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Transforming RNA as a template directing RNA and DNA synthesis in bacteria*

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Attempts were made to approach the mechanism by which the transfer of information is carried by transforming RNA from *Escherichia coli*. Transforming RNA, capable of inducing inherited changes in recipient cells, is used in vitro as a template by two distinct enzymes which mediate the transfer of information from RNA to RNA and DNA respectively. Using transforming RNA as a template, polynucleotide phosphorylase insensitive to rifampicin, synthesizes a product which was characterized as being a "copy" of template RNA. Reverse transcriptase, which can be physically separated from DNA polymerase, transcribes the transforming RNA into a complementary DNA product. According to hybridization experiments, DNA from *Agrobacterium tumefaciens* transformed by *E. coli* transforming RNA seems to contain one or less than one copy of complementary DNA.

1. Introduction

Discoveries of particular RNA species in chromosomes [1], plasmids [2], plants [3–5] and bacteria [6, 10] have opened a new avenue to study the transfer of information in biological systems. Thus "viroid RNA" [3, 5], free from proteins (causing diseases in plants) and transforming RNA inducing transformation in bacteria [6, 7] are both excellent candidates for Temin's provirus [8] and protovirus hypotheses [9]. Our investigations have established that genetic information can be transferred to different bacterial species by a specific transforming RNA found as a product excreted into the culture medium of showdomycin resistant mutants of *Escherichia coli* [6, 7, 11]. Transformants, which appear at a high rate, exhibit physiological and biochemical changes of unexpected magnitude [7] especially illustrated by transformed *Agrobacterium tumefaciens* B_6 [7, 13]. These trans-

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Abbreviations: RNase, pancreatic ribonuclease; DNase, pancreatic deoxyribonuclease; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; XDP, ribonucleoside-5'-diphosphate; XTP, ribonucleoside-5 '-triphosphate; d-XTP, deoxyribonucleodide-5'-triphosphate; Sho-R, showdomycin resistant; PNPase, polynucleotide phosphorylase.

formants have acquired new characteristics expressed in profoundly altered ribosomal RNA (rRNAs), ribosomal proteins and certain enzymes [7, 11, 12]. Unexpectedly, the transformants of the oncogenic strain *A. tumefaciens* B_6 have partially or completely lost their capacity for tumor induction in plants [7].

In order to explain the origin of transforming RNA and the process by which it induces *inherited changes* in recipient bacteria we raised the two following questions:

(1) Does the transforming RNA direct RNA or DNA synthesis in the presence of specific enzymes?

(2) Does the transformation by RNA operate through an RNA to DNA pathway?

The present work deals with studies conducted in order to determine whether transforming RNA can be used in vitro as a template by two distinct enzymes which mediate the transfer of information from RNA to RNA and to DNA respectively. Pathways through which transforming RNA could transform the recipient bacteria are discussed.

The most striking property of transforming RNA (excreted RNA from *E. coli* Sho-R) is that at a low concentration (0.1 μg/ml) it rapidly induces in *E. coli* the transformation of the whole recipient population, leading to stable transformants which express new biological properties [6, 7, 12]. This same RNA fraction also transforms *A. tumefaciens* with high efficiency into partial and complete transformants with characteristics which have never been observed before in wild types of the same species [7, 13]. It is also remarkable that partial transformants of *A. tumefaciens* become completely transformed during further growth in the absence of transforming RNA [15].

The techniques for isolation, purification and characterisation of transforming RNA as well as the procedure for transformation of bacteria have been described elsewhere [6, 10]. It should be added that recipient bacteria are usually harvested before the stationary phase of growth and that the synthetic medium used for transformation should contain a rather low amount of carbon source in order to achieve rapid and efficient transformation of recipient cells.

2. *In vitro replication of transforming RNA*

Excreted RNA and episomal RNA* [6, 10], both carrying the genetic information for transformation differ from all other RNA species found in *E. coli.* In particular, their base ratio G+A/C+U of 1.70 to 2.0 differs considerably from that of RNA's from the wild type (G+A/C+U = 1.0). This characteristic indicates that purine-rich RNA is not complementary to DNA of the same bacterial species. This was already shown by the absence of hybridization between ribosomal RNA's rich in purines from *E. coli* Sho-R and DNA of the same strain. This characteristic implies that the mechanism by which the transforming RNA is synthesized and replicated has to be

* Episomal RNA is a transforming RNA bound to DNA.

different from that proposed for other RNA species. Thus DNA-dependent RNA polymerase should not be the enzyme involved in replication of the transforming RNA. In fact, in vitro experiments show that this is the case.

As an enzyme which would replicate the transforming RNA, polynucleotide phosphorylase (PNPase) was a likely prospect for two reasons. First, PNPase from wild type bacteria grown in the presence of showdomycin has modified properties, i.e. in the presence of equivalent amounts of all four ribonucleoside-5'-diphosphates (XDP) it synthesizes a polymer whose content of purine nucleotides exceeds that of pyrimidine nucleotides [14]. Second, PNPase from *E. coli* M 500 Sho-R, synthesizes in vitro an AGUC polymer in which the amount of purines is twice that of pyrimidines. It should be recalled that "episomal RNA" and excreted RNA from M 500 Sho-R contain purine nucleotides in excess and these two RNA fractions possess an equivalent genetic potential toward recipient cells [6, 10].

Preliminary experiments showed that transforming RNA was not used as a template by DNA-dependent RNA polymerase. We considered the possibility that it was used as a template by the PNPase. If it was, the product synthesized by wild type PNPase in the absence of template RNA might have a different base composition from that synthesized by the same enzyme in the presence of transforming RNA. We were thus led to search for conditions in which PNPase from wild type bacteria (wild enzyme) would replicate the transforming RNA in vitro. Although there is an endogenous activity of PNPase in the absence of any kind of RNA, addition of transforming RNA to the reaction mixture results in a several fold stimulation of the enzyme activity (fig. 1). Remarkably, the stimulating effect with transforming RNA is observed only if all four XDP's are present in the reaction mixture. No effect is observed in the presence of all four XTP's. High enzyme purity is not required, since crude or 250-fold purified preparations respond in the same fashion to the presence of transforming RNA [14].

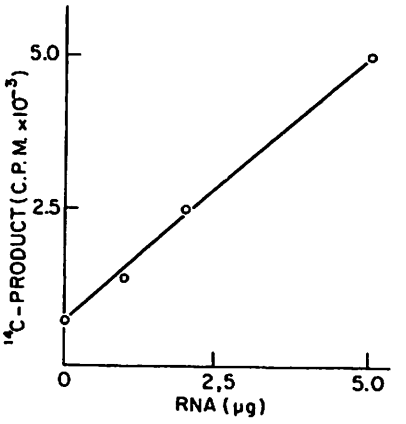

Fig. 1. Effect of transforming RNA on polynucleotide phosphorylase activity. Incubation conditions, see legend to table 1. Transforming RNA excreted by showdomycin resistant *E. coli* was used. Time, 30 min at 36°C.

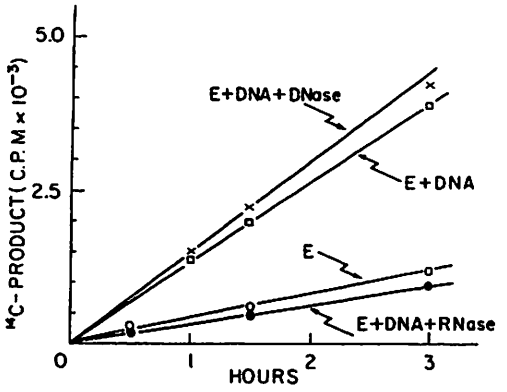

Fig. 2. Effect of episomal RNA on polynucleotide phosphorylase activity. PNPase was isolated and purified from *E. coli* Hfr wild type as described [14]. Incubation conditions, see legend to table 1. All four ^{14}C-XDP were used. Aliquots taken at indicated intervals were mixed with TCA (5%) and the precipitate washed on GF/C glass filters. After drying, the radioactivity was determined in a Packard liquid spectrometer.

The effect of various bacterial RNA species on the endogenous activity of PNPase has been tested. None of these RNA's was stimulatory [14]. However DNA isolated from wild type *E. coli* exhibits a strong stimulatory effect on PNPase activity (fig. 2). But such a DNA preparation has to be purified under appropriate conditions [10] in order to contain no more than 7–8% of RNA (orcinol reaction).* It still carries the episomal RNA from which the excreted RNA originates. The stimulatory effect remains even if the DNA is extensively treated with pancreatic DNase.

TABLE 1

Replication of episomal RNA by polynucleotide phosphorylase in the presence of rifampicin

	Nanomoles of ^{14}C-nucleotides incorporated in 10 min	
	4-XDP	4-XTP
Complete	5.80	0.76
+ rifampicin 10 μg	5.85	0.80
+ rifampicin 20 μg	5.70	--
+ rifampicin 40 μg	5.80	0.70

Incubation mixture contains per 0.2 ml: Tris (pH 8.0) 100 μM; Mg^{2+} 2 μM; XDP 0.25 μM of each, 2.5 X 10^4 cpm. DAN from *E. coli* (wild type) 20 μg. DNase 10 μg. Enzyme (G-200 Sephadex) [14] 100 μg; rifampicin added at the beginning. Time of incubation: 30 mn at 37°C (except when indicated). ^{14}C-product was precipitated with trichloroacetic acid, washed, filtered on GC/F glass filters, dried and radioactivity determined in a Packard liquid spectrometer.

* If DNA preparation contains more than 10% of RNA it does not stimulate the activity of PNPase.

TABLE 2
^{14}C product synthesized by polynucleotide phosphorylase in the presence of RNase

	Moles per 100 moles of nucleotides	
	^{14}C-product with enzyme alone + RNase	^{14}C-product with enzyme + DNA + RNase
A	23.5	31.0
G	50.0	40.5
C	14.3	14.3
U	12.1	14.2
G+A/C+U	2.79	2.51

^{14}C-polymer was synthesized in the presence of RNase (20 μg/0.2 ml) without and with DNA carrying the episomal RNA (see legend to table 1). ^{14}C-polymer was precipitated with TCA, washed several times with TCA (5%), hydrolyzed with N HCl at 100°C for 1 hr. Degraded material was separated and analysed [43].

The effect of increasing concentrations of template RNA is given in fig. 1. With purified PNPase (50–100 μg) the rate of polymer synthesis is linear during several hours. Fragmented RNA (after treatment by pancreatic RNase) has completely lost its stimulating activity. The ^{14}C labelled product, synthesized in the presence of RNase (independently if template RNA is present or not) is mainly made from AMP and GMP nucleotides (table 2).

The absence of effect of *rifampicin* on PNPase activity (table 1) clearly shows

TABLE 3
Base analysis of transforming RNA (associated with DNA) and ^{14}C RNA product

Nucleotides	Moles per 100 moles of nucleotides		
	Polymer with enzyme alone	Polymer with enzyme + DNA	Episomal RNA (*E. coli* wild type)
A	23.4	29.7	29.6
G	26.7	35.2	34.4
C	21.5	17.6	18.0
U	28.4	17.5	18.0
G+A/C+U	1.01	1.80	1.73

The base ratio of transforming RNA (episomal RNA) was determined using a Dowex column as previously described [10]. ^{14}C-polymer synthesized in vitro (see legend to table 1) was precipitated and washed several times with 5% TCA, hydrolysed with 0.3 N NaOH and separated on a Dowex column (20.000 cpm per sample) in the presence of 1 mg of unlabelled ribosomal RNA hydrolysed with 0.3 N NaOH [21]. Ratio of radioactive nucleotides is presented.

that the synthesis of polymer is not catalyzed by DNA dependent RNA polymerase. It is well established that rifampicin inhibits the activity of this latter enzyme [15].

3. *Analysis of the ^{14}C product synthesized in the absence and presence of transforming RNA bound to DNA*

3.1. Base ratio analysis

The ^{14}C labelled product was synthesized with wild type PNPase in the presence of equivalent amount of ^{14}C-labelled XDP and in the presence and absence of transforming RNA. The ^{14}C-product was separated from the enzyme as described in the legend to table 3, and washed. The dialysed ^{14}C product was degraded by alkali and the nucleotides separated on a Dowex-column.

Table 3 shows that ^{14}C-product synthesized in the presence of transforming RNA (episomal or excreted RNA) has base ratios close or identical to those found for transforming RNA itself. In the absence of template RNA, base ratios are completely different. These results show that the replication of the transforming RNA is accomplished by PNPase through a mechanism in which complementarity of bases is not observed.

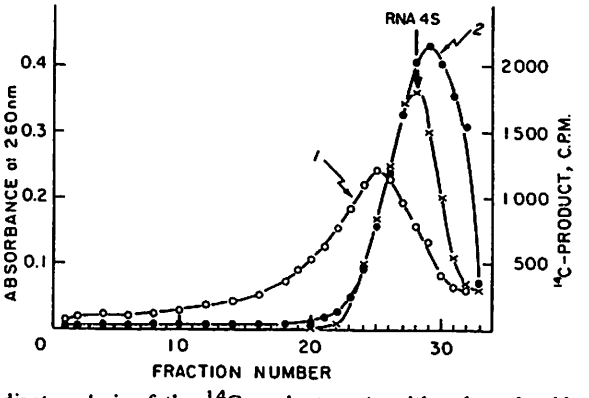

Fig. 3. Sucrose gradient analysis of the ^{14}C-product made with polynucleotide phosphorylase. The ^{14}C product was synthesized as described in the legend to table 1 using four labelled ^{14}C-ribonucleoside-5'-diphosphates. The ^{14}C product was extensively dialysed against 0.2 M KCl in distilled water and sedimented in a 5–20% linear sucrose gradient at 4°C in the Spinco L SW_{39} rotor at 25,000 rpm for 16 hr. Fractions were collected and the radioactivity determined. Transfer RNA^{met} was included as marker. (1) = ^{14}C polymer synthesized in the absence of template. (2) = ^{14}C polymer synthesized in the presence of DNA + DNase.

3.2. Sucrose gradient analysis

Fig. 3 illustrates the profile of the ^{14}C-polymer synthesized in the absence and presence of template (DNA + DNase). 4 S RNA was included as internal marker in a linear sucrose gradient. One sees that ^{14}C polymer synthesized upon template sediments at a position different from that of the ^{14}C polymer made in the absence of template. Further analysis showed that the ^{14}C product is not bound to DNA, that it is not double stranded [16]. However it is rather resistant to pancreatic RNase due to the fact that it contains an excess of purine nucleotides.

4. Transcription of transforming RNA into DNA by reverse transcriptase

Transformation of bacteria by transforming RNA raised the following question: could the transforming RNA be transcribed into DNA? To answer this question it was necessary to determine (a) to what extent transforming RNA was contaminated by DNA and (b) to search for an enzyme fraction which would transcribe the RNA into DNA.

4.1. Does the transforming RNA contain DNA?

Transforming RNA purified as previously described [5] was analysed for DNA content. Even when large amounts of RNA were used the diphenylamine reaction was negative (table 4). However when the excreted RNA was isolated from culture medium in which *E. coli* Sho-R had been grown in the presence of ^{14}C-thymidine, radioactivity was detectable in the RNA preparation and corresponded to a DNA content of around 0.05% on the basis of the specific radioactivity of newly synthesized bacterial DNA. No further attempts were made to characterise the radioactive material. Transforming RNA, centrifuged in Cs_2SO_4 gradient, sedimented in the region of RNA and no detectable amount of U.V. material or radioactivity was found in DNA density region (fig. 4). A small contaminating DNA product, if present in the transforming RNA, could be considered as a product of transcription of the transforming RNA.

4.2. Search for reverse transcriptase

In our preliminary experiments we found that an enzyme fraction, present in the 105,000 *g* supernatant from *E. coli*, was capable of polymerizing deoxyribonucleotides in the presence of transforming RNA used as template, and this suggested that the synthesized product was a DNA like material [17]. It was particularly interesting to find out if such an enzyme fraction from *E. coli* and *A. tumefaciens* would distinguish the transforming RNA from all other RNA species from various sources

TABLE 4
DNA content of template RNA(?) and RNA content of DNA

	Ratio 260/280	U.V. absorption at 260 nm	Orcinol reaction	Diphenylamine reaction
Transforming RNA from *E. coli*	2.06	100 μg	162 μg	not detectable [a]
5.5 S RNA from *Alcaligenes faecalis*	2.15	206 μg	204 μg	not detectable [a]
DNA from *E. coli*	2.1	280 μg	26 μg	256 μg

[a] 400 μg of RNA (on the basis of U.V. absorption) were used for diphenylamine reaction.

Several samples and different concentrations of transforming RNA and *Alcaligenes faecalis* 5.5 S RNA [41] were analysed for DNA content. Agreement between U.V. determination and orcinol reaction are presented. DNA isolated under appropriate conditions [10] always contains RNA.

and especially from DNA. The behavior of reverse transcriptase from *A. tumefaciens* toward *E. coli* transforming RNA was of great interest, since this RNA induces inherited changes in *A. tumefaciens.*

5. *Physical separation of reverse transcriptase from DNA dependent DNA polymerase*

The observation that transforming RNA directed DNA synthesis in the presence of a soluble fraction from *E. coli* [17] strongly suggests that this fraction contained a

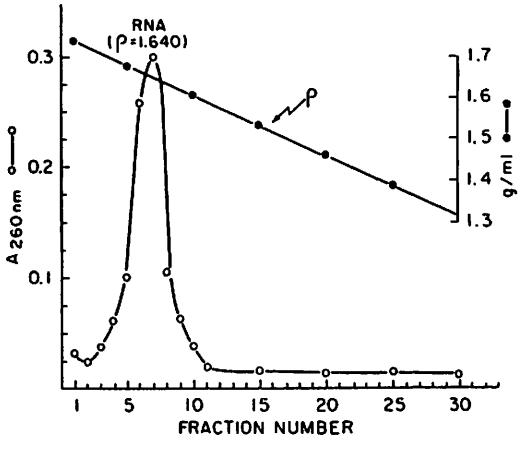

Fig. 4. Centrifugation of transforming RNA in cesium sulfate equilibrium gradient. Transforming RNA (25 μg) in Tris buffer was mixed with Cs_2SO_4 (1.8 g pH 7.3 fin. vol. 3.1 ml) and centrifuged at 20°C (30,000 rpm) for 64 hr in Spinco SW_{39} rotor. Fractions were collected and analysed for refractive index, absorbance at 260 nm and acid-precipitable radioactivity.

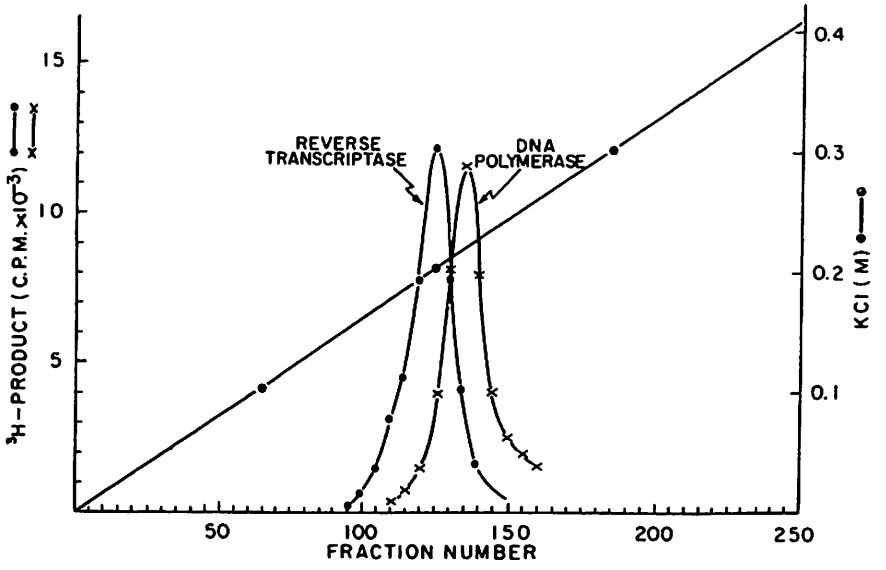

Fig. 5. Chromatography of reverse transcriptase and DNA dependent DNA polymerase on DEAE-cellulose. *E. coli* ML 30 Sho-R cells were grown and disrupted as described [21]. A crude cell extract was centrifuged at 20,000 *g* in the SS1 Servall centrifuge for 30 min. The pellet was discarded. The supernatant was precipitated with $SO_4(NH_4)_2$ (70%) and the precipitate was collected by centrifugation and dialysed against Tris-HCl buffer 10^{-2} M (pH 7.6) and mercaptoathanol (10^{-4} M) containing $MgCl_2$ 10^{-3} M and KCl 0.06 M. This dialysed preparation was chromatographed. 4 g of proteins were applied to the DEAE column (54 × 3 cm) equilibrated with Tris-HCl buffer 10^{-2} M (pH 7.6). After washing the column with the same buffer, it was eluted with a linear gradient: Tris 10^{-2} M (pH 7.6) – Tris 10^{-2} M (pH 7.6) containing 0.5 M KCl. 5 ml fractions were collected and the enzyme activity was assayed with 0.05 ml of each fraction under conditions described in the legend to table 6. Blank values around 100–200 cpm were subtracted. Transforming RNA (2 μg) and thymus DNA (2 μg) were tested as templates.

TABLE 5

Effect of potassium ions on reverse transcriptase activity from *E. coli* and *A. tumefaciens*

Concentration in potassium chloride (M)	Per cent of activity (cpm)	
	E. coli	*A. tumefaciens*
0.01	100	100
0.02	100	74
0.03	97	35
0.04	96	12
0.06	80	9

Incubation conditions, see legend to table 6. ^{3}H d-CTP was used. Excreted RNA (1 μg) from *E. coli* ML 30 Sho-R); enzyme fraction (DEAE, see fig. 6), 50 μg.

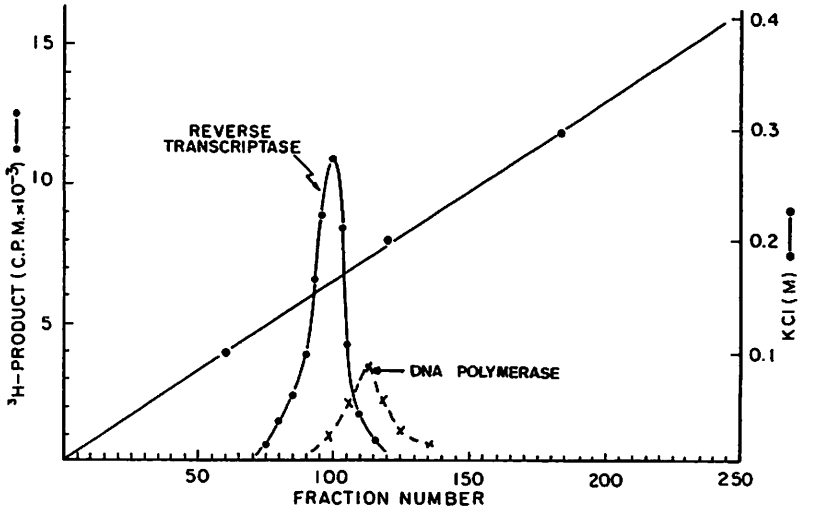

Fig. 6. Chromatography of RNA dependent reverse transcriptase from *A. Tumefaciens* B_6 on DEAE cellulose column. Bacteria were grown in rich medium [10] and the cells broken in the French Press. An ammonium sulfate fraction was prepared as described for *E. coli* in the legend to fig. 5. Chromatography conditions were identical to those described in the legend to fig. 5.

TABLE 6

^{3}H product formation from deoxyribonucleotide-5'-triphosphates in the presence of reverse transcriptase from *E. coli* and *A. tumefaciens*

Reaction mixture	*E. coli* enzyme (pmoles)	*A. tumefaciens* enzyme (pmoles)
Complete	276	30
– transforming RNA	< 1	< 1
– $MgCl_2$	< 1	< 1
– dGTP	72	7
– dCTP	65	8
– dGTP, dCTP, dTP	< 2	< 1
+ RNase (50 μg) preinc.	53	6
+ DNase (10 μg)	< 1	< 1

Incubation mixture (0.2 ml) contains: $MgCl_2$, 2 μM; Tris-HCl buffer (pH 7.65), 25 μM; each deoxyribonucleoside-5'-triphosphate, 5 nmoles + ^{3}H d-TTP (100,000 cpm); RNA 2 μg; enzyme fraction (DEAE) 40 μg (see fig. 5). Incubation 20 min at 36°C. TCA is added to precipitate the ^{3}H product. Precipitates were washed with TCA 5% filtered on Whatman GC/F glass filters, washed, dried, and the radioactivity measured in a Packard liquid spectrometer.

TABLE 7
Activity of reverse transcriptase in the presence of different polymers

	E. coli enzyme (pmoles)	*A. tumefaciens* enzyme (pmoles)
Complete transforming RNA	206	32
Alcaligenes faecalis RNA (5.5 S)	71	–
E. coli rRNA (23 S + 16 S)	< 1	< 1
$tRNA^{met}$	< 1	< 1
rA-dT (pH 7.65)	36	–
rA-dT (pH 9.0, Mn^{2+})	67	–
polyovirus RNA	< 1	< 1
myeloblastosis virus RNA	< 2	–
poly AGUC	< 1	< 1

Incubation conditions, see legend to table 6. ^{3}H d-GTP was used.

reverse transcriptase activity. Evidence for the presence of such an enzyme is presented here. Fractionation of an *E. coli* soluble fraction (see legend to fig. 5) on a DEAE cellulose column shows the presence of an enzyme which actively uses the transforming RNA (and not DNA) as template for DNA synthesis. Separated from DNA dependent DNA polymerase this enzyme behaves like reverse transcriptase found in the RNA viruses. Fig. 6 shows that *A. tumefaciens* also contains a reverse transcriptase and that the activity of DNA dependent DNA polymerase in eluates from DEAE column is very low under our fractionation conditions.

A. tumefaciens reverse transcriptase uses transforming RNA from *E. coli* as a template for DNA synthesis, although with much less efficiency than the *E. coli* enzyme. In contrast to the *E. coli* enzyme, reverse transcriptase from *A. tumefa-*

TABLE 8
Activity of reverse transcriptase and DNA polymerase in the presence of DNA

	E. coli reverse transcriptase (pmoles)	*E. coli* DNA dependent DNA polymerase (pmoles)
Thymus DNA	16	198
Thymus DNA + RNase	8	186
A. tumefaciens B_6 DNA	8	168
A. tumefaciens B_6 DNA + RNase	6	174
E. coli DNA	21	18
E. coli DNA + RNase	7	20
Transforming RNA	206	7

Incubation conditions, see legend to table 6. DNA used 2 μg; ^{3}H d-GTP (100,000 cpm) was used. Transforming RNA 1 μg.

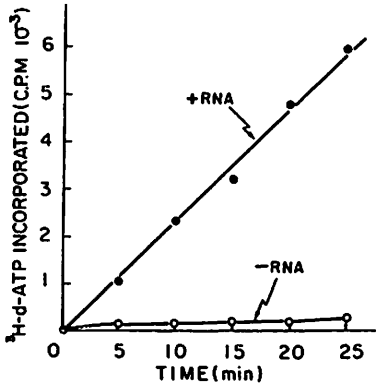

Fig. 7. Synthesis of ^{3}H-DNA product in the presence of transforming RNA as function of time. Incubation conditions, see legend to table 6.

ciens is strongly inhibited by potassium chloride even at rather low concentrations (table 5). However actinomycin D and rifampicin have similar inhibitory effects on the activity of reverse transcriptase from both *A. tumefaciens* and *E. coli.*

6. Specificity of RNA dependent DNA polymerase for RNA templates

Reverse transcriptase transcribes the transforming RNA into a DNA like product most actively in the presence of all 4 d-XTP and Mg^{2+} ions (table 6). The template activity of transforming RNA is destroyed by treatment with RNase. DNA polymer does not accumulate in the presence of DNase. Among various RNA or DNA templates tested (tables 7 and 8), transforming RNA is the best template for reverse transcriptase, although 5.5 S RNA (rich in messenger of transforming RNA) serves as active template for polymerisation of the d-XTP into TCA precipitable material. Time course of enzyme activity is illustrated in the fig. 7.

The amount of ^{3}H-product synthesized in 20 min corresponds roughly to 20% of that of template RNA, although we do not know if all fractions of transforming RNA are transcribed or exclusively one small fraction.

7. Inhibitory effect of actinomycin D, rifampicin and N-demethylrifampicin on reverse transcriptase activity

It has already been shown that actinomycin D [18] and some rifampicin derivatives [28] inhibit the activity of viral RNA dependent DNA polymerase, i.e. of reverse transcriptase. Our results with bacterial reverse transcriptase are similar to those found for the viral enzyme. Actinomycin D at a rather low concentration inhibits the DNA synthesis upon transforming RNA when added to the incubation mixture.

TABLE 9
Activity of reverse transcriptase in the presence of actinomycin D and N-demethyl-rifampicin

	E. coli enzyme		*A. tumefaciens* enzyme	
	pmoles	% inhibition	pmoles	% inhibition
Complete	199	–	26	–
+ actinomycin D 2 μg	120	40	21	18
+ actinomycin D 4 μg	87	56	9.2	61
+ actinomycin D 8 μg	86	55	4.1	84
+ rifampicin 10 μg	106	47	–	–
+ rifampicin 20 μg	62	68	8.1	69
+ N-demethylrifampicin 10 μg	108	45	–	–
+ N-demethylrifampicin 20 μg	89	55	–	–

Incubation conditions, as described in the legend to table 6. ^{3}H d-GTP was used, transforming RNA from *E. coli* ML 30 Sho-R (4 μg). Enzyme fraction (DEAE cellulose) 40 μg. Actinomycin D, rifampicin and N-demethylrifampicin were added at the beginning of the reaction.

Somewhat higher concentrations of rifampicin and N-demethylrifampicin also inhibit RNA dependent DNA synthesis (table 9). In addition to that further evidence (fig. 5) shows that reverse transcriptase is not DNA dependent DNA polymerase.

TABLE 10
Base analysis of DNA synthesized by reverse transcriptase from *E. coli* and *A. tumefaciens*

	Moles per 100 moles of nucleotides			
	Transforming RNA (*E. coli*)	^{3}H-DNA (*E. coli* enzyme)	^{3}H-DNA (*A. tumefaciens* enzyme)	DNA (*E. coli*)
A	31.0	17.3	17.7	24.5
G	33.0	18.8	18.8	24.8
C	18.0	30.8	30.3	24.6
U (T)	17.8	33.1	32.1	26.1
	$\frac{G+A}{C+U} = 1.76$	$\frac{C+T}{G+A} = 1.74$	$\frac{C+T}{G+A} = 1.71$	$\frac{C+T}{G+A} = 1.01$

Incubation conditions (see legend to table 5). Each ^{3}H-dXTP (100,000 cpm) was used in equivalent amount. Acid precipitable material was washed several times. Dialysed ^{3}H-DNA product was hydrolysed in 70% formic acid for 30 min at 173°C and the hydrolysate chromatographed on Whatman 1 paper. Each spot was detected with an ultraviolet lamp, eluted with 0.1 HCl and the radioactivity determined in a Packard liquid spectrometer.

8. Nature of DNA synthesized upon transforming RNA

Data summarized in table 10 and fig. 13 show that transforming RNA is transcribed into the complementary DNA like material in the presence of reverse transcriptase isolated either from *E. coli* or *A. tumefaciens.* This is demonstrated by two sets of results. First, base ratio analysis of the ^{3}H-DNA product (table 10) is close if not identical to that found for transforming RNA. Second, evidence was obtained by annealing the ^{3}H-DNA product with transforming RNA and analysis by equilibrium sedimentation in Cs_2SO_4 density gradient (fig. 13). Synthetic RNA-^{3}H-DNA hybrid sediments in the density region between RNA and DNA as does the enzymatically formed hybrid (see fig. 8).

A synthetic hybrid was not detected when transforming RNA was replaced by ribosomal RNA or bacterial DNA in annealing experiments (fig. 13).

9. Density gradient analysis of the ^3H product synthesized in vitro

To provide evidence that the ^{3}H-heterodeoxypolymer synthesized in vitro under optimal conditions is DNA like it was separated from the enzyme by repeated treatments with chloroform, dialysed and analysed [11]. It was also submitted to equilibrium density centrifugation in Cs_2SO_4; unlabelled transforming RNA and DNA from *E. coli* were used.

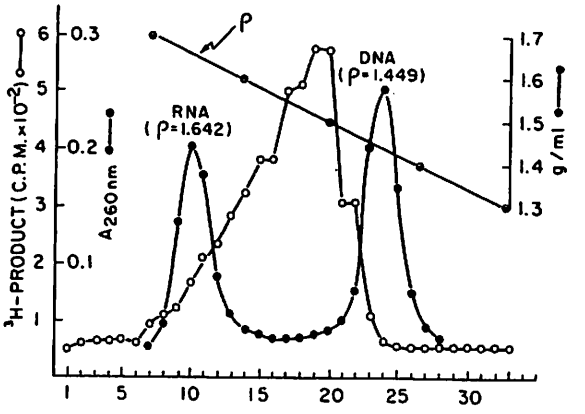

Fig. 8. Centrifugation of the ^{3}H-DNA product synthesized for 10 min (Cs_2 SO_4 equilibrium gradient). The incubation mixture (legend to table 6) in which ^{3}H-DNA was synthesized upon transforming RNA (^{3}H-dTTP and ^{3}H d-GTP were used) was twice treated with chloroform + 0.001 M EDTA, centrifuged, and the aqueous solution dialysed against a solution of 0.1 M KCl in Tris buffer 10^{-2} M (pH 7.6). Around 5500 cpm (TCA precipitable material) was centrifuged (legend to fig. 4). Internal markers, transforming RNA and *E. coli* DNA were included.

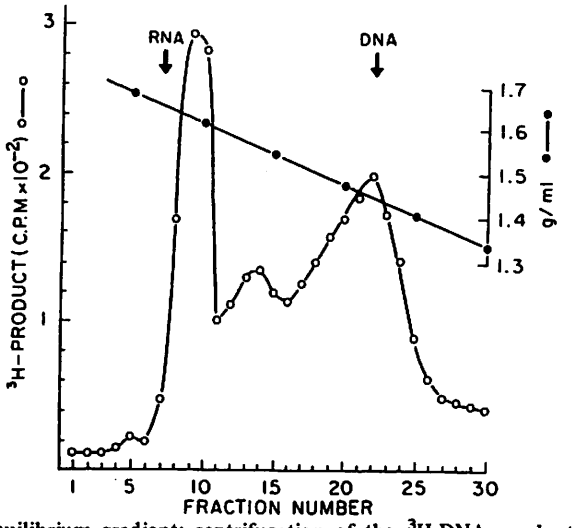

Fig. 9. Cs_2SO_4 equilibrium gradient; centrifugation of the ^{3}H-DNA product synthesized for 30 min. Conditions described in the legend to fig. 8. ^{3}H-DNA product (2300 cpm) was used.

The results of neutral Cs_2SO_4 density gradient sedimentation analysis of the products synthesized during in vitro incubations lasting 10, 30 and 60 min are respectively shown in fig. 8, 9 and 10. In all three cases most of the ^{3}H product is

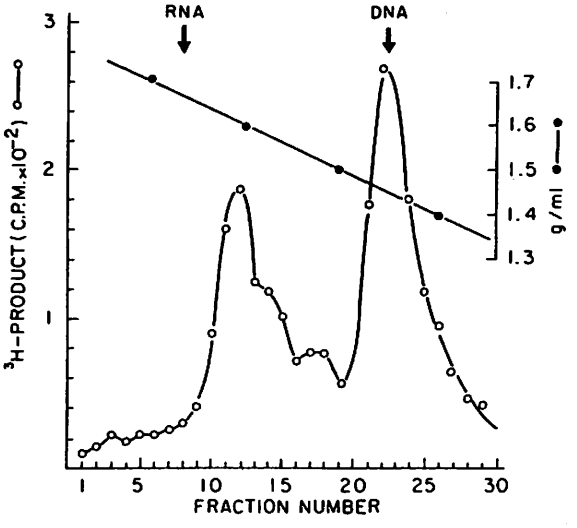

Fig. 10. Cs_2SO_4 equilibrium gradient: centrifugation of the ^{3}H-DNA product synthesized for 60 min. Conditions described in the legend to fig. 8. ^{3}H-DNA product (2400 cpm) was used.

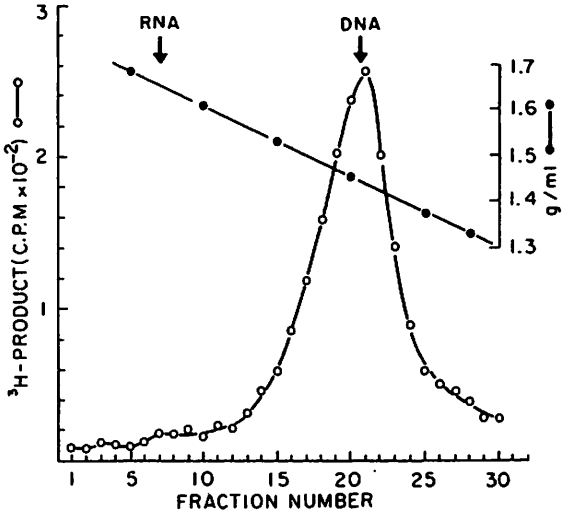

Fig. 11. Profile of the ^{3}H-DNA product in alkaline Cs_2SO_4 equilibrium gradient. The ^{3}H-DNA product was incubated in NaOH 0.3 M at 37°C for 16 hr. For centrifugation see legend to fig. 4.

largely spread over density region between RNA and DNA. Practically no ^{3}H material is found at the density of RNA itself. The heterogenous distribution of the ^{3}H product is due to its existence in the form of RNA–DNA hybrids [19, 20] with different chains lengths.

It is remarkable that the amount of ^{3}H product present in the DNA density

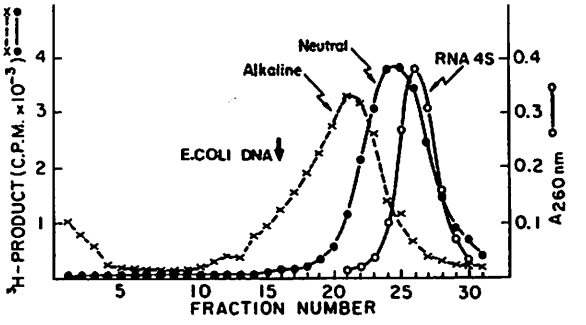

Fig. 12. Sucrose gradient analysis of the ^{3}H-DNA product before and after treatment with alkali. ^{3}H-DNA product was isolated from a 20 min incubation mixture and dialysed as described in the legend to fig. 8. An aliquot was incubated at 37°C with 0.3 N NaOH for 16 hr then layered on a linear 5–20% sucrose gradient containing 0.3 M NaOH + 0.001 M EDTA. A second untreated aliquot was layered on a neutral sucrose gradient. Both gradients were centrifuged at 4°C for 16 hr at 25,000 rpm in the SW 39 Spinco rotor. Fractions were collected, and alkali stable radioactivity determined. The neutral gradient contained $tRNA^{met}$, as RNA marker.

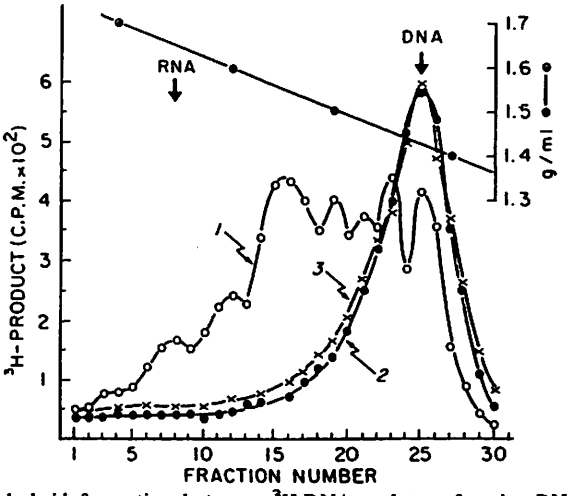

Fig. 13. Synthetic hybrid formation between ^{3}H-DNA and transforming RNA. The ^{3}H-DNA product lasting 10 min was synthesized and isolated from the incubation mixture as described in the legend to fig. 8. Dialysed solution containing the ^{3}H-DNA product was treated with 0.3 N NaOH for 16 hr to eliminate the template RNA, then neutralized before used. The ^{3}H-DNA (5,600 cpm) was incubated at 66°C for 8 hr in 2 SSC: (1) synthetic hybrid, transforming RNA–^{3}H-DNA (50 μg of transforming RNA); (2) ^{3}H-DNA after annealing with *E. coli* DNA (50 μg of *E. coli* DNA); (3) ^{3}H-DNA after annealing with ribosomal RNA(50 μg rRNA).

region increases with the time of incubation. This means that DNA synthesized on the RNA template is progressively released as free DNA.

After treatment of the ^{3}H product–RNA hybrid with alkali, the heterogeneity of the ^{3}H product disappears and the radioactive material is found in the *E. coli* DNA density region (fig. 11). Since the curve (peak) representing the ^{3}H material is rather large this would suggest that the population of DNA is heterogenous.

Sucrose gradient analysis of the RNA–^{3}H-DNA hybrid product before and after treatment with alkali shows (fig. 12) that the alkali-released ^{3}H-DNA from the hybrid sediments between 6 and 7 S. These values correspond to those determined for transforming RNA itself [7]. On this basis one can estimate that the molecular weight of synthesized DNA is close to 1.8×10^5.

10. Search for homology between the ^{3}H-DNA product made in vitro and bacterial DNA

Transcription of transforming RNA into DNA by the reverse transcriptase raised the following question: is there homology between ^{3}H-DNA product synthesized upon transforming RNA and DNA originating from the same strain or from the *A. tumefaciens* transformants?

For hybridization experiments with bacterial DNA the ^{3}H-DNA product was synthesized in the presence of actinomycin D in order to restrict the synthesis to single stranded DNA. Several attempts were made to demonstrate the homology between enzymatically synthesized ^{3}H-DNA product and DNA isolated either from

TABLE 11
Hybridization of ^{3}H-DNA product with bacterial DNA

Input of bacterial DNA (μg)		Input of ^{3}H-DNA (cpm) (0.04 μg)	DNA/ ^{3}H-DNA ratio	Cot [40]	^{3}H-DNA hybridized with bacterial DNA	
					cpm	per cent
E. coli Sho R	100	4200	2.5×10^3	0.8×10^4	280	6.6
	200	4200	5.0×10^3	1.6×10^4	310	7.4
A. tumefaciens transformant	300	4300	7.5×10^3	2.5×10^4	2160	50
	600	4300	1.5×10^4	5.0×10^4	2740	64
A. tumefaciens wild type	300	4200	7.5×10^3	2.5×10^4	145	3.4
	600	4200	1.5×10^4	5.0×10^4	163	3.8

Both ^{3}H-DNA and bacterial DNA dissolved in 0.2 X SSC were denaturated by incubating in 0.3 N NaOH at 37°C for 16 hr. Samples were dialysed against 1 SSC overnight. ^{3}H-DNA (about 0.04 μg, 4300 cpm) was incubated with DNA from various origins in 2 SSC [21] at 66°C for 6 days (in sealed tubes). Separation of single stranded from double stranded DNA was achieved using hydroxyapatite column [39]. ^{3}H-DNA incubated alone gave around 1% of double stranded material. We considered it as negligible. Radioactivity of eluates was determined in a Packard liquid spectrometer. Results are expressed in cpm (per cent) for the ^{3}H-DNA found in hybrid form with bacterial DNA. Recovery from hydroxyapatite column was 85–90%.

E. coli mutant Sho-R or from *A. tumefaciens* transformed by RNA from *E. coli*. The ^{3}H-DNA product was annealed at 66°C ("Cot" values*, table 11) with DNA from *E. coli* Sho-R from which transforming RNA is excreted into culture medium. The most surprising fact is that under described conditions the presence of ^{3}H-DNA–DNA hybrid was hardly detectable when *E. coli* Sho-R or *A. tumefaciens* wild type DNA was used. On the contrary when the ^{3}H-DNA product was annealed with cold DNA from *A. tumefaciens* transformed by *E. coli* RNA, significant binding of labelled DNA was observed (table 11). These results seem to show that in *A. tumefaciens* transformant DNA synthesized upon transforming RNA from *E. coli* is "integrated" into genome DNA. Although it is very difficult to determine the exact amount of the "integrated DNA" into bacterial genome, one can say that there is one or less than one copy per genome. This estimation was calculated by considering the molecular weight of bacterial DNA = 2×10^9 [42] and that of ^{3}H-DNA = 1.8×10^5 (see fig. 13). In addition the above results suggest that transforming RNA is transcribed into DNA when introduced into bacteria belonging to a different species.

11. Discussion and conclusions

Evidence has been presented [7] that an RNA fraction from *E. coli* Sho-R can transfer information to *A. tumefaciens* which is then capable of maintaining the newly acquired characteristics.

* Cot = DNA concentration in moles per liter × time of incubation in seconds [40].

In the present report we attempted to approach the mechanism by which the transfer of information carried by transforming RNA is accomplished. We have suggested elsewhere [10, 11] that transforming RNA, once inside the recipient cells, could be linked by "ligases" to other RNA fractions already associated with bacterial DNA. Thus a large RNA episome could be formed and either replicated, giving rise to RNA's, or transcribed into DNA. This hypothesis was suggested for the reason that in living organisms different molecular mechanisms seem to be involved in genetic inheritance [22]. Transformation by RNA may reveal one of the "hidden" systems in the cells, thus allowing this RNA to be in function.

Results reported here show that the replication of transforming RNA in vitro can be accomplished by a polynucleotide phosphorylase. The activity of this enzyme is strongly stimulated by transforming RNA exclusively in the presence of 4 XDP, while there is no activity with 4 XTP. It is remarkable that under the same conditions transforming RNA's, even that associated with DNA (episomal RNA) is replicated whether or not DNase is present in the incubation mixture. In contrast, RNase abolishes the RNA dependent transcription process. Furthermore, rifampicin and actinomycin D inhibit DNA dependent RNA polymerase but not affect the polynucleotide phosphorylase.

The product synthesized on transforming RNA by PNPase has a base ratio (G+A/C+U about 1.70–2.0), close to that found for transforming RNA. On this basis it should be considered as an identical copy of RNA used as template. Similar results have been recently described for replication of RNA from reovirus. In fact either single stranded or double stranded RNA in vitro is replicated by an enzyme from L-cells infected with reovirus [23]. This system uses the 4 XDP's as substrate, although there is another enzyme which uses the 4 XTP's for replication of reoviral RNA. This observation and ours shows that certain biologically active RNA's can be replicated by appropriate enzymes using the 4 XDP as substrate.

It is remarkable that transforming RNA can also serve as template directing in vitro DNA synthesis by an enzyme which we have identified in bacteria as a reverse transcriptase. Results presented here show that the activity of reverse transcriptase can be distinguished from that exhibited by DNA dependent DNA polymerase. This is true for enzymes partially purified from both *E. coli* and *A. tumefaciens*. Among various RNA's tested, transforming RNA whose directing capacity is destroyed by RNase, is the best template for reverse transcriptase activity. It should be emphasized that an RNA fraction (5.5 S RNA) [17] from *Alcaligenes faecalis* rich in messenger RNA – or perhaps containing episome like RNA – is used as template by reverse transcriptase from *E. coli*. Active DNA preparation used as template for DNA dependent DNA polymerase is inactive with the reverse transcriptase. In some respects reverse transcriptase from bacteria has properties similar to those observed with the enzyme whose existence was predicted and demonstrated by Temin [24] in oncornavirus and in normal uninfected chicken embryos [44]: (1) its activity is RNA dependent; (2) its activity is inhibited by actinomycin D and by rifampicin or N-demethylrifampicin [30]; (3) the ^{3}H-DNA product synthesized in vitro is complementary to the RNA used as template.

From the point of view of transformation of *A. tumefaciens* by *E. coli* transforming RNA, it is important that reverse transcriptase from *A. tumefaciens* transcribes in vitro (and possibly in vivo) *E. coli* transforming RNA into DNA, although with much less efficiency than that exhibited by *E. coli* reverse transcriptase. Something of this sort could happen in vivo during transformation of *A. tumefaciens* by *E. coli* transforming RNA.

The ^{3}H product made in vitro by reverse transcriptase was characterized by several means as being DNA like. The most significant observation is that ^{3}H-product has a base ratio complementary to that of transforming RNA (table 10) whether it is synthesized by *A. tumefaciens* or by *E. coli* reverse transcriptase. The ^{3}H-product is a single stranded DNA (roughly 10% double stranded) which hybridizes efficiently with transforming RNA. However this same ^{3}H-product hybridizes very poorly with bacterial DNA from *E. coli* Sho-R. It was suggested that RNA and reverse transcriptase perhaps constitute a system which may not be directly dependent on the bacterial genome [11].

It was of particular interest to determine whether transforming RNA of *E. coli* was transcribed (in vivo) into DNA during the process of transformation of *A. tumefaciens* and if it was integrated into the DNA of the corresponding transformant. If this was the case one would expect to find a hybrid formed between ^{3}H-DNA synthesized in vitro on transforming RNA and DNA isolated from *A. tumefaciens* transformant. By annealing the ^{3}H-DNA product with DNA from transformant we have detected the existence of one or less than one copy per genome of DNA transcribed from the transforming RNA.

If reverse transcriptase operated in vivo as it does in vitro it would offer tremendous possibilities for a new type of mutation mechanism proposed and demonstrated by Temin [8, 24]. His findings were confirmed and further developed by others [25–30]. Along these lines it is of great importance to report that several workers have shown [31–38] that in vitro initiation for replication of DNA by DNA dependent DNA polymerase isolated from bacteria or higher organisms requires an RNA which is "covalently" associated with DNA itself [37, 38]. This RNA seems not to be synthesized by classical DNA dependent RNA polymerase since the system is not sensitive to rifampicin [37]. RNA required for replication of ϕ-X 174 DNA with *E. coli* enzyme contains an excess of purine nucleotides over pyrimidines (G+A/C+U = 2.4). The existence of such an RNA may be related to our finding that both *E. coli* transforming RNA excreted into culture medium and episomal RNA associated with DNA [10] contain excess purine nucleotides (G+A/C+U about 1.70–2.1). This RNA is transcribed into DNA by the reverse transcriptase from *E. coli* and *A. tumefaciens* but is not used by DNA dependent DNA polymerase. It will be interesting to determine to what extent and in what manner the primer RNA required for DNA replication differs from one system to another. Also, it now seems quite necessary to establish the essential relationship between RNA dependent reverse transcriptase and DNA dependent DNA polymerase, in bacteria namely, if the former functions for initiation of DNA replication.

The dual potentialities of transforming RNA, i.e. replication into RNA and transcription into DNA constitute a possible molecular mechanism in the process of evolution, independently of whether transcribed DNA is integrated in some way into cell genome or remains in the cytoplasm as a cytoplasmic genetic element [11].

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