A NEUROCHEMICAL CREATIONIST CONCEPT BASED ON *IN VITRO* STUDIES OF BRAIN mRNAs OF THREE LUMBER VOLE SPECIES: *Clethrionomys glareolus, Clethrionomys frater* and *Clethrionomys gapperi*

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Abstract

*I carried out a complex comparative neurochemical study of the translation machinery functioning in the brain cells of three conventionally "phylogenetically related" species of wild lumber voles (Clethrionomys glareolus, Clethrionomys frater and Clethrionomys gapperi). 1 found that the cytoplasm of vole brain cells contains one or more oligonucleotide (oligoribonucleotide) factors with molecular weight below 1.0 KD** which are capable of the complete and highly selective inhibition of translation directed by mRNAs which are species-specific templates isolated from analogical tissue (brain) of these so-called "closely related" organisms. This phenomenon was found for the first time using a special cell-free translation system (CFTS) of very different variants of composition consisting of the following main components: (1) post-mitochondrial supernatant (PMS); (2) total cytoplasmic poly(A)$^+$ mRNA or a species-specific poly(A)$^+$ mRNA isolated from the PMS by affinity chromatography on columns with the anti- mRNA$_i$-Fab-(CNBr)-Sepharose, or purified 9S globin or 11S histone specific mRNAs, respectively, and (3) a few samples of the CFTS containing the addition of high or low molecular weight cytosolic compounds isolated from S150 fraction by ultrafiltration in Diaflo UM2 membranes with an exclusion limit of 1.0 KD. All CFTS components listed were isolated separately from the brain tissue of each organism studied.*

A new complex way for constructing and using the CFTS provided enough evidence to suggest the existence of one or more special, and as yet uncharacterized, cytoplasmic oligoribonucleotide factors which efficiently block the cytoplasmic expression of "evolutionally renovated parts" of the genome. These factors seem to be powerful enough to suppress the translation of every mRNA template that is not part of the cell type containing the cytoplasmic suppressors mentioned. Thus they would block the translation of any "novel" mRNA molecules that might have arisen as a result of spontaneous nonlethal gene mutations. This is a case in which gene expression is blocked at the level of mRNA function in the cytoplasm. The origins implications of this finding are discussed.

Introduction

It seems to be quite widely accepted that spontaneous mutation of nuclear DNA can lead to nonlethal or even "useful" modifications of the genome, and that such mutations yield new proteins that ultimately supply new properties to the whole organism, as steps in the ongoing pathway of evolution—see Agadzhanyan and Yatsenko (1984), McIntyre (1985 and 1987), and Gaskil and Pratt (1988).

Analyzing the numerous data which serve as a basis for such a concept of molecular evolution, we must take into account that the majority of these results were obtained by the methodology of cytogenetics and molecular genetics—see Agadzhanyan and Yatsenko (1984), McIntyre (1986), Lefebvre *et al.* (1987 **and** 1988), **and** Umada and Telashima (1988). Nevertheless, it would be a methodological error to believe that phenotypic development is governed only at the transcriptional level where RNA is synthesized from DNA in the nucleus. It is also well known that a large number of concrete pathways exist for regulation of the translation process, directed by messenger RNA-dependent factors inside the cytoplasm—Sheiness *et al.* (1975), Darnell (1978), Katinakis *et al.* (1980), Slater and Burden (1982), Brawerman (1986), Bergman and Brawerman (1987) and Hyden (1988). So, it is logical to assume that there are great possibilities (a majority of which are still unknown) for modulation (inhibition or activation) of polypeptide chain translation. This modulation is dependent upon the templates of mRNA transcribed from so-called "evolutionally origined" DNA sites, *i.e.* novel genes. More specifically, it seems to be a mistake to assume that the origin of a new, "useful," and replicable nuclear gene is sufficient in itself for the renovation of the phenotype or for the formation of a new form of life.

If a molecular mechanism for maintaining constancy of species does exist, this biochemical system must function in the cytoplasm. This is at the level of the cytoplasmic control of gene expression, the level of polypeptide chain translation on the mRNA templates.

We must begin systematic revision of the idea that a single scheme of molecular events is sufficient to cause the evolution of a "natural response" to nonlethal mutagenic spontaneous genome renovation, i.e. the mere origin of the "additional" gene alleles. It seems realistic to search for biochemical evidences where there will be complete suppression of every non-species-specific mRNA by a special organization of the cytoplasmic components surrounding the protein synthesis machinery. If such evidence is obtained, the creationist concept of the origin of the many different and harmoniously functioning forms of life, will be supported by a new argument.

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***'A key to this and other abbreviations is found in Appendix I.**

It may be a fruitful approach to analyze the translational ability of different mixtures in the CFTS consisting of mRNAs and cytoplasmic factor(s) (PMS) isolated from one and the same or from different species of wild animals such as the wild Lumber Voles: *Clethrionomys glareolus (1), Clethrionomys frater (2)* and *Clethrionornys gapperi (3).* According to some modern evolutionist views, these species are closely related phylogenetically through a common biological precursor which went extinct during former "battles for existence" (Agadzhanyan and Yatsenko, 1984), Pelshe and Jarwett (1987) and Lormski and Krawczinska (1987). I have tried to evaluate the possibility that the control of translation is either an evolutionary or an anti-evolutionary evidence.

Animals

Wild lumber voles of the species *Clethrionomys glareolus (1), Clethrionomys frater (2)* and *Clethrionomys gapperi (3)* were purchased from the Petrozavodsk State University Zoological Station. I used only male adult (130-160 g body weight) animals that were kept on a standard vitaminized diet. All animals were starved for 18 h before the experiment and then were killed instantaneously by decapitation. Just after this procedure, the separated heads were immediately placed into liquid nitrogen for 3-4 min. Then, frozen brain tissues from a number of animals of the same species were collected, chopped into thin pieces, and homogenized as described below.

Methods

Pieces of frozen brain tissue (1 x 3 to 2 x 2 mm) were homogenized in three volumes of the medium: 35 mM tris - HCl (pH 8.45), 250 mM sucrose, 50 mM $MgCl_2$, 5 mM KCl, using the Elvehjem-Potter glass homogenizer with a teflon pestle (1,500-1,800 rpm) at 0°C for 10-12 min. After filtration of the homogenate through four layers of gauze the resulting filtrate was centrifuged at 12,000g for 30 min (2°C) for isolation of the post-mitochondrial supernatant (PMS, or S12 fraction). The same portions of filtered homogenates were simultaneously used for isolation of S150 fraction (cytosol). In these cases, the filtrates were centrifuged at 150,000g for 2.5 h, (2 to 4°C).

Total RNA was rapidly and completely extracted from S12 using a guanidine thiocyanate procedure according to Stanley and Fink (1986). Quantitative RNA measurement was carried out as described by Munro and Fleck (1966); protein measurement was performed by the technique of Lowry *et al.* (1951). The resulting total preparation of cytoplasmic RNA was a source for further isolation of poly$(A)^+$mRNA by affinity chromatography on the columns of 1.0 x 8.0 cm packed with the Oligo(dT)-Cellulose using a formamide elution as described by Musajev and Kuznetsov (1988).

In all cases, mRNA samples were precipitated by addition of chemically pure, cold (2°C) ethanol and the resulting mRNA pellets were stored over five weeks (but not more than eight weeks) under a layer of ethanol at -20°C. The nativity and purity of the isolated mRNAs were controlled by ultracentrifugation of mRNA samples in linear 15-30% sucrose gradients–Kuznetsov (1981).

The CFTS on the basis of endogenous mRNAs involved free PMS and exogenous brain tissue poly-$(A)^+$mRNAs which were synthesized and used conventionally with the ATP/GTP and creatine phosphate—creatine kinase ATP-regenerating pair and ^{35}S-L-methionine as a protein labeled precursor—see Goertz (1982). The CFTS from a rabbit reticulocyte lysate cell-free translation system contained the same labeled protein precursor (^{35}S-L-methionine). I used this type of *in vitro* translation model lacking endogenous mRNA for testing the template activity of different purified mRNAs isolated in the experiment. This type of CFTS was made by means of application of a special reagent kit including a cell-free extract and ATP-regenerating system as well as the label and a full set of ^{12}C-L-amino acids dissolved in a buffer (Amersham Radiochemical Centre Kit CFT No. 90). A technique of experiments with such reticulocytic system was described in detail by Pelham and Jackson (1976). All procedures with all types of the CFTS used were conventional—Pelham and Jackson (1976), Goertz (1982) and Kuznetsov and Musajev (1988).

Conjugation of $mRNA_1$ with BSA was carried out in the medium of 2xSSC containing 3 mM tris (pH 8.45), 25 mg $mRNA_1$ per 5.0 ml of conjugation medium containing 3.5 mg BSA per the same volume (5 ml). This conjugation proceeded at room temperature with 45 min incubation under a 100 watt UV-lamp—Solvarssen and Hjerten (1974). The resulting $mRNA_1$/ BSA conjugates separated by Sepharose 6B-CL gel filtration were then additionally purified from conjugation medium components by a three-fold ultrafiltration on the YM1O Diaflo membranes followed by the dissolving of macromolecular complexes in 10 mM tris-HCl (pH 7.45), 20 mM EDTA, 10-12 A_{280} per 1.0 ml of the resulting solution—see Kuznetsov (1981).

The $mRNA_1$/BSA conjugates were used for an immunization of healthy adult male Chinchilla rabbits—Goldfarb and Zamchuk (1975). After finishing a six month schedule of multiple administration of the $mRNA_1$/BSA conjugate preparations both intracutaneously and intravenously—see Lokmediani *et al.* (1973), blood serum containing antibodies (to $mRNA_1$) was collected and defibrinated. A purified $mRNA_1$-(IgM+ IgG) fraction of $mRNA_1$ affinity was isolated using the ethanol precipitation followed by fractionation of the pellets on columns with DEAE- and CM-cellulose, according to Shatsky and Bogdanov (1984).

As a result, total $mRNA_1$-specific highly purified antibodies were obtained. These antibodies were treated with proteinase K, catepsin, pronase and chymotrypsinogen A using a special sequence of enzymatic procedures and different regimes of acetone and ammonium sulphate fractionation with the aim of isolating ab immunoglobulin fragments highly specific for mRNA—see Beaud and Chantrennes (1987). The $mRNA_1$-specific Fab-fragments were immobilized on particles of activated CNBr-Sepharose as described previously Kuznetsov and Traichev (1985). Then 1.5 x 10 cm columns were packed with this immunoaffinity sorbent and equilibrated with 30 mM tris (pH 8.90), 10 mM EDTA, 15 mM glutathione.

Samples of $mRNA_1$, $mRNA_2$, $mRNA_3$, globin 9S mRNA, histone 11S mRNA, and *E. coli* 5S rRNA were applied to these columns at room temperature (45mg

RNA per column). After the immobilization of affinity-binding sequences, all other RNA chains were easily eluted from the column by an equilibrium medium (at 22-25°C). The *immunoaffinity-binding mRNA sequences* were removed from the columns by elution with 70% formamide at 40°C, after which further purification of the templates necessary for *in vitro* translation was carried out by the routine dialysis and rapid ultrafiltration through UM2 membranes—Korn *et al.* (1985).

Thus, by means of affinity chromatography on Fab-$mRNA_1$-Sepharose columns, it should be possible to separate unique, *i.e.* species-specific, mRNA sequences from the sequences often found in mRNAs of different organisms, the so-called "regularly repeated mRNA chains" —see Kuznetsov and Dunlop (1986).

In a separate series of experiments, PMS samples isolated from vole brain tissue (PMS_1, PMS_2, PMS_3) were subjected to treatment with different hydrolyses in order to reveal the nature of the PMS *(i.e.* cytoplasmic) factors which are able to selectively and completely inhibit all similar but not species-specific mRNA-directed translation. To accomplish this, the following enzymatic procedures were performed:

(1) 75 mg Pronase per 1.0 A_{280} PMS in 25 mM tris-HCl (pH 9.10),45 mM $MnCl_2$, incubation time—1 h at 37°C;

(2) 25 mg RNase A (Bovine pancreatic) per 1.0 A_{280} PMS in 35 mM tris-HCl (pH 8.60), incubation time—20 min at 37°C;

(3) 100 units of the RNase T_1 per 1.0 A_{280} PMS in 50 mM tris-HCl (pH 8.00), 20 mM $CaCl_2$, incubation time—15 min at room temperature;

(4) RNase-free DNase–45 mg per 1.0 A_{280} PMS in 35 mM tris-HCl (pH 8.85), 20 mM $MnCl_2$, 5.0 mM glutathione, incubation time—45 min at 37°C;

(5) 200 units Hyalorunidase B per 1.0 A_{280} PMS in 50 mM tris-HCl (pH 9.00), 25 mM EDTA, 7.5 mM NaCl, incubation time—1.O h at 37°C;

(6) Proteinase K (10 mg) per 1.0 A_{280} PMS in 45 mM tris-HCl (pH 8.45), 5 mM EDTA, 2.5 mM NaCl, incubation time—1 h at 37°C;

(7) Micrococcal nuclease S (30 units) per 1.0 A_{280} PMS in 20 mM tris-HCl (pH 8.25), 30 mM $CaCl_2$, incubation time—30 min at 37°C;

(8) Salival amylase R3 (100 units) per 1.0 A_{280} PMS in 45 mM tris-HCl (pH 6.00), 30 mM EDTA, incubation time—1 h at 37°C.

After finishing each of these incubations listed, the reaction was arrested by addition of specific inhibitors for different hydrolyses from human placenta polypeptide extracts—see Walsh *et al.* (1986).

For tentative evaluations of possible inhibitory roles and LMWC of cytosol (S150) of the different animal's brain cells, all the S150 samples were subjected to a rapid fractionation by ultrafiltration using the Diaflo UM2 membrane in the MMC-lA Apparatus as described previously —Kuznetsov (1981). In all cases, the addition of purified HMWC and LMWC fractions of cytosol into different samples of the CFTS equaled 0.15-0.20 A_{280} per 1.0 ml. All these additions were made before the start of the CFTS incubation, just after the template (translatable mRNA) addition.

Radioactivity of liquid samples was measured by placing 5 ml of each into 20 ml of Unisolv-2, dioxane scintillation liquid. Radioactivity of each dry fiberglass millipore filter containing acid-precipitated pellets was determined using 20 ml of ZhS-8 toluol scintillation fluid. For all experiments, I used a Wallac 21104 Liquid Scintillation Counter. All column-developed chromatograms were examined for absorbance at 280 nm or at 254 nm using a LOMO SF48 UV-Spectrophotometer.

For statistical evaluation a non-parametric method for analysis of variance was used. All the data were processed in an HP-9815A mini-computer with a special FORTRAN/PAD program—see Shwernick and Bellinsghausen (1983). In the CFTS tests, the amount of mRNA added was 40 mg: total CFTS volume was 0.335 ml; time of incubation at 37°C was 1 h with a preincubation period of 10 min while the samples were in a warm bath at 37°C before the mRNA or pseudotemplate addition. All other procedures with CFTS samples were conventional and described previously in detail—see Pelham and Jackson (1976), Goertz (1982), and Kuznetsov and Musajev (1988).

Results

As seen in Table I, all brain postmitochondrial supernatants (PMS) used for the CFTS composition contain free *(i.e.* cytosol soluble) components which inhibited translation of mRNAs isolated from the brain of similar (so-called "evolutionally related") animals. But such inhibitors do not influence the translation directed by mRNA templates isolated from cells of distinctly different animals such as 9S globin mRNA from rabbit reticulocytes and 11S arginine-rich histone mRNA from HeLa cells. Also, the cytoplasmic free inhibitors mentioned above do not suppress translation controlled by mRNAs isolated from the very same brain tissue homogenate, *i.e.* from the same species of vole being studied. Low translation with cold CFTS incubation at O°C as well as with those treatments involving *E. coli* 5S ribosomal RNA as a pseudotemplate indicate that translation in the CFTS samples used was well above these control levels.

The preparative isolation of purified species-specific poly(A)$^+$mRNAs accomplished was by affinity chromatography of total cytoplasmic poly(A)$^+$mRNA samples on the columns involving activated Sepharose containing the immobilized species-specific mRNAs-challenged active antibody Fab-fragments. The $mRNA_2^{el}$ and $mRNA_3^{el}$ were significantly inactivated by PMS compounds isolated from the brains of similar "evolutionally related" species of lumber voles—Agadzhanyan and Yatsenko (1984). As for the homologous samples of a CFTS containing the mRNA-free PMS and poly-(A)$^+$mRNA brain tissue from the same vole species, the CFTS samples show a remarkable active translation without any signs of inhibition (Table I). The results summarized in Table II show us that the well-known test-system consisting of a post-microsomal supernatant isolated from the rabbit reticulocyte lysate of Pelham and Jackson (1976) does not contain any inhibitors for translation in a reticulocytic lysate cell-free system. This is true of vole brain, even after addition to this system of preparations of $mRNA_1$, $mRNA_2$, $mRNA_3$, $mRNA_2^{el}$.

The biochemical nature of the proposed cytoplasmic translation blocker(s) was revealed by determination of the fact that these factors are essentially and selec-

Table I. The Template Activity of Different Populations of Brain Messenger RNAs in a CFTS Containing the Fractions of Brain Cells Cytoplasmic Pool (PMS) Isolated from Different Species of Wild Voles, *Clethrionomys glareolus (1), Clethrionomys frater (2)* and *Clethrionomys gapperi (3).*

Composition of the CFTS	^{35}S-methionine incorporation into the acid-insoluble pool, c.p.m. per 1 mg protein (M+ SE)	Temperature of the sample incubation, °C
PMS_1 + $mRNA_1$	61,887 ± 431	37
PMS_1 + $mRNA_1$	91 ± 7	0
PMS_1 + $mRNA_1^{el}$	*none:* no mRNA was eluted from FaB-$mRNA_1$-Sepharose by a first step elution with an equilibrium medium.	
PMS_1 + $mRNA_2^{el}$	2,489 ± 96	37
PMS_1 + $mRNA_3$	55,876 ± 456	37
PMS_1 + $mRNA_2$	51,563 ± 477	37
PMS_1 + $mRNA_3$	88 ± 7	0
PMS_1 + $mRNA_2^{el}$	87 ± 8	0
PMS_1 + $mRNA_3^{el}$	5,088 ± 211	37
PMS_1 + 9S globin mRNA	47,885 ± 456	37
PMS_1 + 11S histone mRNA	50,650 ± 449	37
PMS_1 + *E. coli* **5S** rRNA	76 ± 5	37
PMS_2 + $mRNA_2$	74,665 ± 487	37
PMS_2 + $mRNA_2$	83 ± 6	0
PMS_2 + $mRNA_2^{el}$	123,887 ± 667	37
PMS_2 + $mRNA_2^{el}$	91 ± 8	0
PMS_2 + $mRNA_3$	58,905 ± 444	37
PMS_2 + $mRNA_3$	88 ± 8	0
PMS_2 + $mRNA_3^{el}$	3,076 ± 188	37
PMS_2 + $mRNA_3^{el}$	9 0 ± 7	o
PMS_2 + 9S globin mRNA	50,665 ± 488	37
PMS_2 + 11S histone mRNA	57,887 ± 674	37
PMS_2 + *E. coli* 5S rRNA	76 ± 8	37
PMS_3 + $mRNA_3$	68,903 ± 554	37
PMS_3 + $mRNA_3$	94 ± 6	0
PMS_3 + $mRNA_3^{el}$	112,877 ± 658	37
PMS_3 + $mRNA_3^{el}$	88 ± 8	0
PMS_3 + $mRNA_2$	44,760 ± 387	37
PMS_3 + $mRNA_2$	9 0 ± 7	o
PMS_3 + $mRNA_2^{el}$	2,075 ± 233	37
PMS_3 + $mRNA_2^{el}$	8 4 ± 6	o
PMS_3 + 9S globin mRNA	52,664 ± 503	37
PMS_3 + 11S histone mRNA	59,044 ± 489	37
PMS_3 + *E. coli* 5S rRNA	69 ± 6	37

Mean data from seven separate experiments are listed. For all comparative points presented P <0.05

tively sensitive to the ribonucleases A and T_1. But there is a marked resistance to the action of purified and highly active enzymes including Pronase, Proteinase K, Amylase, Hyaluronidase and RNase-free DNase. This translation inhibitor is also partly sensitive to micrococcal nuclease S (see Table III). Therefore it can be concluded that the factors mentioned are poly - or oligoribonucleotides. Further ultrafiltration of cytosol (S150 fraction) on the membrane with a 1.0 KD exclusion limit and the subsequent testing of the resulting fractions with MW more or less than 1.0 KD and in very different variants of the CFTS samples (Table IV), lead to strong support that this inhibitor, which does express its extremely powerful action only for the suppression of a ***"species-like but non-species-specific mRNAs,"*** is a short chain of oligoribonucleotide with molecular size less than 1.0 KD. As seen from this data, such factors are present in the brain cell cytoplasm of all three vole species studied—(Table IV).

Discussion

It should be clearly noted that a striking difference between the cell types used as sources for isolating messenger RNAs tested in these experiments (globin mRNA from rabbit reticulocytes, HeLa cells histone mRNA and brain mRNAs from a similar species of wild lumber voles), caused a marked resistance of non-brain 9S and 11S mRNAs to the action of the translation inhibitor of oligonucleotide nature identified in the brain cytosol (S150). Therefore, factors in the PMS fraction of the voles' brain selectively and completely suppressed the translation programmed by mRNAs from brains of similar, "potentially related," vole species (Tables I-IV). Specifically, the cytoplasm of brain cells studied contains one or more special oligonucleotide factors of MW less than 1.0 KD and with an extremely high species-associated specificity, as well as a cell-type-associated inhibitory specificity. This action seems to be very powerful and effective in all cases of ***in vitro*** translation with the cell components isolated from vole brain tissue.

High resolution preparative immunochemical isolation of purified species-specific brain poly(A)$^+$mRNAs gives a good possibility that the PMS_2 or $LMWC_2$, for instance, sharply suppress the translation directed by $mRNA_3^{el}$, unlike the 9S and 11S "non-related" mRNAs (Table I). This means that the $mRNA_3^{el}$ was completely inactivated as a species-3-specific ***(Clethrionomys gapperi)*** messenger translatable template by a selective inhibitor which contains in analogical tissue (brain) of so-called "phylogenetically related" organisms such as ***Clethrionomys glareolus*** and ***Clethrionomys frater*** (or-

Table II. The Template Activity of Different Wild Voles Brain Messenger RNAs in a Rabbit Reticulocyte Lysate Cell-Free Translation System Containing the ^{35}S-L-Methionine as a labelled Protein precursor

RNA template tested	Translation activity, ^{35}S-c.p.m. per 1 mg protein (M+ SE)	Temperature of incubation, °C	
$mRNA_1$	**55,871 ± 542**	**37**	
$mRNA_2$	**58,971 ± 556**	**37**	
$mRNA_3$	**52,663 ± 488**	**37**	p >0.05
$mRNA_3^{el}$	61,087 ± 503	37	
$mRNA_2^{el}$	57.886 ± 499	37	
9S globin mRNA	96,563 ± 678	37	p ˚0.05
11S histone mRNA	61,962 ± 652	37	
9S globin mRNA pretreated* with ribonuclease A	122 ± 11	37	
9S globin mRNA	87 ± 9	0	

***20 ug of pancreatic ribonuclease A per 1 mg mRNA, incubation medium: 25 mM tris-HCl (pH 9.0) 15 mM CaC1,/10 mM NaCl; incubation time–20 min at 37°C.**
Mean data of eight separate experiments are listed.

Table III. The Effect of Pretreatment of PMS_3 and PMS_2 by Different Hydrolyses on the Translation Activity in a CFTS Containing These Pretreated PMS Fractions.

PMS sample	Translation activity, ^{35}S-L-methionine incorporation into the *in vitro* synthesized polypeptide chains, c.p.m./mg protein (M ± SE)	
Nontreated PMS_2 + $mRNA_2$		76,872 ± 566
Nontreated PMS_3 + $mRNA_3$	p <0.01	70,099 ± 509
Nontreated PMS_3 + $mRNA_3^{el}$		2,607 ± 188
Nontreated PMS_3 + $mRNA_2^{el}$		2,111 ± 176
(PMS_2 + Pronase) + $mRNA_2$		78,074 ± 573
(PMS_3 + Pronase) + $mRNA_3$		69,733 ± 566
(PMS_2 + Pronase) + $mRNA_3^{el}$	P <0.01	2,555 ± 211
(PMS_3 + Pronase) + $mRNA_2^{el}$		2,650 ± 167
(PMS_2 + RNase A) + $mRNA_2$		90,123 ± 432
(PMS_2 + RNase A) + $mRNA_3$	P >0.05	82,761 ± 603
(PMS_3 + RNase A) + $mRNA_2^{el}$		76,888 ± 487
(PMS_2 + RNase A) + $mRNA_3^{el}$		80,099 ± 543
Nontreated PMS_2 + 9S globin mRNA	P >0.05	67,895 ± 564
Mild RNase treated PMS_2 + 9S globin mRNA*		68,055 ± 542
Nontreated PMS_3 + 11S histone mRNA	p >0.05	75,088 ± 643
Mild RNase treated PMS_3 + 11S histone mRNA*		77,063 ± 600

Analogical data were obtained using the PMS pretreatment with the RNase-free DNase, Hyaluronidase and the salival amylase (see Methods).

Analogical data were obtained using a PMS pretreatment with Proteinase K (see Methods).

Analogical data were obtained using a PMS pretreatment with RNase T_1 and micrococcal nuclease S.

In all cases of the enzymatic pretreatment of the PMS fraction, after the finishing incubation (see Methods), each of the enzymes used was selectively inhibited by a specific human placental peptide factor according to Walsh et al. (1986).

*The treatment with RNAse T_1 was pm-formed
Mean data of eight experiments are listed.

ganisms 1 and 2). The same examples of a species-specific mRNA inhibition by the cytoplasmic factor(s) from the PMS or cytosol fractions isolated from "evolutionally related" animal species (brain tissue) are listed in Tables I and IV On the other hand, the cytoplasmic components of a rabbit reticulocyte lysate CFTS do not influence the translation rate of any of the brain mRNAs studied if they were tested in the *in vitro* system, Table II.

Undoubtedly, further characteristics of the activity and physiochemical properties of cytoplasmic factor(s) mentioned as well as a knowledge of the characteristics of analogical translation species-specific blockers from different tissues, cells and species of animals from "related" and widely separated species will lead to the development of a more fundamental understanding of the cause and mechanism(s) involved in the origin of the great and wonderful diversity seen in all life forms. My research program includes a new series of experiments aimed at trying to solve these origins problems, at least in part.

Now, it is necessary to evaluate the data presented in this paper as new findings for radical criticism of the most widely accepted evolutionary concepts in biochemical and molecular genetics. First, the data indicate an extremely high probability that there exists a brain cell cytoplasmic chemical which is a complete inactivator of the mRNA templates previously transcribed from any "unusual," "strange." or unique gene. By "unusual" I mean a gene that might result from a nonlethal mutagenesis such as the spontaneous mutation process often postulated as one of the main molecular causes of biological evolution—see Griffith and Palmiter (1980), Agadzhanyan and Yatsenko (1984), O'Brien *et al.* (1985), McIntyre (1985 and 1986) and Hecht (1987).

It should be taken into account that the cytoplasmic level of genome expression may be a keystone in the objective analysis of the so-called problem of the "renovated genome." It should be noted that this term was created and frequently used by a famous authority in the field of molecular neurogenetics, H. T. Sarcar (1978). He later originated the concept that there are widespread cases of newly formed genes inside the total genome infrastructure (chromatin) just after the origin of these new genes. He thought such inactivation was the result of nonlethal chemical modification of "old" genes or as a result of some more "crude" changes during nonlethal, spontaneous, and environmentally caused mutagenesis—see Sarcar *et al.* (1984 and 1987). As for possible mechanisms for such "intragenome inactivation" of "new genes," it was assumed that the major inhibitory role was played by non-histone chromatin proteins having a non-random position inside the chromatin—see Eayrs *et al.* (1986), Hrubstein *et al.* (1986), and Farquhar and Chebeaud (1987).

Concerning the molecular genetic concept of "concealing" novel, "unusual" and "unexpectedly changed" genes it should be noted that, theoretically, this "concealing" at the level of the nucleus would not guarantee that translation of the same "changed genes"-transcribed mRNAs would be limited in the cytoplasm. It does not guarantee the absence of corresponding mRNA templates in the cytoplasm nor does it preclude the incorporation of these mRNAs into translatable polysomes. Does such a process of translation limitation exist or not? To some extent I hope to answer this question by using the biochemical approach I developed for studying the fate of mRNA templates placed into a surrounding cytoplasmic environment taken from similar but not biologically identical cells as compared to that taken from cells which were a source for isolation of the messenger RNA being tested, Tables I-IV As a whole, the first results of the application of my approach show that all "unusual" species-non-specific mRNAs from brain cells of similar but not the same organism were not translated. The genes which may be the source of such messengers in all probability would not be expressed at the cytoplasmic level. This simple consequence of molecular events does indicate

Table IV. The Effect of Addition of Low Molecular Weight Compounds (LMWC) and High Molecular Weight Compounds (HMWC) Total Fractions Isolated from Wild Voles Brain Cytosol (S150) by a Rapid Ultrafiltration on the DIAFLO UM2 Membrane into the Functionated CFTS Samples Prepared on the Basis of Wild Voles Brain PMS Lacking of Endogenous mRNAs and mRNA Preparations Isolated from the Same PMS Fractions

CFTS Composition*	Translation activity, ^{35}S-L-methionine incorporation into the acid-insoluble pool, cpm/mg protein (M ± SE)
PMS_1 + $mRNA_1$ + $HMWC_1$	68,952 ± 651
PMS_2 + $mRNA_2$ + $HMWC_2$	77,098 ± 543
PMS_3 + $mRNA_3$ + $HMWC_3$	67,776 ± 602 P >005
PMS_1 + $mRNA_1$ + $LMWC_1$	69,014 ± 574
PMS_2 + $mRNA_2$ + $LMWC_2$	78,034 ± 651
PMS_3 + $mRNA_3$ + $LMWC_3$	68,981 ± 598
PMS_1 + $mRNA_2^{el}$	2,876 ± 77
PMS_1 + $mRNA_1$ + $LMWC_1$	2,322 ± 64
PMS_1 + $mRNA_2$ + $HMWC_1$	50,887 ± 299 P <0.05
PMS_1 + $mRNA_3^{el}$	4,077 ± 321
PMS_1 + $mRNA_3$ + $LMWC_1$	3,876 ± 266
PMS_1 + $mRNA_3$ + $HMWC_1$	48,557 ± 508

*All samples of HMWC and LMWC were added into the CFTS to a final concentration of 20 A_{260} per 1.0 ml. Mean data of eight experiments are listed.

CFTS Composition	Translation activity, ^{35}S-cpm/mg protein (M ± SE)
PMS_2 + $mRNA_3^{el}$	2,650 ± 421
PMS_2 + $mRNA_3$ + $LMWC_2$	4,761 ± 322 P <0.05
PMS_2 + $mRNA_3$ + $HMWC_2$	55,889 ± 450
PMS_3 + $mRNA_2^{el}$	3,076 ± 245
PMS_3 + $mRNA_2$ + $LMWC_3$	5,876 ± 466 P <0.05
PMS_3 + $mRNA_2$ + $HMWC_3$	46,668 ± 409
PMS_2 + 9S globin mRNA	51,754 ± 564
PMS_2 + 11S histone mRNA	60,022 ± 600 P >005
PMS_2 + 9S globin mRNA + $HMWC_2$	51,000 ± 588
PMS_2 + 9S globin mRNA + $LMWC_2$	52,088 ± 459
PMS_3 + 9S globin mRNA	53,876 ± 544
PMS_3 + 11S histone mRNA	62,077 ± 578 P >005
PMS_3 + 9S globin mRNA + $HMWC_2$	54,083 ± 544
PMS_3 + 11S globin mRNA + $LMWC_3$	60,122 ± 582

Mean data of 10 experiments are listed
All samples of HMWC and LMWC were added into the CFTS to a final concentration of 20 A_{260} per 1.0 ml

that the mere existence of a new gene, a gene previously absent from the total genome, is not sufficient for complete expression of this gene since the corresponding mRNA templates may be fully inactivated by specific cytoplasmic low molecular weight soluble factors.

I am proposing that such factors exist within the cytoplasm of most cells and tissue and because of them, a known "Species Main Peculiarities Conservatism''—see Mahony *et al.* (1975)—actually occurs. Intraspecies variability is indeed a fact and this phenomenon is distinctly different from any "genuine evolutionary changes."

The mRNAs for proteins that are distinctly different from the type of cells under study (for example 9S globin mRNA within the CFTS from PMS of wild vole brain tissue) have no affinity with the inhibitory brain cytoplasmic translation control factors identified in this present study. At the same time, the appearance in the brain cell cytoplasm of one wild vole species of new or "unusual" species-specific mRNAs from similar cells is followed by complete inactivation of the latter templates by small cytoplasmic oligonucleotide factors. These serve especially to prevent the expression of mRNAs that were previously nonexistent inside this highly "balanced" system. There is a complex interrelationship of non-random components forming a mutually functional infrastructure—see Kuznetsov (1985 and 1988).

It is difficult to form fixed conclusions from the foregoing data. However, I present a hypothesis in which some soluble and as yet unidentified, cytoplasmic factor(s) controlling the translation process are responsible for the selective inhibition of the expression of messenger RNAs that have appeared as a result of spontaneous non-lethal mutation. If this hypothesis is true, the factors governing species-specific cytoplasmic translational control may be called the molecular cytoplasmic system for preservation of phenotypic constancy or conservatism. It is one of the systems for preserving the constancy of the main features of a species of living organisms. I mean that the living types which were created may undergo minor modifications but will be generally kept unchanged as long as the earth exists.

A few additional words are needed concerning the choice of tissue types studied. It is well known that in the majority of mammalian cells not more than 7-10% of the total genome may be simultaneously involved in the state of active expression—see Gorkin *et al.* (1982) and Slater and Burden (1984). However this phenomenon is much more marked in the case of brain cells. The proportion of mammalian brain cell genes being transcribed at any time is usually no more than 3.0%—see Cupello *et al.* (1985), McLeod and Stahl (1986), Sieliwanowicz (1986), Bleachey *et al.* (1987), and Lormyck and Roostekija (1988). There are at least two points of view that have been adopted concerning the nature of this brain-specific regulation:

(1) One view takes into account that approximately 90% of mammalian brain nuclear DNA sequences are not unique chains. They code for messenger mRNAs and serve for a transcription control of the distanced DNA sites by binding with steroids, cyclic nucleotides and some other biogenic effecters—see Burden and Katinakis (1982), Barthels and Lim (1986), and Arheim-Zwieler *et al.* (1987);

(2) Another view takes into account that 30-45% of the mammalian brain cell genes are actively expressed during the early stages, as opposed to the later stages of ontogenesis. They are usually inhibited when the animal tested is mature—see Medvedev (1983 and 1986), Gornsheck *et al.* (1985), Smaller-Braudy (1986), Palatnik and Shimon (1987), Larski and Yagihl (1987) and Borquest and Piermont (1988).

These two opinions both find some experimental support. Nevertheless, the origin and biological role of the majority of DNA sequences in the total brain cell genome is still obscure and is a mystery.

The data presently listed do not exclude the possibility that these numerous, multiple, and unexpressed

mammalian brain genes are able to undergo transcription to the fullest extent but are not expressed at the translational (mRNA) level because of mechanisms discovered and partly discussed in this study. This additional hypothesis needs careful and impartial examination, and I hope to stimulate serious interest in the world wide society of researchers for this important aspect of the biological control of species constancy.

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Appendix I:

Abbreviations

$mRNA_1$, $mRNA_2$, $mRNA_3$ — total cytoplasmic poly-(A)–containing messenger RNAs isolated and purified from the brains of three tested organisms, respectively; *Clethrionomys glareolus (1), Clethrionomys frater (2)* and *Clethrionomys gapperi (3).*

$mRNA_1^{el}$, $mRNA_2^{el}$, $mRNA_3^{el}$ — mRNA preparations mentioned above after their elution from the columns packed with an anti-$mRNA_1$-FaB_1-CNBr-Sepharose by an equilibrium solution. These mRNA fractions consist of only a rare, unique, species-specific messenger RNA sequence.

CFTS — cell-free translation systems.

PMS (S12) — Post-Mitochondrial Supernatant, i.e. a special subcellular fraction resulting after a removal of mitochondrial pellets obtained by the centrifugation of a tissue homogenate.

S150 (Cytosol) — a subcellular fraction isolated directly from brain tissue homogenate by centrifugation following collection of the supernatant (S150).

PMS_1, PMS_2, PMS_3, $S150_1$, $S150_2$, $S150_3$ — PMS and cytosol (S-150) isolated from different animals of the *Clethrionomys* taxonomic group (brain tissue).

MW — molecular weight.

KD — kilodalton.

EDTA — sodium ethylenediaminotetraacetate.

HMWC and *LMWC — High Molecular Weight Compounds* and *Low Molecular Weight Compounds* which have been completely separated by a rapid ultrafiltration procedure from the S150 fractions.

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