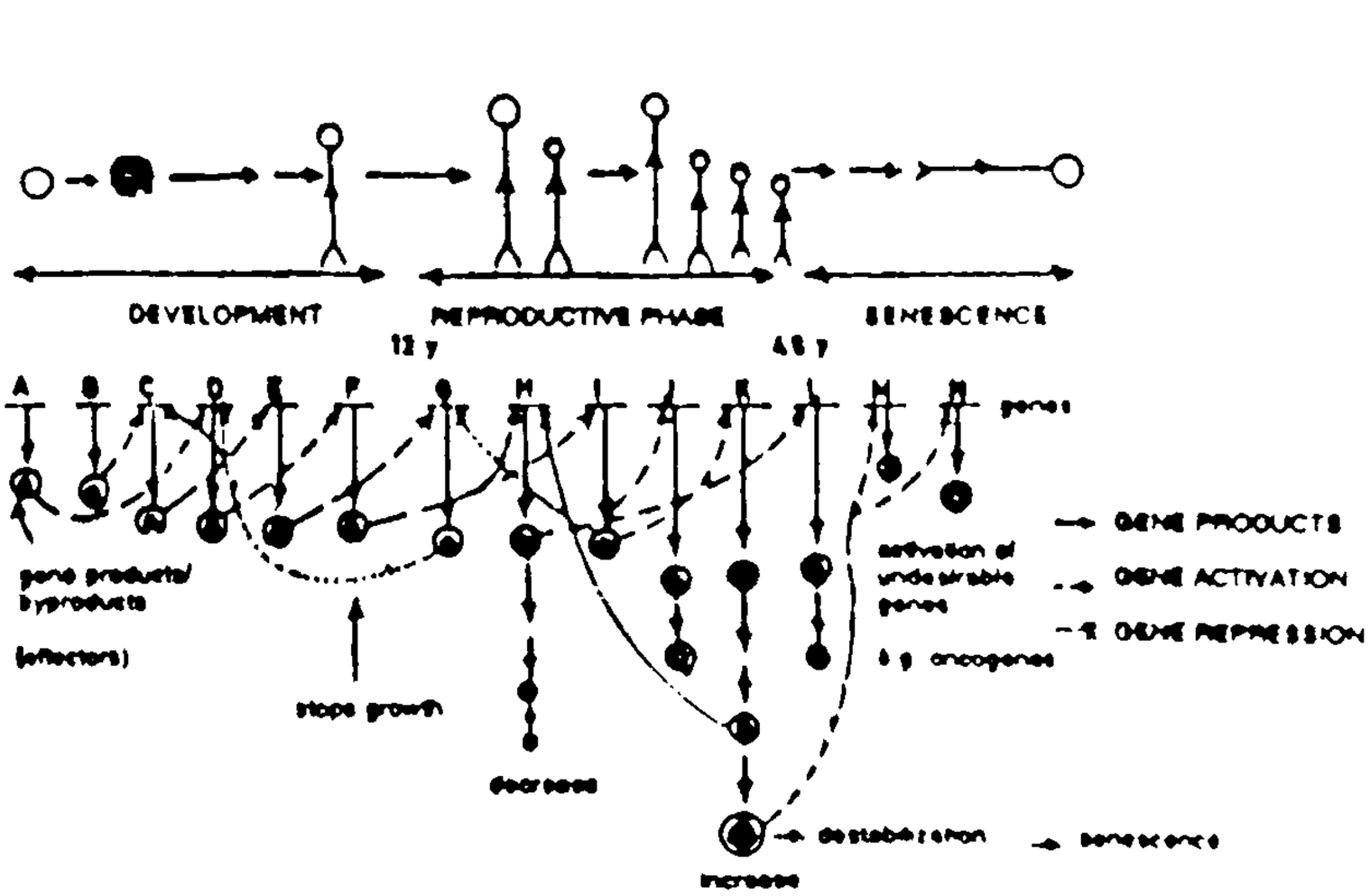

Figure 1. Model for ageing (top)–Representation of various phases of the life span: development, reproduction, senescence. (Bottom)–For clarity, the number of active genes has been kept at a minimum, and genes that are permanently repressed are not shown. Developmental and reproductive phases are controlled by unique genes, A-F, and G-L, respectively. No specific genes for senescence are depicted. Development occurs by sequential activation of genes, A-F, the product of gene A switching on gene B and so on. Some genes of developmental phase, E and F, switch on certain unique genes, G and H, belonging to early reproductive phase. These genes then switch on sequentially other genes. The organism attains reproductive ability when required levels of gene products are formed. Continued reproduction may deplete certain factors which may be necessary for keeping certain essential genes of reproductive phase active. Continued reproduction may also cause accumulation of certain gene products beyond a certain level, resulting in activation of certain undesirable genes, M and N, whose products may cause autoimmune diseases, or activate oncogenes causing cancer. Thus the decline in various functions that begins after a certain stage of reproductive phase may cause destabilization of the homeostatic functioning of genes of reproductive phase or adulthood, and lead to ageing and death. (Source: Kanungo[1,5])

sion of genes changes after adulthood, (ii) whether these changes can be reversed by various cellular factors, and (iii) whether one can manipulate the ageing process by manipulating the expression of genes, and delay or defer the onset of ageing. The following data from this laboratory support the theory.

Changes in enzymes

Isoenzymes

Several workers had shown earlier that the levels of many enzymes decrease after adulthood. Though each enzyme is generally coded by one gene, there are several enzymes which exist in multiple molecular forms called isoenzymes. They are made up of two types of protein chains or subunits, each chain being coded by a gene. Though all isoenzymes catalyse the same reaction, there are subtle differences in their kinetics. Changes in the levels of isoenzymes that catalyse a specific step may destabilize the fine control of a metabolic path because of the differences in their affinities for the substrates and effectors. This may alter a specific function.

Kanungo and his co-workers studied the isoenzymes of lactate dehydrogenase (LDH), and alanine amino-transferase (AAT) of the rat to get some insight into their molecular changes during the life span, because changes in their levels may throw light on the changes in the expression of the corresponding genes. LDH has five isoenzymes: M_4, M_3H_1, M_2H_2, M_1H_3 and H_4. It catalyses the reaction, pyruvate $\rightarrow$ lactate, and is essential for anaerobic glycolysis by which energy is derived in the absence of oxygen. M_4-LDH is more efficient for this reaction. Singh and Kanungo[7] found that M_4-LDH is significantly lower in the brain, heart and skeletal muscle of old rats. Hence the cells in these tissues become more aerobic or dependent on oxygen and, therefore, are more vulnerable to death when oxygen supply is cut off due to a clot in the blood vessel supplying the tissues. This is one of the reasons for the higher frequency of heart attack and brain damage in old age.

Cytoplasmic AAT (cAAT) is necessary for amino acid metabolism. It is a dimer made up of two types of subunits, A and B, each coded by a gene. It has two isoenzymes, cAAT-A and cAAT-B. Kanungo and Patnaik[8,9] found that cAAT-A is present in the liver of the rat during its early developmental period. In the adult, both cAAT-A and cAAT-B are present. In the old, only cAAT-B is present. This shows that the genes for A and B get expressed sequentially during the life span. The A gene is expressed during the developmental phase, and is switched off during adulthood, and then B gene gets expressed from adulthood onwards (Figure 2). Such changes may alter the metabolism of the alanine and α-ketoglutarate which may, in turn, affect the activity of the Krebs cycle.

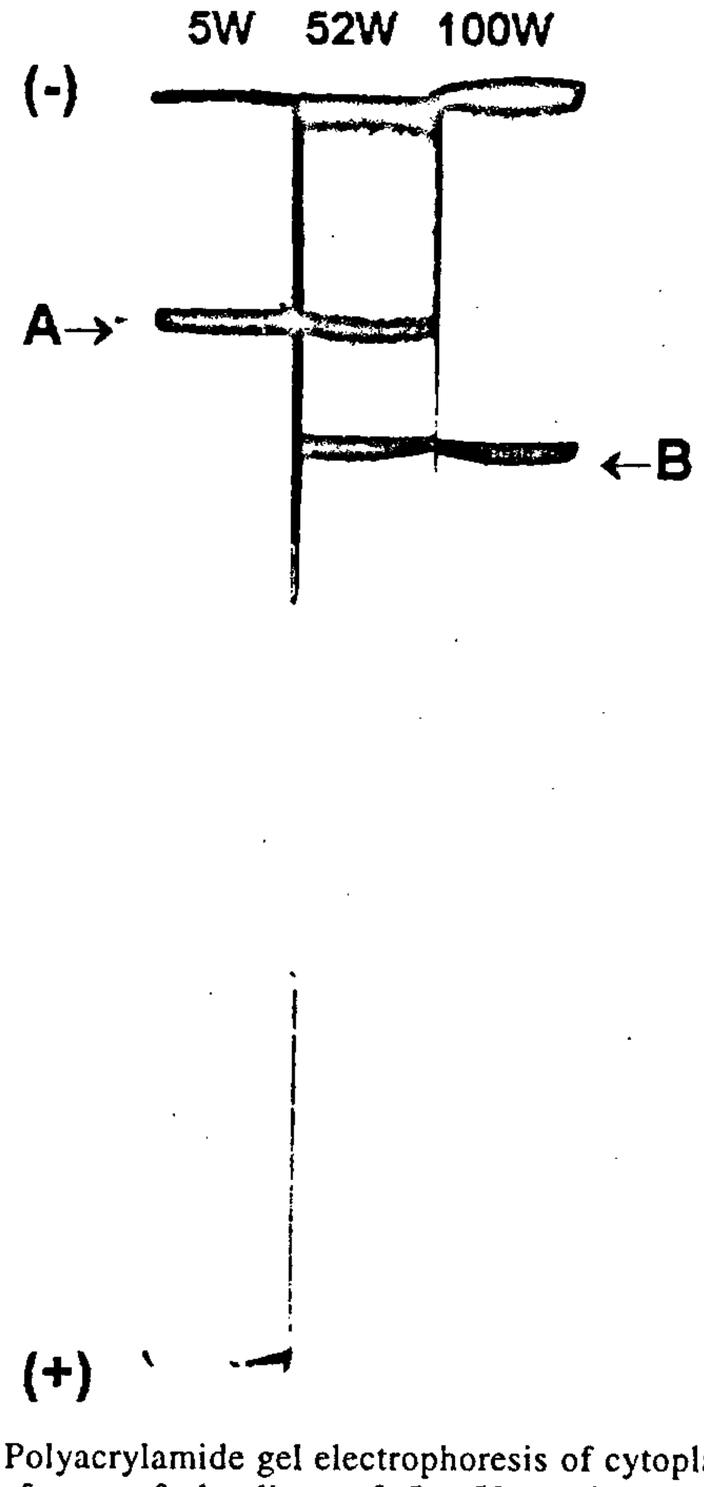

Figure 2. Polyacrylamide gel electrophoresis of cytoplasmic alanine amino-transferase of the liver of 5-, 52- and 100-week-old rats (Source: Kanungo and Patnaik[8]).

Induction of enzymes

The levels of several enzymes decrease after adulthood. This may either be due to a decrease in transcription of the respective genes, or a decrease in translation of their mRNAs to proteins. To find out if these alterations are reversible, the enzymes, acetylcholinesterase (AChE) and cholineacetyl trans-ferase (CAT), were induced in the brain of rats of various ages by the steroid hormone, 17β estradiol, which is known to act on genes after binding to its receptor and other proteins. It was found that the levels of both the enzymes are lower in the brain of normal old rats, but their levels get elevated to adult levels after administration of estradiol. Hence the changes in the levels of enzymes seen after adulthood can be manipulated by regulating the rate of transcription of their genes using hormones which act on genes[10,11]. It was further shown that the level of the receptor protein to which 17β estradiol binds decreases in the brain with increasing age[12]. This may contribute to the lower expression of AChE and CAT genes, and hence lower levels of AChE and CAT enzymes in old age, resulting in decreasing brain function.

Primary structures of proteins do not change with age

The lower levels of enzymes in old age may be due to lower expression of their genes, or due to increasing levels of error containing enzyme molecules as postulated in the 'error' theory of ageing[13,14]. This theory postulates that the frequency of incorporation of 'errors' into RNAs and proteins during information transfer steps like transcription and translation, respectively, increases with age, and results in increasing levels of 'error' containing proteins. Such proteins would be partially or totally inactive. Particularly, errors in proteins that are involved in protein synthesis like RNA polymerase II and aminoacyl-tRNA synthetases would amplify such errors and lead to 'error' catastrophe, leading to decline in cell function and cell death. The latter possibility was tested by purifying proteins from young and old rats and analysing them by peptide mapping, kinetics and immunodiffusion using antisera against the proteins. By all these criteria it was shown that there are no apparent differences between the primary structure of a protein of young and old rats[15]. This not only contradicts the 'error' theory, but also shows that the decrease in the levels of enzymes seen in old age is not due to changes in the primary structures of proteins, but is due to the decrease in expression of their genes.

Expression of genes

Though RNA polymerase II that binds to the TATA region of the gene along with other transcription factors (proteins) (TFs) is responsible for basal transcription of a gene, the rate of its transcription is regulated by specific *trans*-acting factors (nuclear proteins) that bind to specific *cis*-acting elements (DNA sequences) in the *non*-coding 5′ flanking (promoter) region of the gene and interact with the TFs. Alterations in the levels of these proteins and their modifications influence the rate of transcription.

We used fibronectin (FNT) gene of the rat, and vitellogenin (VTG) and ovalbumin (Ov) genes of the bird for finding out if the *trans*-acting factors undergo alterations during ageing. FNT gene is expressed in the liver and codes for a protein that is required for several functions such as differentiation, morphogenesis, cell–cell interaction, wound healing, tumour metastasis, etc. We first found by Northern hybridization technique that the expression of the gene in the liver declines after about 20 weeks of age of the rat[16].

Whether or not the levels of the *trans*-acting factors that bind to specific sequences of the promoter of the FNT gene change after adulthood in the rat was examined by gel mobility shift assay. The promoter of the gene has several interesting *cis*-acting elements including CCAAT, GGGCGG as well as responsive elements for cAMP and heat shock proteins that take part in its transcriptional regulation. The following 25-mer synthetic DNA that contained the cAMP responsive element (CRE), TGACGTCA, of the FNT gene was

5′ AATTCCCCG **TGACGTCA**CCCGGAC-3′
3′-GGGG**CACTGCAGT**GGGCCTGTTCGA-5′

labelled by ^{32}P and incubated with nuclear extract of the liver of young, adult and old rats. Gel shift assay was carried out to find out the proteins that bind to the 25-mer DNA. It was found that three nuclear proteins bind to the 25-mer DNA in the young. The levels of these proteins are far lower in the old. Since the transcription of the FNT gene in the liver is also lower in the old, the decreasing levels of the nuclear *trans*-acting factors may be responsible for the decreasing degree of transcription of the FNT gene (Figure 3)[1,17]. Hence induction of the FNT protein in old rats by stimulation of transcription of its gene may accelerate the healing of wounds in the old.

Several cellular factors are known to modulate the rate of expression of genes. A single factor may alter the expression of one or more genes which may or may not be related. The levels of several factors, especially of steroid hormones, change after adulthood. These hormones are known to regulate the expression of genes. They bind to specific receptors, and the complexes then bind to specific *cis*-acting elements in the promoter. There are usually several *cis*-acting elements in the promoter of a gene, each acting as a module. Interactions between *trans*-acting factors bound to one or more modules and the transcription factors bound to the transcription start site cause subtle changes in the expression of a gene. Each hormone also may act on several genes, so it may have in-

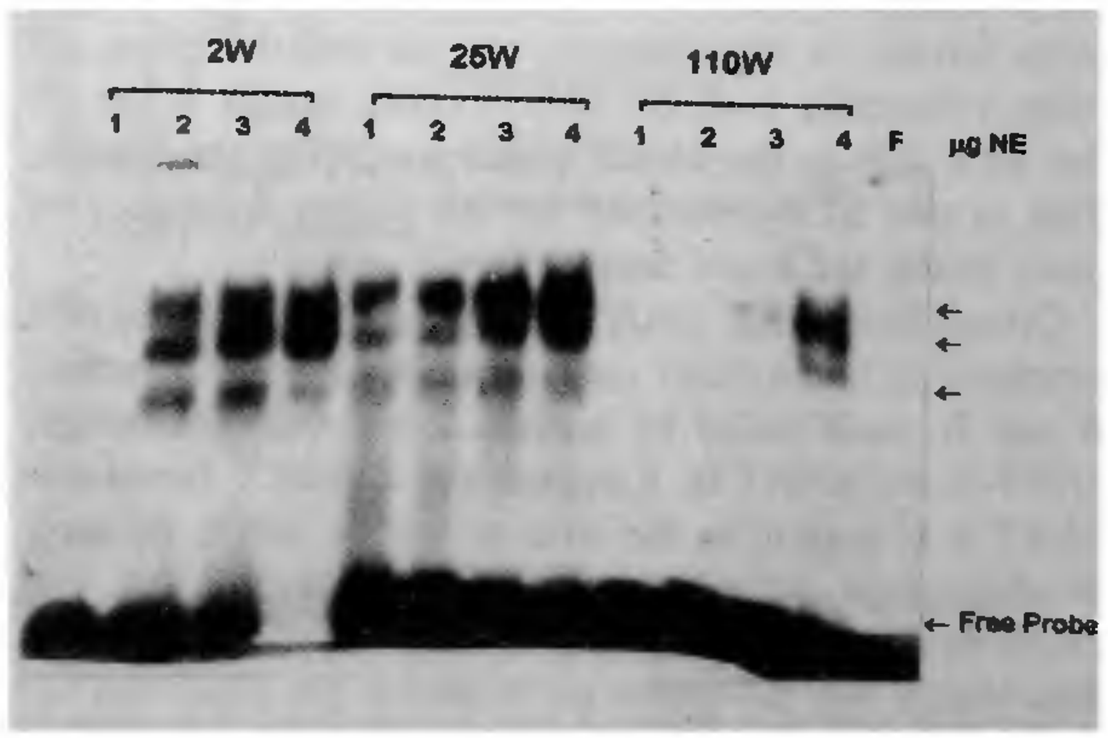

Figure 3. Gel mobility shift assay of 25-mer dsDNA containing the cAMP response element (CRE), TGACGTCA, present in the promoter region of the fibronectin gene[1]. Nuclear extract (1–4 µg) of the liver of 2-, 25- and 110-week-old male rats was titrated with 25-mer ^{32}P-labelled dsDNA containing the CRE. Three nuclear proteins are present in immature and adult rats, but the levels of these proteins are significantly lower in the old (Source: Kanungo[1]).

fluence on several functions in a complex, multicomponent organism. Changes in the hormone levels would, therefore, destabilize several functions, and affect the homeostatic functioning of various organs, and lead to ageing.

To get an insight into the gene–function relationship, it is necessary to correlate the activity of a gene (the genotype) with the activity of a specific function (the phenotype) carried out by an organ. It would throw light not only on the basic mechanism of ageing at the level of genes, but also help in the development of techniques to manipulate a specific function and defer the ageing process. It was reasoned that genes whose timing and rate of expression run parallel with the timing of the onset, peaking and cessation or the rate of a specific function during the life span of an organism may serve as appropriate models. The changes in the function should be measurable in order to correlate it with the changes in the expression of the gene responsible for this function. This would directly correlate the change in a phenotype with that of a genotype, and establish the relationship of a gene with the function it controls.

Hence, the genes that code for proteins needed for egg production in the bird, Japanese quail, were chosen for this study for the following reasons. Ovalbumin (Ov), the egg white protein, is synthesized in the oviduct. Vitellogenin (VTG), the egg yolk protein, is synthesized in the liver. The initiation, peaking and cessation of expression of their genes are expected to run parallel with the initiation, peaking and cessation of egg formation and laying which can be monitored. Also, the promoter regions of these genes have interesting sequences, *cis*-acting elements, estradiol responsive element (ERE) and progesterone responsive element (PRE), to which the steroid hormones, 17β-estradiol and progesterone, bind respectively, along with their receptors and other *trans*-acting nuclear proteins to stimulate the expression of these genes. The genes are highly induced by estradiol. These genes have other *cis*-acting elements such as CCAAT and NFI which are also known to bind to nuclear proteins and modulate expression of these genes. The levels of the two hormones decline after adulthood, and the progesterone/estradiol ratio is the highest in the adult bird when peak egg laying occurs, and it is considerably lower in the old which does not lay eggs[18]. Hence it may be possible to correlate the changes in the rate of expression of the two genes during the life span with an important phenotypic function, egg laying.

Japanese quail has a life span of only about two years. It begins to lay eggs from 8 to 10 weeks which peaks between 20 and 30 weeks, declining thereafter, and ceasing at 60 weeks. This phenotypic change can easily be monitored, and its correlation with the two genes responsible for the synthesis of the two vital egg proteins, ovalbumin in the oviduct and VTG in the liver, can be established.

Total RNA from the oviduct of 8, 20 and >60 week old birds was resolved in 1% denaturing agarose gel, transferred to nytran membrane and hybridized to ^{32}P-labelled cDNA probe of Ov gene. The level of Ov-mRNA was found to be far greater in the adult than in the immature and the old[19].

Gel mobility shift assay was carried out with the following synthetic 20-mer ds DNA corresponding to the promoter sequence –120 bp to –100 bp of the Ov gene to find out if the nuclear factors that bind to it change with age. The ds DNA was ^{32}P-labelled and incubated with nuclear extract of the oviduct of 8, 20 and >60 week old birds.

5′ AATTTCTAA**CCAAT**CCCATTAAA-3′
3′-AGATTGGGTTAGGGTAATTTTCGA-5′
20 mer synthetic ds DNA containing CCAAT sequence

Figure 4 shows that there are atleast two nuclear protein factors that bind to this sequence, and their levels are the highest in the adult when the transcription of the Ov gene is also the highest[19]. Hence there is a direct correlation between the levels of the *trans*-acting factors and the expression of the Ov gene during the life span of the bird.

Total RNA was extracted from the liver of immature (2–3 week) birds, resolved on 1% formaldehyde denaturing agarose gel and hybridized with ^{32}P-labelled VTG cDNA. No VTG mRNA was seen at this age. However, when these birds were administered 17β estradiol (E), and sacrificed after 48 h, the VTG gene was expressed. Progesterone (P) did not induce the gene, but when E + P were administered together, the gene was significantly expressed[20]. In the adult bird (20–25 week),

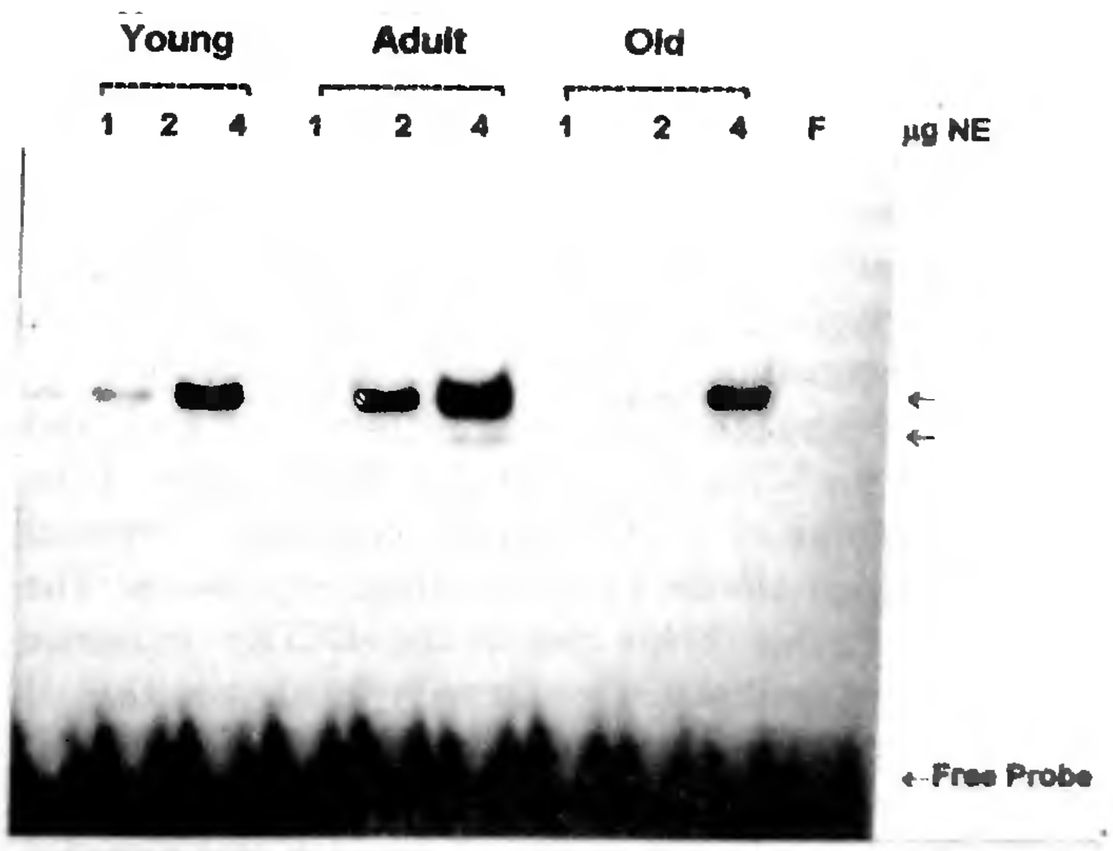

Figure 4. Gel mobility shift assay with the 20-mer DNA containing the CAAT-box of the Ov gene promoter and nuclear extract (NE) of the oviduct of young, adult and old Japanese quails. The ^{32}P-labelled 20-mer dsDNA was incubated with 1,2 or 4 µg of NE of the oviduct of birds of different ages. The DNA-proteins complexes were resolved on 5% polyacrylamide gel. F-Free probe (Source: Upadhyay *et al.*[19]).

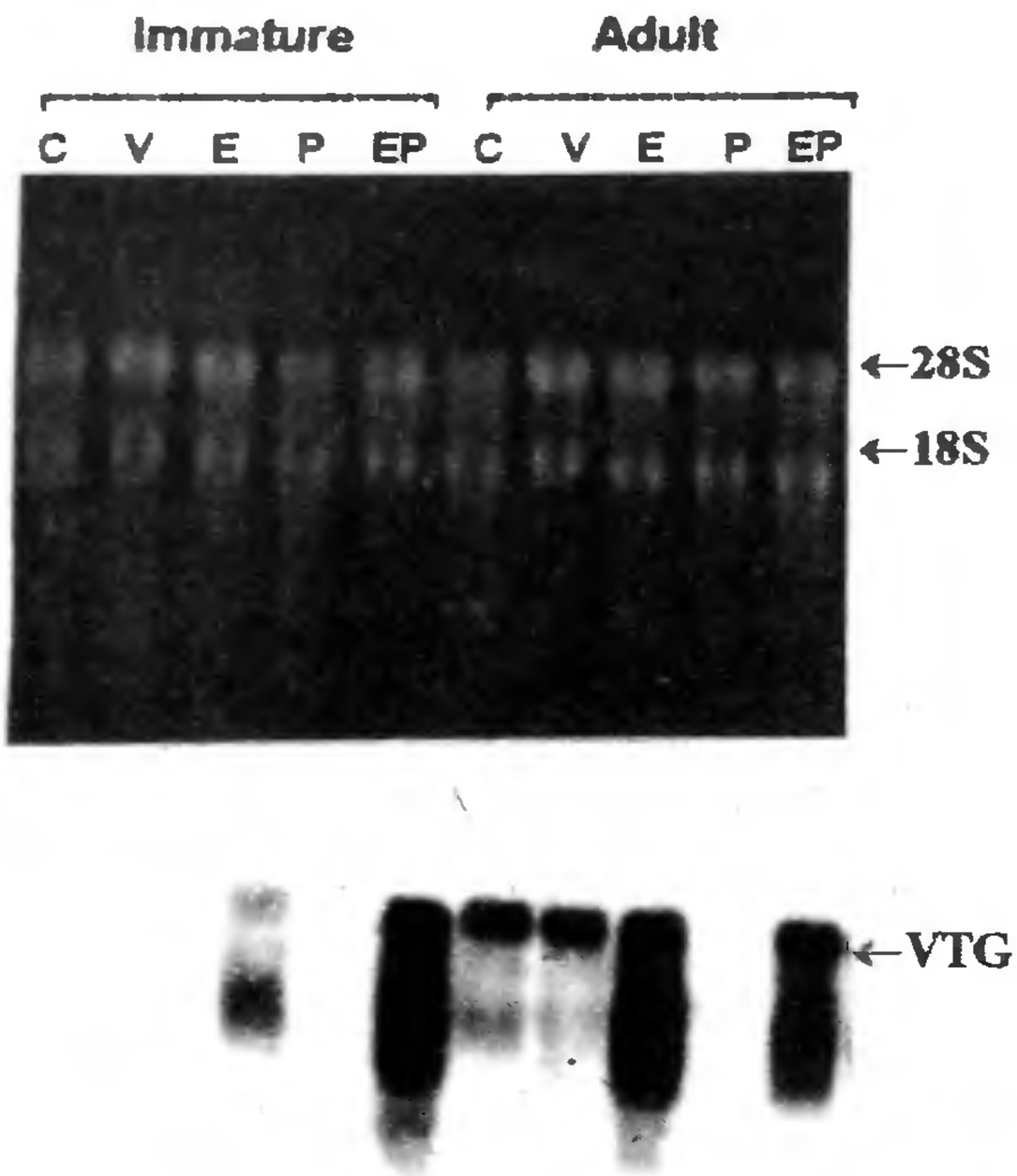

Figure 5. Northern blot hybridization of total RNA (15 μg) of liver of Japanese quail on formaldehyde denaturing gel electrophoresis and hybridized with ^{32}P-labelled VTG cDNA. RNA was purified from the birds 48 h after administration of either estradiol (E) or progesterone (P) or E + P, (a) Immature bird (2–3 week); (b) adult bird (20–30 week). C-control; V-vehicle (Source: Gupta *et al.*[20]).

VTG-mRNA was expressed and its level increased greatly after estradiol administration[21]. Progesterone, however, completely repressed its expression. Estradiol and progesterone together induced its expression, but less than that by estradiol alone. Thus progesterone has an inhibitory effect on the expression of the VTG gene, both in the immature and the adult birds (Figure 5).

A -CCGG- sequence is present between the ERE and the PRE of the VTG gene promoter. Methylation of the internal cytosine of -CCGG- in the promoter of several genes has been shown to inhibit their expression. The restriction enzyme, MspI cleaves the -CCGG- sequence whether it is methylated or unmethylated, but Hpa II cleaves it only if it is unmethylated. Hence the methylation status of the VTG promoter can be studied using the two enzymes. High m.w. DNA of the liver was digested by EcoRI, or EcoRI + Msp I or EcoRI + Hpa II, the DNA fragments were resolved on 1.5% agarose gel and hybridized to a 1.3 kbp VTG-DNA fragment of the promoter region (–1484 to –75 bp) that encompasses the ERE and the PRE. It was seen that in the adult bird, Hpa II cleaves the 1.3 kbp fragment of the promoter at the -CCGG- site[21]. Hence the -CCGG- sequence is unmethylated in the adult in which the gene is expressed and egg is formed. We have further shown that in the immature bird which does not lay eggs and in which the gene is not expressed, the -CCGG- sequence is methylated. In the old bird which also does not lay eggs, the sequence gets methylated (to be published).

Immature (2–3 weeks) and adult (20–25 weeks) birds were administered estradiol or progesterone or E + P to find out if the *trans*-acting factors that bind to the *cis*-acting elements are induced by the hormones. The promoter region of the VTG gene was restricted with enzymes to obtain a 112 bp fragment that contained the ERE, and a 149 bp fragment that contained the PRE. The ^{32}P-labelled fragments were incubated separately with nuclear extract of the liver, and gel shift assays were carried out. It was seen that in the control immature and adult birds only one protein factor binds to the ERE. In estradiol-treated birds its level is greatly induced as also in E + P treated birds (Figure 6). In progesterone-treated birds, the factor is also induced in the immature, but in the adult, greater retardation of the factor is seen. It indicates that after progesterone administration either the factor gets modified or an inhibitor is synthesized, that binds to the factor, and hence retards its mobility. Later we have shown that the levels of these factors are either lower in old birds, or the factors are modified (to be published).

Another factor that may contribute to the decline in gene expression during ageing, especially in post-mitotic cells such as neurons and muscle cells that stop dividing soon after birth, is the increasing condensation of the chromatin. This has been shown to occur in the brain of the rat. When nuclei of the brain are incubated

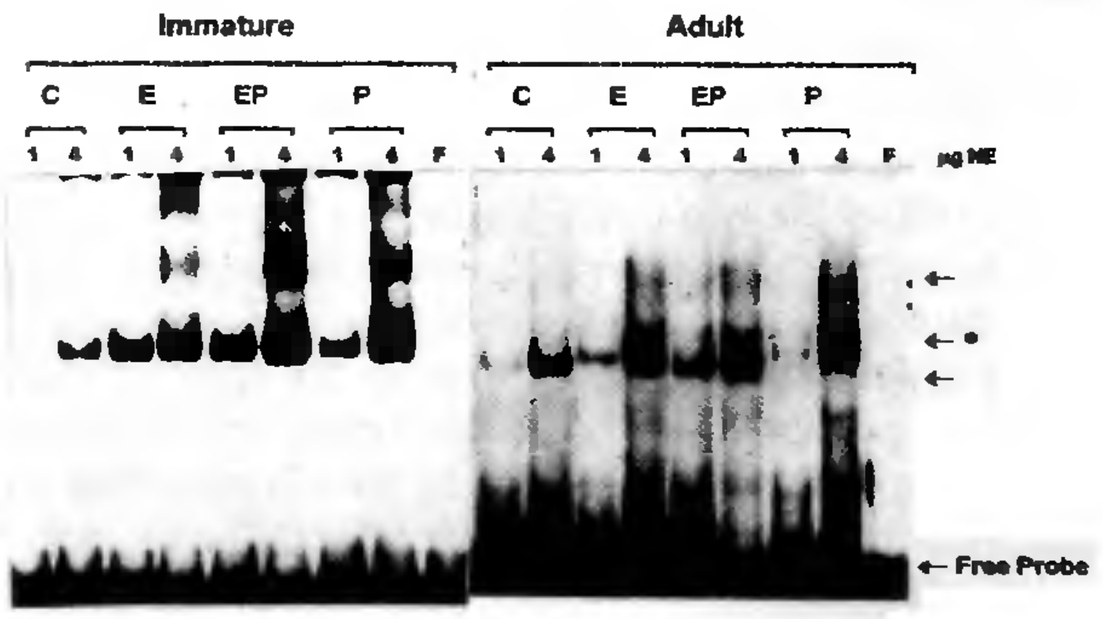

Figure 6. Gel mobility shift assay of the 112 bp dsDNA fragment of the promoter region of the VTG gene containing the ERE. One and 4 μg nuclear extract (NE) of the liver of immature (2–3 week) and adult (20–30 week) Japanese quail were incubated for 10 min with ^{32}P-labelled VTG-ERE. NE was prepared from the birds 48 h after administration of estradiol (E) or progesterone (P) or E + P (Source: Gupta *et al.* [21]).

with DNAase I that digests the DNA and produces fragments of 10 bp and its multiples, less of 10 bp fragments are produced in the old[22]. When acetylation of histones in the chromatin is carried out using nuclei of the brain, the degree of acetylation is significantly lower in the old brain. Also, the degree of transcription in these nuclei is greatly decreased[23]. Condensation of the chromatin would not only hinder the accessibility of the transcription start sites of genes to transcription factors that are required for transcription but also of *cis*-acting elements to *trans*-acting factors that are required for regulation of transcription. Hence both the transcription and the degree of regulation of the expression of genes would decline as a function of age in these cells.

Conclusions

The above studies have shown that the expression of genes in various organs is regulated by *trans*-acting nuclear protein factors that bind to specific *cis*-acting elements present in their promoters. The decline in the expression of genes that occurs after adulthood and leads to ageing is due to the decline in the levels of these factors or due to their modifications. These factors are inducible by specific hormones and other effectors whose homeostatic balance and levels are destablized due to various types of stresses that the organism encounters during its adulthood. It is possible, therefore, to extend the period of expression of the genes by maintaining the levels of the effectors and hormones. This would extend the period of activity of various functions, and thereby prolong the period of adulthood or defer the process of ageing.

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