



0531-5565(95)00004-6

LIFE SPAN PREDICTION FROM THE RATE OF AGE-RELATED DNA DEMETHYLATION IN NORMAL AND CANCER CELL LINES

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Abstract—A method has been proposed for the “Hayflick Limit” prediction by the analysis of the 5-methylcytosine content in DNA at earlier and later cell passages. The following facts were used as the basis of the method: (i) the rate of m^5C loss from DNA remains approximately constant during cell divisions and it does not depend on the cell donor age; (ii) this rate is inversely proportional to the “Hayflick Limit” as well as to the life span of cell donor species; (iii) the period corresponded to loss of all m^5C residues from the genome coincides with or somewhat exceeds the “Hayflick Limit” of normal cells. The prognosis of the “Hayflick Limit” has usually been found in good agreement with the experimental data for the various human, hamster, and murine normal and cancer cell lines. The age-related m^5C loss may result from accumulation of the m^5C -to-T+C transition mutations occurring with DNA methylation in every division of normal cells. In cancer cells, DNA methylation system begins to operate as if in the opposite direction increasing the m^5C content in genome. The method proposed may be used for early detection of precrisis and cancer cells.

Key Words: DNA methylation, 5-methylcytosine (m^5C), age-related m^5C loss, m^5C -to-T+C transitions, “Hayflick Limit”, cell aging and immortalizing

INTRODUCTION

IN THE CLASSIC study of Hayflick (1965), the division of animal normal cells in culture has been found to be usually limited, and the number of cell doublings until cessation of proliferation was termed the “Hayflick Limit”. One of the possible mechanisms counting cell divisions may be related to gradual shortening of telomeric DNA sequences with each cell division (Counter *et al.*, 1992). DNA methylation can be hypothesized as the other such mechanism.

DNA methylation is a natural enzymatic modification, which in eukaryotes proceeds by DNA(cytosine-5)-methyltransferase [MTase: EC 2.1.1.37] during and after DNA replication (Adams *et al.*, 1990). This enzyme transfers CH_3 groups from S-adenosyl-L-methionine to the C5 atom of cytosine residues to produce 5-methylcytosine (m^5C ,

(Received 3 March 1994; Accepted 25 November 1994)

*C) at *CG and *C⁵TG sites of DNA (Woodcock *et al.* 1987). This DNA modification system is typical for the majority of eukaryotes but far from all species (Mazin *et al.*, 1984). DNA methylation may affect many genetic processes, although the true biological function of this modification is not yet clear.

The m⁵C residues were noticed to be the subject to deamination to "minor" thymine, and that is why they are "hotspots" for m⁵C→T transitions in DNA (Coulondre *et al.*, 1978). Methylation of a cytosine at a CG site is through a many-fold increase in the potential for CG→TG or CG→CA mutations (Cooper and Youssoufian, 1988; Sved and Bird, 1990; Mazin, 1993c, 1994). Therefore 75-80% of the CG sites were lost from vertebrate DNA during evolution (Josse *et al.*, 1961; Mazin and Vanyushin, 1987a,b). It has been found recently that deamination of m⁵C residues may proceed both spontaneously and directly during the very DNA methylation, and this reaction is catalyzed by the same MTase (Ivanetich and Santi, 1992; Shen *et al.*, 1992; Mazin *et al.*, 1985; 1993c). Thus, m⁵C→T transitions evidently are a result of the DNA methylation as such, and MTases are the generators of a special kind of mutations in cells (Mazin, 1993c).

It has been shown in many laboratories that gradual loss of m⁵C from DNA takes place with cell aging both in vivo (Wilson *et al.*, 1987; Mays-Hoopes, 1989; Helden and Helden, 1989) and in vitro (Table 1). The animal genome may lose the bulk of, if not all, m⁵C residues during the life span (Mazin, 1993a), and this phenomenon usually coincides with the "Hayflick Limit" of aging cell lines (Mazin, 1993b). Age-dependent m⁵C loss was recently explained by accumulating in DNA of m⁵C-to-T+C transitions, which occur with every cell division as a result of DNA methylation (Mazin, 1993a-c). In the present report this phenomenon is considered as one of the possible molecular mechanisms underlying the "Hayflick Limit".

THEORY OF THE METHOD

Let us consider some results of the m⁵C→T substitutions at CG sites of DNA. If during replicative DNA methylation (Fig. 1, 1) m⁵C deamination takes place (Fig. 1, 2) then mismatched G-T pairs will occur in DNA. These mispairs are usually corrected by the special G/T-repair system (Brown and Jiricny, 1987) to restore the initial G-C pairs in DNA (Fig. 1, 3). Occurring hemimethylated CG sites are a good substrate for maintenance MTase and will be modified postreplicatively (Fig. 1, 4). As a result, some portion of newly formed m⁵C residues will be deaminated again (Fig. 1, 5), and the cycle (5→2→5) may be repeated several times until the next S-phase (II). Symmetrical methylated and nonmethylated CpG duplexes will occur in DNA after the next DNA replication round (Fig. 1, 7). Hence, m⁵C→C substitutions will be accumulated in DNA with each cell division.

It has been found that 1-2% of G-T mispairs by this or other reasons are not repaired at all (Brown and Jiricny, 1987) and preserved in DNA until the next replication (Fig. 1, 8). In addition, about 8% of G-T mispairs may be repaired not into G-C but into A-T pairs by mistake (Fig. 1, 6). In both cases, instead of *CG, new TG and CA dinucleotides appear after DNA replication (II), and CG→TG+CA mutations take place in DNA (Fig. 1, 8).

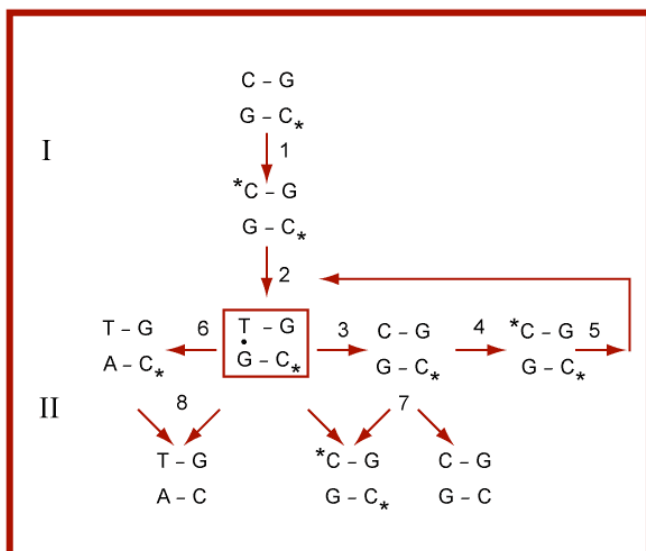


FIG. 1. Scheme of m⁵C→T+C substitutions occurring as result of replicative (1) and postreplicative (4) DNA methylation, of m⁵C deamination (2 and 5), and of G/T-repair mistakes (6 and 8) during a cell cycle (I-II).

The $m^5C \rightarrow T$ transitions are, in essence, irreversible, as the probability of inverse mutations is very small, and will be continuously accumulated in the genome with time. That is why cytosine methylation in DNA of long-lived organisms would lead to the following main results: (i) loss of m^5C from DNA with aging (Fig. 1, 7), and (ii) gradual accumulation of $*CG \rightarrow TG + CA$ mutations in DNA (Fig. 1, 8) (Mazin, 1993a-c; 1994). The m^5C loss from DNA with cell divisions can be described by the function:

$$M_n = M_0(1 + k/100)^n \quad (1)$$

where M_0 is the m^5C content in DNA at the present moment, M_n is the content after a certain number (n) of cell population doublings (PD), and k is the rate of m^5C loss from DNA in relative percents per one PD.

TABLE 1. AGE-RELATED DYNAMICS OF DNA METHYLATION IN NORMAL AND CANCER CELLS

Cell lines	$m^5C \times 100 / (m^5C + C)$, % ^a			Number of PD ^b		Rate of m^5C loss, ^c %/PD (k)	"Hayflick Limit", PD		References
	M_0	M_n	$(M_n - M_0)$ %	n_0	n_i		H^d	Experiment	
Human								70-100	1, 2
MRC-5	3.60	1.80	-50.0	20	56	-1.39	72	69-71	3
MRC-5	4.82	3.35	-30.5	18	39	-1.45	69		4
MRC-5, imm.						-0.002	>50000		4
MRC-5, imm.						0.014	∞		4
MRC-5	3.50	0.30	-91.4	20	43	-3.97	25		5
MRC-5, imm.	4.00	4.00	0.0	650	750	0.0	∞	>750	5
IMR-90	3.25	2.55	-21.5	18	48	-0.72	139	100	1
IMR-90	3.35	2.24	-31.1	25	61	-0.86	116	65-70	6
IMR-90, imm.	2.60	2.72	4.4	139	170	0.14	∞	>170	6
T1	3.00	2.37	-21.0	8	36	-0.75	133		1
A2 (11)	54.0 ^e	48.0	-11.1	17	58	-0.27	370	62	7
DS (36)	58.5 ^e	48.0	-18.0	17	53	-0.50	200	60	7
J089 (68)	55.0 ^e	51.6	-6.4	14	31	-0.38	263	41	7
J088 (76)	52.0 ^e	44.5	-14.4	16	42	-0.55	182	47	7
T98G, imm.	2.70	2.94	8.2	421	451	0.27	∞	>451	6
Hamster								25-30	1, 2
SHE	2.90	1.75	-39.7	0	25	-1.59	63	25	1
SHFB			-42.5		17	-2.50	40		8
SHEB, imm.			0.0			0.0	∞		1
FAF-28	4.37	3.29	-24.7	3 ^f	17 ^f	-1.76	57		9
CHO, imm.	3.21	3.15	-1.9	1	6	-0.38	263		10
Mouse								10-15	2
C3HME	3.75	0.85	-77.3	0	5	-15.46	6	6	1
C3YCIV, imm.	2.95	3.64	19.0	5	23	1.05	∞		1
3T3, imm.	3.60	3.75	4.0	6	17	0.36	∞		1
10T1/2, imm.	2.60	2.75	5.5	6	17	0.50	∞		1
PCC3/A, imm.	4.20	3.80	-9.5	1	30	-0.33	303		11

References: 1) Wilson and Jones, 1983; 2) Holliday, 1986; 3) Fairweather *et al.*, 1987; 4) Matsumura *et al.*, 1989; 5) Fairweather *et al.*, 1985; 6) Grey *et al.*, 1991; 7) Shmookler-Reis and Goldstein, 1982; 8) Fairweather, 1989; 9) Chochlov *et al.*, 1988; 10) Palitti *et al.*, 1990; 11) Fabricant *et al.*, 1979.

^aLevel of cytosine methylation in cell DNA: M_0 - at an earlier passage (n_0), M_n - at a later passage (n_i).

^bPD, number of cell population doublings.

^cRate of age-related changes in m^5C content in DNA; k from equation (2).

^d H , prognosis of "Hayflick Limit" from equation (3).

^ePercents of methylated CCGG sites in DNA, in brackets, age of cell donors (years).

^fAge in days.

The theoretical graphs of DNA hypomethylation are shown in Fig. 2. If $k < 0$ m^5C content is seen to decrease faster, the higher the rate of m^5C loss, and vice versa. So, if $k = -20\%$ the loss of 50% of m^5C residues proceeds after 3 PD (graph 1), if $k = -5\%$ after 14 PD (graph 2), and if $k = -1\%$ only after 70 PD (graph 3). Thus, eventually most of m^5C residues would be lost from the DNA of actively dividing cells (Fig. 2).

It is obvious that the initial part of graphs 1-3 may be approximated by the direct line in the first approach. If, for convenience, we assume the function of the m^5C loss from DNA to be close to linear, then the rate of DNA demethylation can be determined according to the linear regression:

$$M_n = M_0 + nk \quad (2)$$

And, the potential number of PD up to 100% m^5C loss, that is, the limit of DNA demethylation (H), can be calculated from the equation:

$$H = \frac{100n}{M_n - M_0} \quad (3)$$

The H limit has the same physical meaning as the “Hayflick Limit.”

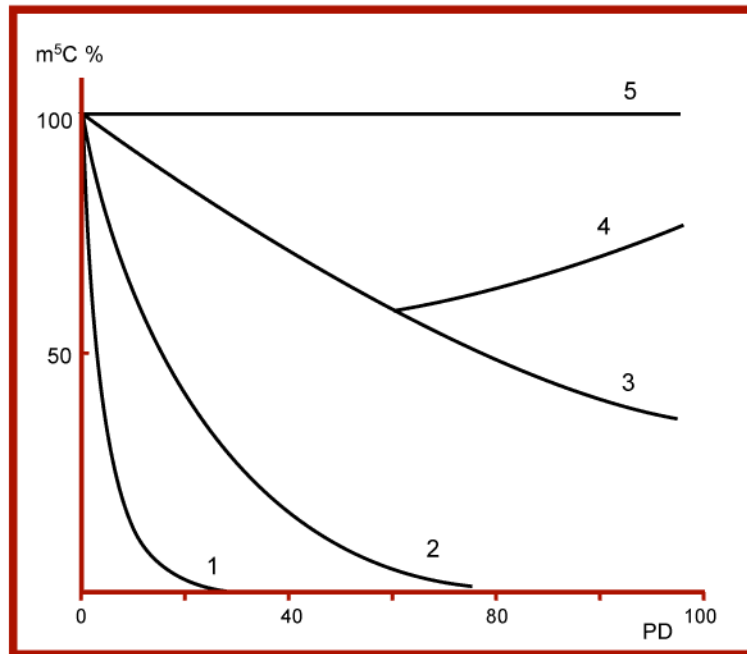


FIG. 2. Dynamics of age-related DNA methylation. Theoretical graphs calculated from equation (1) for different rates of m^5C loss: 1) $k = -20\%$, 2) $k = -5\%$, 3) $k = -1\%$, 4) $k = 0.5\%$, 5) $k = 0\%$ per one cell population doubling (PD).

METHOD

The level of cytosine methylation ($m^5C \times 100 / (m^5C + C)$) in total DNA can be determined by any method usually used for m^5C analysis (Table 1). First of all the m^5C content should be found in DNA isolated from cells before cultivation or during the earliest passages (M_0). Then, after a certain number (n) of cell population doublings the m^5C content in DNA is determined again (M_n). The rate of age-related m^5C loss from DNA (k) may be found according to the function (1) or (2), and the DNA demethylation limit (H) - from the formula (3). The more experimental points we have, the more precise is the determination of the H value and of the expected "Hayflick Limit".

In Table 1, I included all known data with the exception of TM and J069 human fibroblasts from the paper of Shmookler-Reis and Goldstein (1982). The reliability of these results seem to be doubtful because the method used was not very precise, the number of passages between assessments was relatively small, and the initial DNA methylation level was also lower.

RESULTS

The level of cytosine methylation in DNA of normal cell cultures is lowered considerably with age (Table 1). In DNA of human diploid fibroblasts (DF) MRC-5, the m^5C content decreases by 50% in 36 PD or by 30.5% in 21 PD, and the rate of m^5C loss from DNA (k) is equal to -1.39% and to -1.45% per PD, respectively. In human DF IMR-90 this rate is -0.72% or -0.86%, and in murine embryonic cells C3HME -15.46% per PD (Table 1). The two latter examples may be approximated by theoretical graph 3 and 1, respectively (Fig. 2).

The rate of m^5C loss from DNA remains more or less constant during the period of active cell division in culture (Wilson and Jones, 1983; Fairweather *et al.*, 1985; 1987; Matsumura *et al.*, 1989; Gray *et al.*, 1991). In this case there is an analogy with the linear dependence of the age-related DNA hypomethylation in tissues of adult animals (Wilson *et al.*, 1987; Mays-Hoopers, 1989; Mazin, 1993a). Hence, the linear eqn (2) is quite adequate to the experimental data.

The rate of DNA demethylation in human DF lines A2, DS, T089, and T088 does not depend on the age of the cell donor (Table 1). If time is determined in days, this rate drops by 20 times with a decrease in the portion of dividing cells in the population (Fairweather, 1989). By analogy, during animal embryogenesis the rate of DNA demethylation falls by a factor of 29 to 96 in adult organisms of various species (Mazin, 1993a). Thus, the rate of m^5C loss is likely to reflect the intensity of the replicative DNA methylation itself (Fig. 1, 1) and therefore to depend mainly on the proliferative activity of the cell population (Mazin, 1993a).

If cells become immortal the m^5C loss from DNA usually slows down quickly (Table 1). Thus, as a result of SV40 transformation of MRC-5, a stable level of DNA methylation is observed during more than 100 passages (Fairweather *et al.*, 1985). This situation corresponds to theoretical graph 5 where $k = 0$, $M_n = M_0$ for any n , and H becomes infinitely large (Fig. 2). Moreover, this process can change direction, and instead of m^5C loss, a gradual increase in the m^5C content is observed in the DNA of cancer cell lines (Table 1).

For instance, in precrisis AG3204 cells derived by SV40 transformation of IMR-90, the rate of m^5C loss from DNA initially decreases from -0.86% to -0.67% per PD (Gray *et al.*, 1991). Then, m^5C is no longer lost, and its accumulation in DNA is observed in transformed cells AG2804 at the rate of 0.14% per PD. In Fig. 2 theoretical graphs 3 and 4 may describe this experimental situation. Another example is related to the murine cells C3HME, which lose 77% of m^5C in only 5 PD and then stop to divide (Table 1). After spontaneous transformation the m^5C content in DNA of C3YCIV cells increases at the rate of 1.05% and reaches the initial value during 18 PD. The m^5C content in DNA of immortal cells increases at a constant rate, as a rule (Table 1). It is intriguing that the dynamics both of DNA methylation and of the telomeric DNA shortening, in principle, change with cell immortalization in the same direction (Counter, *et al.*, 1992).

The rate of m^5C loss from DNA markedly varies in cell cultures from different species (Table 1). The average rate for human normal DF (without data for MRC-5 (Fairweather *et*

al., 1985) is -0.76%, for hamster cells -2.05%, and for murine cells C3HME - 15.46% per PD. By analogy, this rate for adult mice is 10.2% of m⁵C loss per year and their maximum life span is about 3.5 years, for rats 8.8% and 4 years, for hamster 2.6% and 8 years, for cows 1.5% and 30 years, and for human 0.18% and 120 years, respectively (Mazin, 1993a). The life span of mice is 34 times shorter than humans, and their genome loses m⁵C in vivo 57 times faster, and in vitro 20 times faster than human cells do. Hence, an inverse correlation is observed between this rate and the life span of animal species (Wilson *et al.*, 1987).

The data in Table 1 support an earlier conclusion that the rate of DNA demethylation in cell cultures is usually inversely proportional to their "Hayflick Limit" (Wilson and Jones, 1983). Murine embryonic cells C3HME have a maximal rate of m⁵C loss and are capable of only 6 PD. As well as in vivo, the m⁵C is lost from the DNA of mouse cells 57 times faster than from DNA of human cells, and on average the "Hayflick Limit" of the mouse C3HME cells is 22 times shorter than that of human lines (Table 1).

Knowing the rate of age-related m⁵C loss (*k*), it is easy to calculate using eqn (3) the potential limit of DNA demethylation (*H*) in a corresponding cell line (Table 1). For instance, the *H* prognosis for MRC-5 is equal to 69-72 PD, and it is in good accord with the experimental estimates of their "Hayflick Limit", 69-71 PD. The predicting *H* limit for IMR-90 runs as high as 116-139 PD and are somewhat higher than the "Hayflick Limit", 65-100 PD. The differences between the *H* value and the "Hayflick Limit" observed in human lines A2, DS, J089, and J088 can be partly explained by the m⁵C analysis only at CCGG sites of DNA. In murine cells C3HME, capable of only about 6 PD, all the m⁵C residues are lost also during 6 PD.

Hence, the experimental estimates of the "Hayflick Limit" coincide with, or are somewhat lower than, the *H* prognosis calculated from the rate of age-related DNA demethylation (Table 1). Evidently, most of the cells in a population can reach the "Hayflick Limit" long before their genome loses all m⁵C residues. The situation with aging in vitro and in vivo is similar: the animal genome may lose the bulk of, if not all, m⁵C residues during its life span (Mazin, 1993a,b).

DISCUSSION

The natural process of enzymatic DNA methylation can be considered as an ideal "counter" of cell divisions. A half of m⁵C residues arises in each round of DNA replication (Fig. 1, 1) as a result of maintenance methylation of the daughter DNA chain. Up to 30-50% of newly formed m⁵C is usually deaminated to thymine residues (Fig. 1, 2) (Gulp *et al.*, 1970; Mazin *et al.*, 1985; Mazin, 1993c). About 10% of m⁵C→T transitions are incorrectly or no repaired (Fig. 1, 6 and 8) (Brown and Jiricny, 1987), and gradually accumulate in the genome with each cell division. Complete exhaustion of the m⁵C reserve in DNA seems to reflect accumulation of some critical load of the *CG→TG+CA mutations in the genome (Mazin and Vanyushin, 1987a,b; Cooper and Youssoufian, 1988). They can disturb many genetic functions and result in aging of cells, organs, and of the entire organism (Mazin, 1994).

The simplicity with which this mechanism functions should be especially stressed. Only one special gene coding for MTase is required. Accumulation of m⁵C→T transitions in DNA is intrinsically irreversible, discrete, and finite (Mazin, 1993c). This mechanism is a part of the DNA replication system (Leonard *et al.*, 1992), and automatically counts one cell cycle after another like a clock. It can stop cell proliferation when a critical number of the mutations accumulates and the m⁵C reserve in the genome is depleted.

The m⁵C content in mammalian DNA is usually equal to 1 mole % or about 3x10⁷ residues per genome (Table 1). If the efficiency of the G/T-repair system is about 90% (Brown and Jiricny, 1987), the loss of all m⁵C residues from DNA may correspond to the accumulation of about 3x10⁶ *C→T transitions per genome or 3x10³ per each site during the life span. Thus, at least one such mutation, on average, may take place in every gene by the end of life span, and this may cause a "catastrophe of errors." Analysis of mutations has shown that CG→TG+CA transitions contribute up to 30-40% of all single-base substitutions observed among human genes (Cooper and Youssoufian, 1988). Methylation of the CG sites in the human clotting factor IX gene may generate from 50 to 70% of 750 point mutations causing hemophilia B (Mazin, 1995). However, these estimates may reflect only a part of the m⁵C-dependent mutations because another part of m⁵C residues may be at the *C_TG sites of human DNA (Woodcock *et al.*, 1987). So much may be a direct contribution

of gene methylation to overall mutability.

The rapidly increasing number of m⁵C→T mutations can activate, with age, some protooncogenes, like p53 (Jones *et al.*, 1992). Cell transformation may start some "anti-aging" mechanism that activates *de novo* DNA methylation or in other ways lead both to growth or stabilization of the m⁵C content in DNA and to the immortalization of cells (Table 1). This DNA modification, as well as telomere shortening, allows us to consider cell aging and immortalization as two sides of the same coin (Counter *et al.*, 1992; Mazin, 1994).

The idea that DNA methylation is an endogenous generator of mutations is a point in support of the concept of the accumulation of stochastic errors with aging (Szilard, 1959). On the other hand, DNA methylation can be regarded as a special genetic program that is inherited within genome of germ-line cells (Hayflick, 1965; 1991). DNA, especially from female germ cells, is methylated to a far lesser degree than that of somatic cells (Driscoll and Migeon, 1990), and this may protect them from the CG-mutagenesis. Establishment of this genetic program begins at the earliest stages of embryonal development presumably from the moment of *de novo* DNA methylation (Mazin, 1993a; 1994). Then this mechanism may work automatically and essentially does not require any additional regulation.

It may be concluded that enzymatic DNA methylation combines rather naturally both main concepts of aging and can be considered as a genetically programmed mechanism for accumulating mutations with age.

Acknowledgments — This work was supported by the Russian State Programs Frontiers in Genetics and the Human Genome. The author thanks Irina Frenkel for help in preparation the manuscript.

REFERENCES

- ADAMS, R.L.P., BRYANS, M., RINALDI, A., SMART, A., and YESUFU, H.M.I. Eukaryotic DNA methylation and their use for in vitro methylation. *Philos. Trans. R. Soc. London, Ser. B* 326, 189-198, 1990.
- BROWN, T.C. and JIRICNY, J. A specific mismatch repair event protects mammalian cells from loss of 5-methylcytosine. *Cell* 50, 945-950, 1987.
- CHOCHLOV, A.N., KIRNOS, M.D., and VANYUSHIN, B.F. Level of DNA methylation and in vitro cellular aging. *Isvest. Acad. Sci. USSR. Ser. Biol.* 3, 476-478, 1988.
- COOPER, D.N. and YOUSOUFIAN, H. The CpG dinucleotide and human genetic disease. *Hum. Genet.* 78, 151-155, 1988.
- COULONDRE, C., MILLER, J.H., FARABAUGH, P.J., and GILBERT, W. Molecular basis of basesubstitution hotspots in *E. coli*. *Nature* 274, 775-780, 1978.
- COUNTER, C., AVILION, A.A., LEFEUVRE, C.E., STEWART, N.G., GREIDER, C.W., HARLEY, C.B., and BACCHETTI, S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* 11, 1921-1929, 1992.
- CULP, L.A., DORE, E., and BROWN, G.M. Methylated bases in DNA of animal origin. *Arch. Biochem. Biophys.* 136, 73-79, 1970.
- DRISCOLL, D.J. and MIGEON, B.R. Sex difference in methylation of single-copy genes: Implications for X chromosome inactivation, parental imprinting and origin of CpG mutagenesis. *Somat. Cell. Mol. Genet.* 16, 267-282, 1990.
- FABRICANT, J.D., WAGNER, E.F., AUER, B., and SCHWEIGER, M. 5-methylcytosine content in DNA during differentiation in mouse teratocarcinoma cells. *Exp. Cell. Res.* 124, 25-29, 1979.
- FAIRWEATHER, D.S. DNA methylation falls in vitro because of cell division not duration of culture. *Clin. Sci.* 76, Suppl. 20, 12, 1989.
- FAIRWEATHER, D.S., FOX, M., and MARGISON, G.P. DNA methylation: A new theory of aging. *Clin. Sci.* 69, Suppl. 12, 53-54, 1985.
- FAIRWEATHER, D.S., FOX, M., and MARGISON, G.P. The in vitro life span of MRC-5 cells is shortened by 5-azacytidine-induced demethylation. *Exp. Cell. Res.* 168, 153-159, 1987.
- GREY, M.D., JESCH, S.A., and STEIN, G.H. 5-azacytidine-induced demethylation of DNA to senescent level does not block proliferation of human fibroblasts. *J. Cell. Physiol.* 149, 477-484, 1991.
- HAYFLICK L. The serial cultivation of human diploid cell strains. *Exp. Cell. Res.* 37, 614-636, 1965.
- HAYFLICK L. Aging under glass. *Mutat. Res.* 256, 69-80, 1991.
- HOAL-VAN HELDEN, E.G. and HELDEN, P.O. Age-related methylation changes in DNA may reflect the proliferative potential of organs. *Mutat. Res.* 219, 263-266, 1989.
- HOLLIDAY, R. Strong effect of 5-azacytidine on the in vitro lifespan of human diploid fibroblasts. *Exp. Cell. Res.* 166, 543-552, 1986.
- IVANETICH, K.M. and SANTI, D.V. 5,6-dihydropyrimidine adducts in the reactions and interactions of pyrimidines with proteins. *Progr. in Nucl. Acids and Mol. Biol.* 42, 127-156, 1992.
- JONES, P.A., RIDEOUT III, W.M., SHEN, J.-C., SPRUCK, C.M., and TSAI, Y.C. Methylation, mutation and cancer. *Bioessays* 14, 33-36, 1992.
- JOSSE, J., KAISER, A.D., and KORNBERG, A. Frequencies of nearest neighbor base sequences in DNA. *J. Biol. Chem.* 236, 864-875, 1961.
- LEONHARDT, H., PAGE, A.W., WEIER, H.-U., and BESTOR, T.H. A targeting sequence directs DNA

- methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* 71, 865-873, 1992.
- MATSUMURA, T., HUNTER, J.L., FAROOQ, M., and HOLLIDAY, R. Maintenance of DNA methylation level in SV40-infected human fibroblast during their in vitro limited proliferative life span. *Exp. Cell. Res.* 184, 149-157, 1989.
- MAYS-HOOPES, L. Age-related changes in DNA methylation: Do they represent continued developmental changes? *Int. Rev. Cytol.* 114, 181-220, 1989.
- MAZIN, A.L. A role of enzymatic methylation of regulatory elements in the control of gene activity in different groups of organisms. *Mol.Biol. (Moscow)* 26, 244-263, 1992.
- MAZIN, A.L. The genome loses all 5-methylcytosine during the lifespan: How is this related to accumulation of mutations with aging? *Mol.Biol. (Moscow)* 27, 160-173, 1993a.
- MAZIN, A.L. The loss of all genomic 5-methylcytosine coincides with "Hayflick Limit" of aging cell lines. *Mol.Biol. (Moscow)* 27, 895-907, 1993b.
- MAZIN, A.L. A mechanism of replicative and postreplicative DNA methylation as a generator of mutations in cells. *Mol.Biol. (Moscow)* 27, 965-979, 1993c.
- MAZIN, A.L. Enzymatic DNA methylation as an aging mechanism. A review. *Mol.Biol. (Moscow)* 28, 21-51, 1994.
- MAZIN, A.L. Methylation of the factor IX gene is the main cause of mutations responsible for hemophilia B. *Mol.Biol. (Moscow)* 29, 73-92, 1995.
- MAZIN, A.L., GIMADUTDINOV, O.A., TURKIN, C.I., BURTCEVA, N.N., and VANYUSHIN, B.F. Nonenzymatic DNA methylation by S-adenosylmethionine results in formation of 5-methylcytosine and minor thymine residues from cytosine moiety. *Mol.Biol. (Moscow)* 19, 903-913, 1985.
- MAZIN, A.L., MUCHOVATOVA, L.M., SHUPPE, N.G., and VANYUSHIN, B.F. The absence of 5-methylcytosine in DNA of *Drosophila melanogaster* and *D. virilis*. *Dokl. Acad. Sci. USSR* 276, 760-762, 1984.
- MAZIN, A.L. and VANYUSHIN, B.F. CpG suppression in DNA. I. Methylated and nonmethylated compartments of the genome in eukaryotes with a different content of 5-methylcytosine in DNA. *Mol.Biol. (Moscow)* 21, 543-551, 1987a.
- MAZIN, A.L. and VANYUSHIN, B.F. CpG suppression in DNA. II. Methylated and nonmethylated genes of vertebrates. *Mol.Biol. (Moscow)* 21, 552-561, 1987b.
- PALITTI, F., COZZI, R., D'ERME, M., BONA, R., and PERTICONE, P. Methylation level and damage in mammalian cells in vitro. *Abstr. Meet. Ital. Biochem. Soc.* 134-137, 1990.
- SHEN, J.-C., RIDEOUT III, W.M., and JONES, P.A. High frequency mutagenesis by a DNA methyltransferase. *Cell* 71, 1073-1080, 1992.
- SHMOOKLER-REIS, R.J. and GOLDSTEIN, S., Variability of DNA methylation patterns during serial passage of human diploid fibroblasts. *Proc. Natl. Acad. Sci. USA* 79, 3949-3953, 1982.
- SVED, J. and BIRD, A. The expected equilibrium of the CpG dinucleotide in vertebrate genome under a mutational model. *Proc. Natl. Acad. Sci. USA* 87, 4692-4696, 1990.
- SZILARD, J. On the nature of the aging process. *Proc. Natl. Acad. Sci. USA* 45, 30-45, 1959.
- WILSON, V.L. and JONES, P.A. DNA methylation decreases in aging but not in immortal cells. *Science* 220, 1055-1057, 1983.
- WILSON, V.L., SMITH, R.A., MA, S., and CUTLER, R. Genomic 5-methylcytosine decreases with age. *J. Biol. Chem.* 262, 9948-9951, 1987.
- WOODCOCK, D.M., CROWTHER, P.J., and DIVER, W.P. The majority of methylated deoxycytidines in human DNA are not in the CpG dinucleotides. *Biochem. Biophys. Res. Commun.* 145, 888-894, 1987.