

REVERSE TRANSCRIPTASES IN BACTERIA: SMALL RNAs AS GENETIC VECTORS AND BIOLOGICAL MODULATORS

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ABSTRACT

In 1970, with my group at the Pasteur Institute in Paris, I observed that showdomycin resistant *Escherichia coli* cells excrete into their culture medium a small RNA, about 160 nucleotides long, rich in purine bases. This RNA transforms wild bacteria (*E. coli* and *Agrobacterium tumefaciens*) into transformants which exhibit new stable biochemical and physical characteristics. Thus *A. tumefaciens* once transformed by *E. coli* transforming RNA, partially or often totally loses its oncogenic potentialities and acquires new properties. It appeared that reverse transcriptase might give transforming RNA the means of integrating into the genome of the transformed organisms the modifications it vectored. We demonstrated (1971-1972) that bacterial reverse transcriptase was involved in this process. In the course of our studies on bacterial transformation, we devised a technique which led to the discovery, first of RNA free reverse transcriptase in bacteria, then of RNA-bound reverse transcriptase which is easily distinguishable from DNA- dependent-DNA polymerase. In 1989 several american scientists rediscovered reverse transcriptase in *E. coli*.

INTRODUCTION

The discovery of reverse transcriptase in bacteria

In 1971, I informed my colleagues at the Pasteur Institute in Paris that my group had just discovered a reverse transcriptase in bacteria. But, together with Nobel prize winner Jacques Monod, head of the department, fellow researchers at once voiced their

reprobation: this finding was utterly unacceptable, a shocking heresy, and untrue, in view of the dogma which prevailed at the time, of DNA supremacy over all cellular processes including transfer of genetic information.

The research which, in 1970, had led up to our discovery dealt, on the one hand, with the involvement of short chain RNA primers in DNA replication and, on the other hand, with the part played by RNAs as vectors of information in bacteria as well as inductors of plant tumors (Beljanski *et al.*, 1971a, 1972). These studies had enabled us to demonstrate that heritable information could be transferred to different bacterial species by specific RNA which was excreted by *E. coli* showdomycin-resistant cells (Beljanski *et al.*, 1972; Beljanski and Manigault, 1972).

Transformants exhibited new and stable biochemical and physical characteristics (Beljanski *et al.*, 1972; Beljanski and Manigault, 1972). This was spectacularly evidenced in *Agrobacterium tumefaciens*, a soil bacterium which causes malignant tumors in plants, and which, once transformed by *Escherichia coli* transforming RNA, partially or most often totally lost its oncogenic potentialities and acquired new properties. We published our results on bacterial transformation in 1971 (Beljanski *et al.*, 1972) and on the discovery of reverse transcriptase in bacteria early in 1972 (Beljanski, 1972). Other papers followed (Beljanski and Beljanski, 1974; Beljanski, 1974), in several of which we showed that these small RNA carriers of genetic information could be transcribed into complementary DNA by endogenous bacterial reverse transcriptase; this DNA could then be integrated into the genome of the recipient cell.

However, in a minireview which appeared in *Cell* in 1989, H. Varmus stated: "... The argument for the ubiquity of reverse transcription and for its fundamental role in the generation of contemporary forms of life has suffered from the lack of any evidence for reverse transcription in procaryotic organisms". At about the same time, H. Temin also held that reverse transcriptase had seemed until then not to exist in bacteria (Temin, 1989a). And both these Nobel prize winners attributed the discovery of reverse transcriptase in bacteria to American scientists who had lately published their observations, in that same year (Lim and Mass, 1989; Lampson *et al.*, 1989).

As soon as these enthusiastic announcements of the discovery of reverse transcriptase in bacteria had appeared in scientific journals, I wrote to Varmus and Temin, asking for recognition of the work we had published from 1972 to 1978. While Varmus avoided answering clearly, a short "retrocitation" by Temin, mentioning our research, was finally published in *Nature* (Temin, 1989b).

The present paper deals with the research which enabled us to discover reverse transcriptase in bacteria and with the properties of this enzyme and its role in bacterial transformation.

Small transforming RNA in bacteria

As early as 1970, we found that showdomycin-resistant (Sho^R) *Escherichia coli* excreted into its culture medium a small RNA. After bacteria had been centrifuged out of the culture medium, this 6S RNA was isolated and analysed by gel electrophoresis (Figure 1). It differed from classic RNAs of the wild type strain. Readily absorbed by wild type *E. coli*, it conferred specific characters of the donor Sho^R to the recipient wild type. For this reason, we named it *transforming RNA*. That an RNA was indeed involved, and not a DNA, was proved by the fact that transformation was totally suppressed by RNase, but unaffected by DNase (Beljanski and Plawecki, 1973).

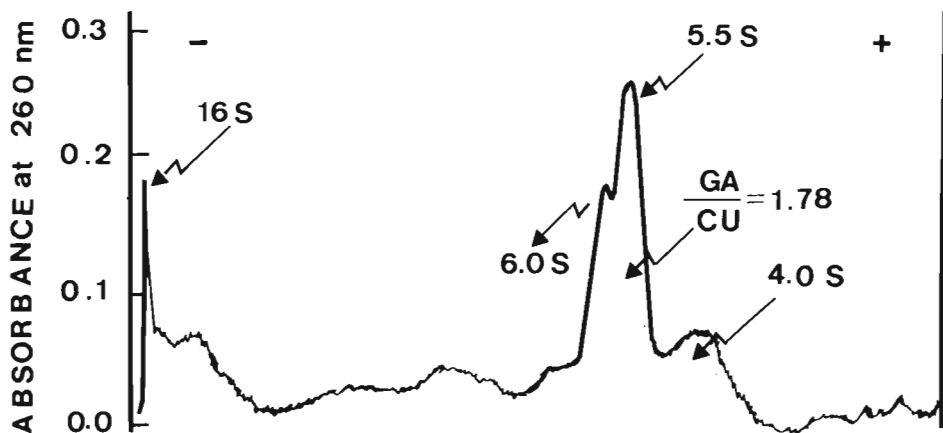


Figure 1 - Densitometer tracing (in the Cary spectrophotometer at 260.7 nm) of RNAs excreted by *E. coli* ML 40 Sho^R . RNAs were purified as described (Beljanski and Manigault, 1972) and separated by electrophoresis on polyacrylamide gel (4.5 %). Length of gel: 62 mm. RNA fractions were eluted from sliced gels with SSC (0.15 NaCl-0.015 sodium citrate) at 4°C for 24 hr and dialyzed against distilled water. In separate experiments the nucleotides were analyzed on a Dowex 1 x 2 column, 200-400 mesh (Beljanski *et al.*, 1972).

Transforming RNA is much richer in purines ($G+A/C+U = 1.76-2.0$) than classic wild type RNAs ($G+A/C+U = 1.0$) (Beljanski *et al.*, 1971a, 1972). When centrifuged on a Cs_2SO_4 gradient, 6S RNA sediments in the same region as other RNAs and contains no DNA. It does not hybridize with DNA of the bacterial strain it was derived from. It may be found not only free in the culture medium, but inside the bacteria too, where it

may be bound somewhat loosely to DNA (as an "episome" see later) and, also, bound to reverse transcriptase.

Polynucleotide phosphorylase copies transforming RNA

Surprisingly, we observed, *in vitro*, that transforming RNA could be used as a template by polyribonucleotide phosphorylase (PNPase), provided that the four ribonucleoside -5' diphosphates (NDPs) were present in the incubation medium (Beljanski and Plawecki, 1973). While in polyAGUC synthesized by wild type PNPase from equal amounts of NDPs the ratio of the latter is 1:1:1:1, polyAGUC synthesized under identical conditions in the presence of 6S RNA has a G+A/C+U ratio of 1.75, that is, practically identical with that of 6S RNA (where G+A/C+U = 1.80). In addition, the rate of synthesis increases several fold. RNase suppresses 6S RNA template activity. NTPs cannot replace NDPs. Rifampicin, which inhibits DNA-dependent RNA polymerase, does not affect PNPase. This enzyme can use neither ribosomal, transfer and poliovirus RNAs nor polyAGUC as templates. On a sucrose gradient, C¹⁴-labeled products synthesized in the presence and in the absence of 6S RNA do not sediment together.

When, instead of the wild strain enzyme, Sho^R PNPase is used, the product synthesized in the presence of equal amounts of the four NDPs is an AGUC containing twice as large an amount of purine as of pyrimidine bases. Modified *E. coli* PNPase has thus synthesized an RNA which does not hybridize with bacterial cell DNA.

Subsequent experiments showed us that under specifically modified conditions, mainly high temperature (70°C), even wild type PNPase could synthesize, from equal amounts of the four NDPs, small purine-rich RNAs (G+A/C+U = 1.5 to 1.8). These RNAs proved active as primers for *in vitro* replication of various bacterial and mammalian DNAs; yet, under the same experimental conditions, they had little effect on phage DNA replication (Beljanski and Plawecki, 1975). We were later to investigate this specificity and obtain a whole range of small RNA primers for viral, bacterial, plant and mammalian DNAs (Beljanski and Plawecki, 1975).

Part of this work showed us that when Sho^R synthesizes small purine-rich RNA under normal conditions, but with the added presence of RNase A, the resulting product can prime replication of several DNAs (Beljanski and Plawecki, 1975). We may thus note, in passing, that PNPase and RNase A, both capable of withstanding a wide range of temperatures, may have played a decisive role in the formation of cell constituents during the early manifestations of life on earth. PNPase is the only enzyme to use NDPs and not NTPs, that is, simpler building blocks. In any case, the acknowledged rusticity of PNPase and RNase A speaks for their primitive character and very ancient origin.

Uptake of labeled transforming RNA by recipient bacteria and transformation procedure

After isolation and characterization, C¹⁴-uracil labeled transforming RNA was incubated for 1 hour with *E. coli* K12 Hfr as a recipient. Table I shows that half the labeled RNA was taken up by recipient cells. Leftover RNA could be totally precipitated by trichloroacetic acid, indicating that this RNA had not been degraded before entering the cells. Neither was it degraded inside the bacteria; if it had, most of the radioactivity would have been found in the ribosomes, which comprise roughly 70% of bacterial RNA: this was shown not to be the case (Table I).

Table I - Distribution of ¹⁴C-uracil transforming RNA absorbed by wild-type *E. coli*.

Analyzed material	¹⁴ C-uracil, CPM in TCA precipitable material	
Washed bacteria	18.460	
Washed bacterial debris	3.621	
Membrane fractions	4.990	18.151
Ribosomes (70 S)	1.180	
105,000 x g supernatant	8.360	
DNA isolated directly from washed bacteria (18.370 CPM) and purified as described	1.552	

Note: 200 ml of an exponentially growing culture (10^8 cells/ml) were incubated at 37°C with shaking in synthetic medium containing glucose (Beljanski *et al.*, 1972) in the presence of 40 µg of ¹⁴C-uracil-labeled transforming RNA (36,640 cpm). After 1 hr incubation, cells were collected by centrifugation at 15,000 x g for 30 min. and washed with fresh culture medium. The presence of labeled RNA (TCA precipitable) was determined in the supernatant and in different fractions after labeled bacteria had been broken up by grinding with alumina, and different fractions had been separated (from Beljanski and Manigault, 1972).

Transformants were obtained in the following way: wild type *E. coli* was incubated (2×10^7 cells/ml for 1 hour at 37°C) in fresh synthetic medium supplemented with 0.1 to 2 µg active RNA/ml. The presence of transformants may be easily demonstrated by comparing the results of two different analytical techniques. The total RNA content of a sample may be measured using UV absorption at 260 nm. A less direct way of analysing RNA is to detect ribose using the orcinol reaction. Yet (we were among the first (Beljanski, 1949) to show this, in 1948-9) only purine nucleotides are involved in this reaction (Beljanski, 1949). It becomes, in fact, a convenient technique for ascertaining the purine content of an RNA. Thus the ratio of results obtained with these two

methods gives a measure of the relative purine contents of various RNA samples. The greater the amount of purines, the higher the "ribose/UV" ratio: when the ratio is higher than for the wild type, transformation has taken place in the recipient strain (Table II).

Table II - Ribose/UV ratio of endogenous RNA of *E. coli* transformants and mutant M 500.

Recipient bacteria	Transforming RNA	Ribose / UV ratio	Difference (%)
<i>E. coli</i> K-12 Hfr.	no	0.64	-
	+ 0.1 µg	0.90	40
	+ 2.0 µg	0.93	45
	+ 2.0 µg + RNase (20 µg)	0.65	1.5
	+ 2.0 µg + DNase (20 µg)	0.91	42
<i>E. coli</i> -RV (8)	no	0.67	-
	+ 0.1 µg	0.89	32
	+ 0.2 µg	0.95	40
	+ 2.0 µg + RNase	0.69	2.8
	+ 2.0 µg + DNase	0.93	38
	+ 2.0 µg + Pronase ^a	0.96	40
	+ 0.1 µg + r-RNA (10 µg)	0.93	45
<i>E. coli</i> Sho ^R mutant M 500	no	0.94	-

Note: Exponentially growing bacteria were collected by centrifugation and reincubated at 37°C (2×10^7 cells/ml) in 5 ml of synthetic medium containing glucose and 0.1 to 3 µg of transforming RNA. After 30 min. or 2 hr, bacteria were collected and washed three times with 10 ml of 5% TCA solution, and exogenous RNA is extracted by heating the suspension (in 2 ml of 10% TCA) at 100°C for 20 min. After centrifugation the supernatant was used for determination of UV absorption at 260 nm and for orcinol reaction (Beljanski, 1949). Ribose/UV ratio (arbitrary ratio) - divisions read at 670 nm (ribose)/divisions read at 260 nm. aRNA preincubated with 200 µg of pronase for 1 hr.

No transformation occurs when transforming RNA is pretreated with pancreatic RNase. DNase changes nothing. The modified ribose/UV ratio for endogenous transformed cell RNA is close to, or identical with, that of Sho^R bacteria which excrete transforming RNA (Beljanski *et al.*, 1971a; Beljanski and Manigault, 1972). All required

assays were carried out with transformants. Ribosomal RNA did not compete with transforming RNA and pronase had no effect.

Transformation of wild type *E. coli* may also be obtained by simply growing together, in the same incubation medium, 2% Sho^R and 98% wild type cells. Both strains have the same growth rate at 37°C. After 10 to 16 hours, the whole population exhibits Sho^R strain characteristics (Beljanski *et al.*, 1971a).

Characteristics of E. coli transformants

1) Transformants have the same ribose/UV ratio as a homogeneous Sho^R cell population (checked by repeated plating and analysis). Transformant clones are stable and no revertants appear.

2) Just like Sho^R cells, transformants excrete transforming RNA into the culture medium.

3) Both ribosomal RNAs (23S and 16S) of transformants contain more purine than pyrimidine nucleotides (Beljanski and Manigault, 1972).

4) Transformant-derived PNPase synthesizes *in vitro*, from equimolar amounts of the four NDPs, a polyAGUC containing more purine than pyrimidine nucleotides, whereas polyAGUC synthesized under the same conditions by wild type PNPase contains equal amounts of both. An excess of purine nucleotides (in synthesized products is characteristic of Sho^R cell PNPase (Beljanski and Plawecki, 1973).

5) Showdomycin resistance itself is only poorly transferred (Beljanski and Plawecki, 1973). However, transformants acquire showdomycin resistance much more rapidly than wild type *E. coli*.

Interspecific transformation

As mentioned in the Introduction, we demonstrated that the action of *E. coli* transforming RNA extends to a completely different bacterial species, the soil bacterium *Agrobacterium tumefaciens*, which causes malignant tumors in plants and which we used extensively in further research.

We codified the procedure for transformation of this bacterium as well as the criteria for quantifying the effect of transforming RNA (from *E. coli* ML30 Sho^R strain). The inhibitory effect of RNase was demonstrated and other necessary assays were carried out.

The required incubation time for *A. tumefaciens* B6 transformation is some what longer than for *E. coli*. The percentage of transformants increases with time, so that the

process may begin after six hours but may take 24 hours to affect the entire population (in some experiments, though, total transformation only took 16 hours; transformation time depends on many factors, such as size of inoculum, etc.). Observing this "progressive and cumulative transformation", we were reminded of the similar fashion in which tumor induction proceeds in plants (Braun, 1951).

Totally transformed strains have completely and permanently lost their oncogenic properties (B₆-Tr1, Table III). No tumors are produced in plants inoculated with these strains. These total transformants, when plated on solid medium, give colonies twice as large as the wild strain; transformed strain homogeneity was checked by cloning.

Table III - Oncogenic properties of *A. tumefaciens* B₆ wild-type, transformants B₆-Tr-1, B₆-Tr-4, and B₆-Tr-4-A in the pea.

	No. of bacteria per wounded host	Confidence limit of the average of adjusted tumor weights (cg) (9)	No. of plants
B ₆ wild-type*	1.2 x 10 ⁸	14.9 < 17.3 < 19.7	42
B ₆ -Tr-1	1.2 x 10 ⁸	No tumors	34
B ₆ -Tr-4	1.3 x 10 ⁸	9.3 < 10.8 < 12.3	34
B ₆ wild-type [†]	At saturation	26.0 < 28.9 < 31.8	46
B ₆ -Tr-1	At saturation	No tumors	37
B ₆ -Tr-1	(Clones at saturation)	No tumors	100
B ₆ -Tr-4-A	(Clones at saturation)	7.1 < 8.0 < 8.9	45

Note: Confidence interval of 1%. Method for inoculation of bacteria (Beljanski *et al.*, 1971a). Tumor-inducing capability of wild-strain B₆ and transformants was routinely tested in several experiments with pea seedlings. The absence of oncogenic properties in the total transformant B₆-Tr-1 was also observed with *Kalanchoe daigremontiana* and *Datura stramonium*. *, Two independent series of experiments.

Partial transformants also appear. Their properties are intermediate between those of the wild type and of total transformants, and tumors induced in plants inoculated with partial transformants weigh much less than those inoculated with wild type. No transformants are produced when either a mixture of purine-rich nucleotides or various synthetic polyribonucleotides are used in place of transforming RNA. Spontaneous transformants have never been described. No revertants appear.

Characteristics of A. tumefaciens transformants

Major specific tests applying to *A. tumefaciens* are summarized in Table IV. Some characteristics of the wild strain are conserved in transformants, but new ones have been acquired (Beljanski *et al.*, 1972, 1974). Among these are a number of striking chemical modifications (Figure 2). The extent of transformation may be appreciated when densitometer tracings of ribosomal RNAs and ribosomal proteins of total transformants and of wild type are compared. In addition, nucleotide composition of transformant ribosomal RNA differs largely from that of the wild type (Table V). Consequently, we expected, and found, modifications of the synthesis and activities of several enzymes in transformants (Beljanski and Manigault, 1972).

Table IV - Characteristics of *Agrobacterium tumefaciens* wild type B₆ and transformants.

	Conditions of growth		Synthetic growth		3 Keto-lactose formation	Serological test
	Aerobic	Anaerobic	Medium 63 ^a	Medium stoll ^b		
Wild type B ₆	+	no	no	+	+++	+++
B ₆ -Tr-4 transformant	+	no	no	+	+++	++
B ₆ -Tr-1 transformant	+	no	no	+	+ (delayed)	-
<i>E. coli</i> ML 30 Sho ^R	+	+	+	no	no	no

Note: Test for 3 keto-lactose, considered as specific for *Agrobacterium tumefaciens* (Beljanski *et al.*, 1972), was performed on bacteria grown for 36 hr at 30°C as one large spot on solid Stoll medium containing 2% of lactose. The yellow color (3 keto-lactose) appears around grown colonies. Serological test was done with the anti-B₆ serum. ^aSynthetic medium 63 routinely used for growth of *E. coli* (Beljanski *et al.*, 1971a). ^bStoll medium (Beljanski and Manigault, 1972) is more specific for *A. tumefaciens*.

Discovery of free reverse transcriptase in bacteria

In the course of our studies on transforming RNA, we had demonstrated that, *in vitro*, it could be transcribed into complementary DNA (cDNA) (Beljanski *et al.*, 1972). Our hybridization experiments (Beljanski and Plawecki, 1973) showed us that DNA from *A. tumefaciens* transformants contained one or several copies of such cDNA. Thus, after PNPase has ensured reproduction of transforming RNA, a reverse transcriptase must take over and, by transcribing this RNA into DNA, turn its message into proper genetic

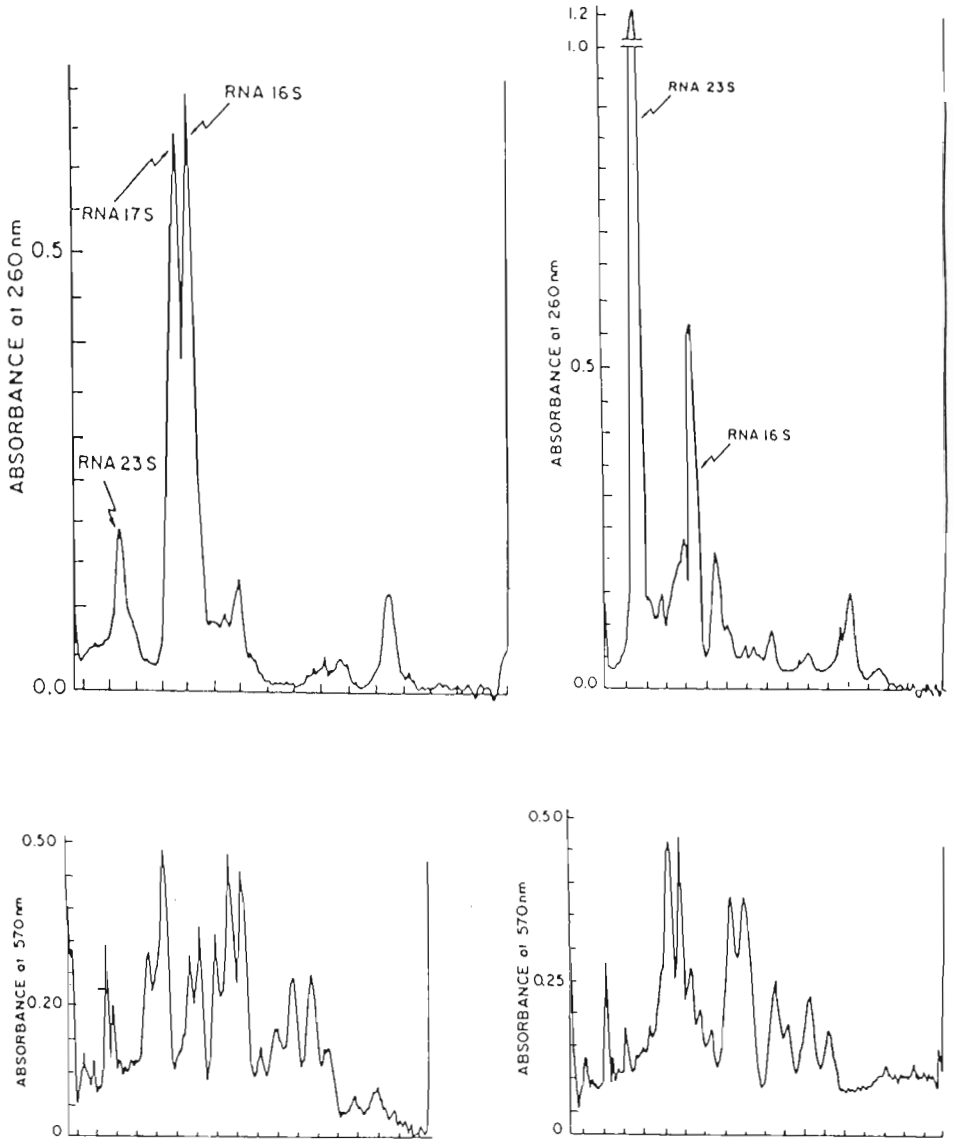


Figure 2 - Densitometer tracings of ribosomal RNAs and ribosomal proteins of wild-type B₆ and transformants (*A. tumefaciens*). Ribosomal RNA and ribosomal proteins after polyacrylamide gel electrophoresis. Conditions described elsewhere (Beljanski and Manigault, 1972). Above, left: r-RNA of wild type B₆; right: r-RNA of B₆-Tr-1. Below, ribosomal proteins; left: of wild-type B₆; right: of B₆-Tr-1 (Beljanski *et al.*, 1972).

information. In this way, our experiments on intra and interspecific bacterial transformation with the help of this small RNA logically prompted us to search for a reverse transcriptase in bacteria.

Table V - Nucleotide composition of r-RNAs of *Agrobacterium tumefaciens*, wild-type B₆ and transformant B₆-Tr-1.

Nucleotide	Transforming ARN <i>E. coli</i> ML 30 Sho ^R	(mol. per 100 mol. of nucleotides)			
		wild type		Transformant	
		23 S	16 S + 17 S	23 S	16 S + 17 S
A	30.3	26.0	25.2	30.6	29.3
G	33.5	30.4	29.8	33.3	31.4
C	18.3	24.7	23.5	19.6	20.6
U	17.8	18.9	21.5	16.5	18.7
G+A/C+U	1.76	1.27	1.22	1.77	1.56
G+C/A+U	1.05	1.20	1.16	1.03	1.08

And we did find this bacterial enzyme. We demonstrated that, in *E. coli* (Beljanski, 1972; Beljanski and Beljanski, 1974) as well as in *A. tumefaciens* (Beljanski *et al.*, 1974; Le Goff *et al.*, 1976), it could be either RNA-free or RNA-bound. These two forms can easily be distinguished from each other, and from DNA-dependent DNA polymerase, after fractionation of ribosome-free bacterial extracts on a DEAE-cellulose column (Figure 3).

RNA-free reverse transcriptase requires the four d-NTPs and transforming RNA for DNA synthesis, which is measured after precipitation by trichloroacetic acid (Table VI). In contrast, RNA-bound reverse transcriptase requires, for DNA synthesis, the four d-NTPs but no transforming RNA (Table VII). In both cases, omission of one or of two of the d-NTPs leads to a considerable decrease in product formation. If only one d-NTP is available, it will not be polymerized by the enzyme. Neither ribosomal nor transfer RNAs are used as templates by *E. coli* RNA-free reverse transcriptase, for which the most efficient template proved to be transforming RNA excreted by the bacteria.

Template activity of transforming RNA is destroyed by preincubation with RNase. The low amount of product formed in the presence of *E. coli* DNA disappears when the preparation is pretreated with pancreatic or T1 RNase, indicating that it must

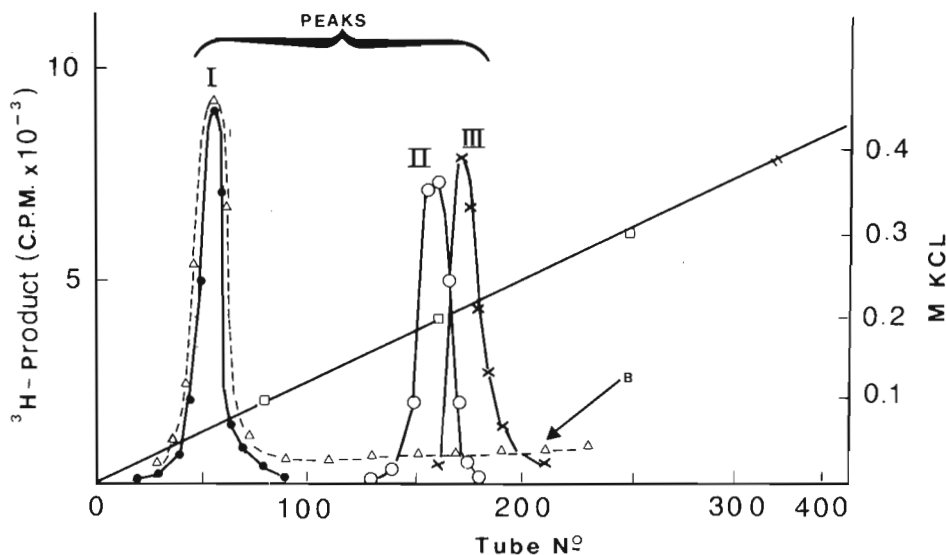


Figure 3 - Isolation of the RNA-bound reverse transcriptase on DEAE-cellulose column (see Materials and Methods section). Peak I RNA-bound reverse transcriptase without exogenous RNA or DNA; RNA-bound reverse transcriptase plus exogenous RNA or DNA. Peak II RNA-free reverse transcriptase. Peak III, DNA-dependent DNA polymerase. The activity of each fraction was determined in the absence and in the presence of exogenously added RNA template or DNA (each separately used) as described in the Materials and Methods section under Enzyme Assay (Beljanski and Beljanski, 1974).

have originated with some remaining DNA-bound RNA ("episome"). ^3H -product synthesis occurs neither in the presence of DNase nor in the absence of transforming RNA.

Table VI - Amount of ^3H d-AMP integrated into macromolecular product after 20 min. under various conditions (Beljanski, 1972).

pmoles		pmoles		Inhibition (%)
Complete medium	411	Complete medium	418	-
- transforming RNA	< 1	+ DNase, 5 μg	< 1	99
- MgCl_2	< 2	+ RNase, 20 μg without preinc.	230	40
- d-GTP	136	+ RNase, 20 μg , preinc.	43	90
- d-CTP	125	+ showdomycin, 50 μg	105	75
- d-GTP, d-CTP, d-TTP	< 1	+ showdomycin, 50 μg (enzyme M)	420	0

Activity, as evidenced by the amount of synthesized ^3H -product, increases with enzyme concentration (Figure 4), and RNA concentration (Figure 5). The ^3H -product synthesized under optimal conditions (20 min. incubation) and separated from the template is resistant to RNase, pronase and KOH; however, it is degraded by pancreatic DNase (Table VIII).

Table VII - Activity of RNA-bound reverse transcriptase in the presence of different templates (Beljanski, 1972).

	pmoles of ^3H -ATP and ^3H -TTP incorporated in 20 min.	
Complete medium + transforming RNA	402	656
Complete medium + wild-type <i>E. coli</i> excreted RNA	392	400
Complete medium + <i>E. coli</i> 23S + 16S RNAs	< 1	< 1
Complete medium + total <i>E. coli</i> transfer RNA	< 1	< 1
Complete medium + <i>A. faecalis</i> 5.5 S RNA	145	136
Complete medium + PolyAG + PolyUC	< 1	< 1
Complete medium + viral RNA (avian myeloblastosis)	< 2	-
Complete medium + wild-type <i>E. coli</i> DNA	98	-
Complete medium + wild-type <i>E. coli</i> DNA pretreated with RNase	2	-

The ^3H -product synthesized *in vitro* by *E. coli* reverse transcriptase on a transforming RNA template was separated from the enzyme and characterized by physical and chemical methods (Beljanski and Beljanski, 1974). Figure 6 shows that it overlaps RNA and DNA density regions (Cs_2SO_4 gradient at pH 7.65). This heterogeneity is expected for RNA-DNA hybrids. However, a certain amount of labeled material sediments in the DNA density region, indicating the presence of free DNA.

Table VIII - Properties of ^3H -product synthesized in the presence of transforming RNA (Beljanski, 1972).

Treatment	Acid-precipitable ^3H -product (cpm)	%
None	1,230	100
With RNase, 50 μg , 10 min. at 20°C	1,197	99.5
With DNase, 50 μg , 10 min. at 20°C	250	20
With pronase, 10 min. at 20°C	1,226	100
With 0.3 M KOH, 30 min. at 80°C	1,210	100

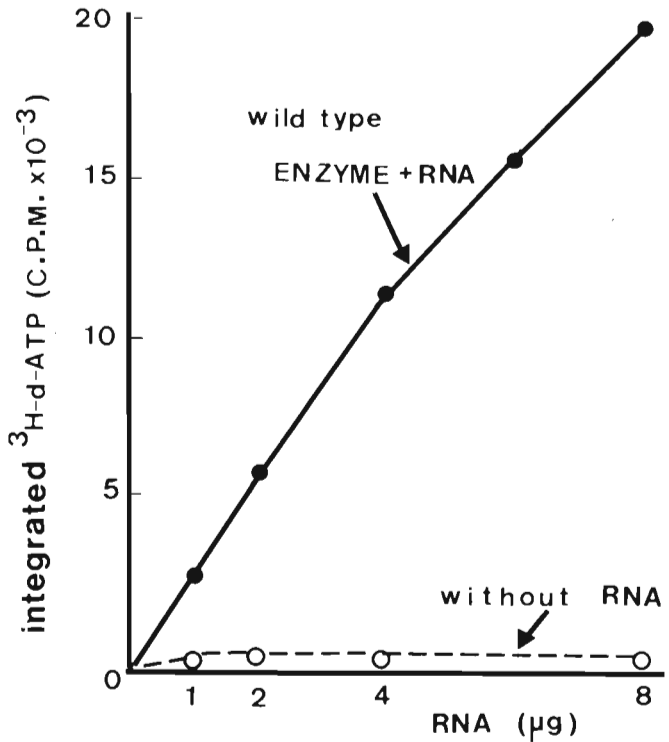


Figure 4 - Wild-type *E. coli* enzyme incubated for 20 min. with and without M 500 mutant transforming RNA (from Beljanski, 1972).

Discovery of RNA-bound reverse transcriptase in bacteria

In addition to our findings concerning RNA-free reverse transcriptase in *E. coli* and *A. tumefaciens*, we described in detail, 18 years ago, a procedure for the combined isolation and characterization of an RNA-bound reverse transcriptase, as a complex which can be distinguished from RNA-free reverse transcriptase and from DNA polymerase I, II and III (Beljanski, 1973; Beljanski and Beljanski, 1974).

When a ribosome-free bacterial extract is passed through a DEAE-cellulose column, three peaks of labeled product are found in the eluted fractions; all these peak protein fractions polymerize d-NTPs into an acid-precipitable product (Figure 3).

Peak I exhibits a high activity which is not modified by addition of *E. coli* transforming RNA; it corresponds to RNA-bound reverse transcriptase. Peak II corresponds to RNA-free reverse transcriptase: polymerization is achieved only when an RNA template (transforming RNA) is added. Peak III is DNA-dependent DNA polymerase I and requires a DNA template.

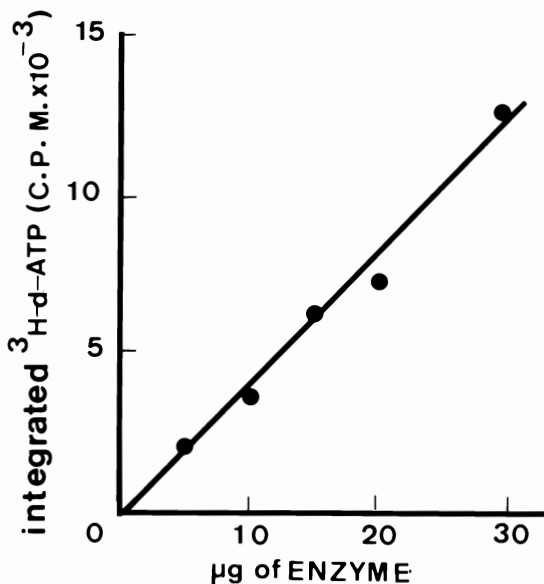


Figure 5 - Wild-type *E. coli* enzyme incubated for 20 min. with 4 µg of M 500 mutant transforming RNA (from Beljanski, 1972).

Peak I enzyme activity is high (Figure 3), provided the incubation medium contains the four d-NTPs; d-NDPs are not polymerized by this enzyme. Heating the enzyme for 10 minutes at 100°C completely destroys its activity, and treating with RNases for 20 minutes at 24°C leads to a considerable decrease of TCA-precipitable ³H-product. Peak I fraction is however partly resistant to RNase A (and, though to a lesser extent, to RNase T1); this may be explained by the fact that, being purine-rich (G+A/C+U = 2.0; see Table IX), the enzyme-bound RNA is less easily degraded by these RNases. *E. coli* DNA, calf thymus DNA ("native" or "activated") and poly(rA)-poly (dT) do not modify RNA-bound reverse transcriptase activity.

Active RNA-bound reverse transcriptase was separated into its RNA and protein components in order to study their respective properties. The RNA contains no DNA. After treating with periodate, it is found to be completely inactive when reincubated with the protein fraction, showing that the 3'OH group of RNA is essential to reverse transcriptase activity. Periodate has no effect on the DNA template of DNA dependent DNA polymerase.

The protein obtained after treating the RNA-bound enzyme with RNase (A and T1) and passing through a Sephadex G200 column (Table II) requires an RNA template, but not a DNA template, for its activity.

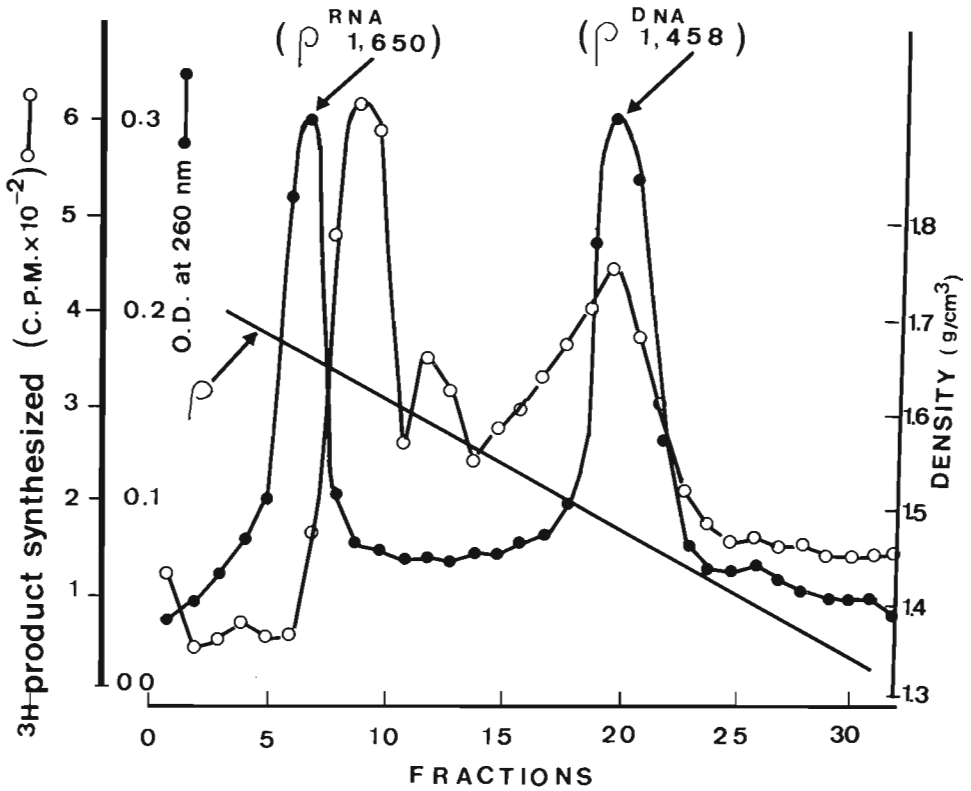


Figure 6 - Cs_2SO_4 gradient (density: 1.550 g/cm³, pH 7.7. Amount of ^3H -product synthesized after 30 min., + RNA and DNA markers. Centrifugation: 30,000 rpm for 60 hours at 20°C (from Beljanski, 1972).

Characteristics of reverse transcriptase-bound RNA

It was of obvious interest to separate and characterize the RNA which was bound to reverse transcriptase.

- 1) The separated RNA preparation contained no detectable amount of DNA.
- 2) In this RNA, G+A/C+U = 2.1. It was thus as purine-rich (Table IX) as *E. coli* transforming RNA (Beljanski, 1973; Beljanski and Beljanski, 1974).
- 3) The size of reverse transcriptase bound RNA was measured using both gel electrophoresis and sucrose gradient centrifugation. A narrow range of RNAs appeared, having similar molecular weights with an average value of about 6S, that is, containing around 165 nucleotides (the slight discrepancies between different samples is probably due, in part, to the fact that synthesis of protein-bound RNA is not always completed). This value corresponds to that of transforming RNA excreted by Sho^R *E. coli*.

Table IX - Base ratio analysis of RNA-bound to reverse transcriptase and ^3H -DNA synthesized by this enzyme.

Nucleotides	RNA bound to reverse		synthesized ^3H -DNA			<i>E. coli</i> DNA	
	transcriptase		Expt. I ^b	Expt. II ^b	Expt. III ^c		
	Expt. I	Expt. II					
A	32.5	31.2	17.3	17.0	18	24.5	
G	36.0	35.1	18.7	18.8	18.3	24.8	
C	16.0	16.6	30.9	32.2	31.2	24.6	
U (T)	15.5	17.0	33.1	34.0	32.5	26.1	
G+A/C+U -	2.1	1.99	C+T/G+A -	1.74	1.98	1.76	1.01

^a Base ratio of RNA was determined as described in Materials and Methods. (Beljanski *et al.*, 1971c; Beljanski and Beljanski, 1974). ^3H -DNA was synthesized at 36°C for 20 min.

^b Base ratio determined from the amount of each DNA-integrated H^3 -dNTP.

^c H^3 -DNA was synthesized, purified, and analyzed as described (Beljanski and Plawewski, 1973).

We mentioned that we had made prior experiments that had led us to the surprising finding that not only Sho^{R} but also wild type *E. coli* contained such a small RNA, which, under normal circumstances, remained in some way attached to bacterial DNA, most probably in the form of a more or less loosely bound piece of RNA or "episome". Its size, base ratio and also its transforming potentialities closely resembled those of Sho^{R} -excreted transforming RNA (Beljanski, 1973; Beljanski *et al.*, 1974). We demonstrated that, once freed from DNA by a cleaving enzyme, this "episomal RNA" may be transcribed into cDNA by bacterial reverse transcriptase. This path leads to stable and heritable transformation of *A. tumefaciens* (Beljanski *et al.*, 1972).

We also investigated the tumor-inducing principle contained in *A. tumefaciens*. Contrary to the common belief that it is contained in a plasmid (i.e., in plasmid DNA), we found the tumor-inducing principle to be pure, 5S to 6S RNA, which, in the bacterium, was seen to be bound either to DNA or to reverse transcriptase (Beljanski *et al.*, 1971b). In the course of later research, we also demonstrated the presence of reverse transcriptase in plant cells (Beljanski *et al.*, 1974), in the mold *Neurospora crassa* (Dutta *et al.*, 1977), and in fertilized fish eggs (Beljanski *et al.*, 1988).

DISCUSSION AND CONCLUSION

The discovery we made in 1971-1972 of reverse transcriptase in bacteria was well documented by experimental data.

1) We showed that transforming RNA is transcribed by the enzyme into cDNA. Transforming RNA possesses self-priming regions and is likely to have the 3'OH looped terminus, which is required for transcription^(*).

2) After chemical and physical analysis of transforming RNA, we determined the required conditions for purification of reverse transcriptase from bacterial extracts and for transcription of transforming RNA into cDNA.

3) We showed that bacterial reverse transcriptase could not be confused with DNA-dependent DNA polymerase I, II and III (Beljanski and Beljanski, 1974).

4) Having shown that *E. coli* transforming RNA remains undegraded in the culture medium as well as after uptake by *E. coli* or after it has been absorbed by *A. tumefaciens*, we performed experiments demonstrating that a DNA transcript of this RNA is integrated into the genome of the recipient bacterial cell (Beljanski and Plawecki, 1972).

In our first paper on the topic (Beljanski *et al.*, 1971, 1972), we described the drastic alteration of various RNAs and proteins (ribosomal proteins and enzymes) in transformants; this implied that the purine-rich transforming RNA was a highly autonomous entity capable of escaping genomic DNA control. We considered it as an extra-chromosomal determinant which was expressed under specific conditions only.

Transforming RNA could migrate from one cell into another, inducing in its host profound biochemical and, thereby, physiological modifications. In *E. coli*, transformants, in turn, started excreting transforming RNA, and this explained the fact that no revertants were ever observed. But revertants did not appear either in quite a different bacteria, *A. tumefaciens*, which, after absorbing transforming RNA, had its properties altered to the point of completely and permanently losing its oncogenic potentialities in plants. Yet *A. tumefaciens* transformants did not excrete transforming RNA: the message the latter carried must then have become integrated into the recipient bacteria's DNA. These were valid motivations for searching for a bacterial reverse transcriptase, which indeed we soon found.

A number of years after we had published our findings, the presence of reverse transcriptase in *E. coli* was confirmed by several authors (Romashchenko *et al.*, 1977; Vorob'eva *et al.*, 1982; Grabkina *et al.*, 1983). Much more recently, in 1989, several American scientists "rediscovered" reverse transcriptase in bacteria and H. Varmus then declared: "the procaryotic void is filled: bacteria too have reverse transcriptases".

(*) In this respect, we may mention that, in *E. coli* H12 carrying the CX28 fragment, an RNA transcript of approximately 6S (170 bases) has been found, which has a stable stem and loop structure at the 3'OH terminus (Mizuno *et al.*, 1983). This small RNA is involved in blocking translation of a specific messenger RNA (our own studies showed small RNAs to be highly versatile indeed).

Together with S. Dutta, we showed years ago that an endogenous RNA-bound reverse transcriptase could also be derived from the microsomal pellet fraction of the eucaryotic fungus *N. crassa* (Dutta *et al.*, 1977). Here too, the enzyme-free RNA template as well as the synthesized cDNA sedimented between 5 and 6S on sucrose density gradients. ³H-DNA was complementary to template RNA, as shown by hybridization data.

The recent discovery of multicopies single-stranded DNA (msDNA) in the bacteria *Myxococcus xanthus* (Dhumdale *et al.*, 1987) and in *E. coli B* (Lim and Mass, 1989) led to the demonstration that reverse transcriptase is required for the formation of branched DNA-RNA compounds both *in vivo* and *in vitro* (Lim and Mass, 1989; Lampson *et al.*, 1989). Bacterial RNA which primes msd locus transcription (msdRNA) is comprised of 77 bases in *M. Xanthus* and 82 bases in *E. coli*. DNA is comprised respectively of 162 and 144 base pairs. The transforming RNA we discovered in 1971 contains 160 to 165 bases, and reverse transcriptase synthesizes from it, *in vitro*, cDNA of equivalent size.

We had also reported, in the course of our extensive research on small RNAs, that a 65 nucleotide RNA fragment (obtained by degradation of *E. coli* ribosomal RNA with U2 RNase) may be used *in vitro* as a self-priming template by *E. coli* RNA-free reverse transcriptase to form cDNA (Beljanski *et al.*, 1978). *In vivo* this U2 RNA fragment is highly active as a regulator of plant tumor formation (Le Goff and Beljanski, 1979). A few years later, American workers showed that U3 RNA, which is abundant in the nucleoli of avian and mammalian cell nuclei, can serve *in vitro* as a template for avian myeloblastosis virus (AMV) reverse transcriptase, which then synthesizes a 75 base pair cDNA. This takes place in the absence of any RNA or DNA primer (Bernstein *et al.*, 1983).

Many observations strongly suggest that reverse transcription goes on in many cells of numerous species. Among striking examples of this, we may mention the identification of retroviral sequences in the genome of various species (Bishop, 1978), the existence of groups of short repetitive genomic DNA sequences (Arsdell *et al.*, 1981), and the integration of heritable preroetroviral DNA sequences in human germline progenitor cells (Schmid and Jelinek, 1982).

We note, in contrast, the case of pseudogenes (Jagadeeswaram *et al.*, 1981; Poiesz *et al.*, 1981), which are genes from which precise, short introns have disappeared, preventing expression. Such pseudogenes are known, for instance, in globin.

It might be assumed that failure to synthesize these introns is due to some hitch in the combined activities of small RNAs and reverse transcriptase, underlining how crucial these chemical entities are to life processes.

In the extensively studied insect *Drosophila melanogaster*, choice material of geneticists for many years, an RNA-bound reverse transcriptase has been demonstrated

(Shiba and Saigo, 1983), as well as cytoplasmic, nonviral particles containing 4S, 4.5S and 6S RNA. This strongly supports some of the findings we reported many years ago (Beljanski, 1973; Beljanski and Beljanski, 1974).

It has been suggested that in *Drosophila*, reverse transcriptase might be responsible for transposition of movable genetic elements (transposons) (Shiba and Saigo, 1983). Yeast transposons have been shown to transpose via an intermediate RNA. In this group, the pattern of sequence inheritance in progeny Ty insertions follows the predictions of a model involving retroviral reverse transcriptase.

Small RNAs and reverse transcriptase thus appear to be intricately and universally involved, independently or together, in genetic processes. From the time we started to investigate small RNAs, over thirty years ago, to this present day, many of these molecules have been accurately characterized (Beljanski and Le Goll, 1986). However, their roles in cells of numerous species must still be definitively ascertained before their possible phylogenetic relationships and their importance for cell life can truly be elucidated. In the past, few scientists have investigated the extent to which small RNAs may be transcribed into DNA, *in vitro* and *in vivo*. The discovery of pseudogenes has brought this problem to the fore: some regions of pseudogenes are transcribed into small RNAs. It is believed at present (but, as far as we know, not definitely proved) that small RNAs, via their transcription into DNA, are the sole candidates for the creation of new genes and pseudogenes (the latter, as we have just seen, set up a sort of cycle by synthesizing in turn small RNA molecules). Study of the numerous small RNAs suggests that a number of them may be interrelated and that a relationship may also exist between several small and large RNAs. In this line of thought, it is believed that pseudogenes may result from retrotranscription of intact or degraded messenger, ribosomal or small nuclear RNAs; in this way a close link might be established between large and small RNA molecules. Whatever the case may be, small RNAs appear to be largely conserved throughout evolution (Beljanski and Le Goff, 1986).

Further search for reverse transcriptase activity in normal mammalian and human cells has become a necessity in view of all its possible genetic consequences. A reverse transcriptase activity may be detected in normal human placenta cells, provided that well-defined assay conditions are respected (Nelson *et al.*, 1981). Detection of the enzyme, in eucaryotic as well as in procaryotic cells, may indeed prove quite difficult.

Yet use can be made of specific factors which regulate reverse transcriptase activity. Although, in this paper, we emphasized transcription of small RNAs, the enzyme of course also transcribes much larger molecules such as messenger RNAs, large amounts of which may accumulate in certain cells, for instance in fertilized eggs.

Not long ago, together with a Chinese group headed by Prof. M.C. Niu, we were looking for reverse transcriptase in fertilized fish eggs. A high enzyme activity could be expected, and yet conventional assays yielded disappointingly low values. I then had the

idea that some unrecognized factor, such as a mineral element, could be required for enzyme activity. We finally chose iron on the strength of Needham's observation as far back as 1935 (Needham, 1935) that fertilized eggs contain large amounts of it. In the presence of iron, transcription was spectacularly enhanced and we could readily demonstrate the existence of a reverse transcriptase in fish eggs as well as its ability to transcribe messenger RNAs into cDNAs (Beljanski *et al.*, 1988). Unfertilized eggs already contain important quantities of messenger RNAs of maternal origin (Raff *et al.*, 1972; Niu, 1981). Fertilization modified the membrane in such a way that iron may enter the egg; iron then stimulates reverse transcription. This regulation is of primary importance for embryonic development.

In the course of his studies, Niu has also reported that when purified messenger RNA from rabbit red blood cells is injected into goldfish eggs, it induces the production of cDNA sequences even when the egg nucleus is removed (Niu *et al.*, 1989). These DNA transcripts hybridize to cloned cDNA of rabbit globin messenger RNA. They seem to become integrated into chromosomes of the developing eggs. The question must then be asked: which part of the messenger RNA is transcribed into a DNA sequence? Are these transcripts involved in the egg in the biological activities of gene activation?

Along these lines, it is of interest to mention a recent successful attempt by Zucchi *et al.* (1989) to compensate genetic alteration in a defective *Aspergillus nidulans* strain. The protoplasts of this strain were incubated with total RNA derived from another *A. nidulans* strain, bearing wild type alleles for the same genetic markers. Genetic alterations were corrected. Yet which RNA took part in the process, and whether it had or not priorly been degraded, was not ascertained.

In this regard, Buchman and Berg (1988) recently demonstrated that recombinant simian SV 40 virus, carrying rabbit B globin cDNA, failed to express this B globin sequence unless an intron was included in the transcription unit; addition of globin introns IVS 1 or IVS 2 caused a 400-fold increase in globin expression. As we pointed out earlier in this text, intron DNA might be derived from transcription of small RNAs.

These varied examples should call our attention to the part reverse transcriptase must have taken in evolution and to the close mutual dependency of this enzyme, small RNAs, and the genome.

RESUMO

Em 1970, juntamente com meu grupo de trabalho no Instituto Pasteur, em Paris, eu observei que células de *Escherichia coli* resistentes a showdomycina, excretavam dentro de seu meio de cultura, um RNA pequeno, aproximadamente com 150 nucleotídeos de comprimento, rico em bases de purina. Este RNA transforma bactérias selvagens (*E. coli* e *Agrobacterium tumefaciens*) em transformadores que exibem novas

caractéristiques biochimiques e físicas estáveis. Portanto, *A. tumefaciens* depois de ser transformado pelo RNA transformador de *E. coli*, parcial ou totalmente perde suas potencialidades oncogênicas e adquire novas propriedades. Pareceu que a transcriptase inversa poderia dar ao RNA transformador meios para integrar ao genoma dos organismos transformados as modificações esesestransmitidas por ele. Nós demonstramos (em 1971-1972) que a transcriptase inversa de bactérias estava envolvida com esse processo. No decorrer de nossos estudos sobre transformação bacteriana, nós desenvolvemos uma técnica que levou a descoberta, primeiro da transcriptase inversa livre de RNA em bactérias, e depois da transcriptase inversa ligada ao RNA, a qual facilmente se distingue da polimerase DNA - dependente - DNA. Em 1989 vários cientistas americanos redescobriram a transcriptase inversa em *E. coli*.

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