DNA COPIES OF VIRAL RNA IN RAT CELLS TRANSFORMED BY ROUS SARCOMA VIRUS (RSV).

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SUMMARY. Using a modified hybridization method at least 50 p cent of the input 70 S RNA from avian tumor virus (RSV) could be annealed with DNA from RSV transformed rat cells, and only 2-3 p cent with DNA from normal cells. The mean number of viral genome copies does not seem to exeed 1 or 2 per tumor cell genome.

According to the theory of Temin (1) the RNA of oncornaviruses is transcribed into DNA copies (provirus DNA) in the infected cells. To date many attempts at demonstrating the existence of provirus DNA by the use of molecular hybridization have been far from conclusive (see discussion). However as shown by Britten and Kohne (2) the conventionnal methods generally utilized can reveal but repetitive sites in the DNA of eukaryotes. Hill and Hillova (3,4) have recently discovered that the DNA from XC rat cells transformed by the Prague strain of RSV (P-RSV) can reproduce P-RSV in chick fibroblasts. It is shown here that using an improved hybridization method a great part of the provirus DNA molecule can be detected in the XC-cell DNA preparations utilized by Hill and Hillova, although each cell seems to contain only 1 or 2 copies of the viral genome on the average.

MATERIALS AND METHODS. Avian Myeloblastosis Virus (AMV) produced by cultivated leukemic cells labelled with ^{32}P, was purified and 70 S viral RNA isolated following the published methods (see ref 5). Embryonic chick fibroblasts were cultivated and infected with P-RSV (see ref 4). After the 5th culture passage, exponantially growing cells were labelled with carrier free ^{32}P (100 µCi per ml) for 18 hr and 70 S RNA was isolated from virus of the culture medium by the procedures applied to AMV. The cells were utilized for preparing ribosomal RNA (^{32}P rRNA) which was extracted from the cytoplasmic phase (5) and filtered through nitrocellulose membranes in 6xSSC (1xSSC = 0.15 M NaCl, 0.015 M Na citrate). XC-cell DNA was prepared as specified elsewhere (4) and rat liver DNA was

purified by the same methods. Non labelled interphase-RNA (In-RNA) from rat liver was prepared as indicated before (5).

Hybridization. DNA dissolved in 0.1xSSC was denatured by incubating for 18 hr at 37°C in 0.3 N NaOH. After neutralization its mean sedimentation coefficient was of about 10 S as shown by analytical ultracentrifugation. Denatured DNA was precipitated with ethanol and dissolved in 2xSSC at the final concentration of 250 A 260 units per ml (about 10 mg) and micro quantities of ^{32}P RNA were added. Aliquots of 0.20-0.25 ml were distributed in sealed tubes and incubated at 66°C at high Cot values (Cot = DNA concentration in moles per liter X time of incubation in seconds, ref 2). Following the method of Melli et al (6) each sample was diluted with 2xSSC to the final DNA concentration of 100 µg per ml and the largest part was incubated with ribonuclease (RNAase) panc A (Worthington, 40 µg per ml) for 30 min at 37°C. After treating with trichloracetic acid at 0°C, the radioactivity in acid-precipitable and acid-soluble fractions was measured in a low back grownd (0.2 - 0.3 cts/min) Tracerlab counter. In the modified method instead of being precipitated DNA was loaded on several nitrocellulose filters. The filters were incubated with RNAase, washed at 55°C in 2xSSC and dried up for counting.

RESULTS. Hybridization experiments were performed using DNA from XC-cells or rat liver and 3 different preparations of 70 S ^{32}P AMV RNA and the annealing rates were estimated by the method of Melli et al (6). Table I shows some typical results. For Cot values of 1-1.5 x 10^4 the proportion of acid-precipitable RNAase resistant ^{32}P RNA bound to XC-cell DNA (30-35 p cent) was constantly superior to that bound to liver DNA (14 - 18 p cent). However because of the results obtained at time 0, similar to those obtained after incubating with E coli DNA at high Cot values, any precise estimation of the hybridization rates appeared hazardous.

The modified method using nitrocellulose filters gave more reliable results (see table I B), since in the presence of non labelled In-RNA from rat liver only negligible amounts of RNAase resistant ^{32}P RNA were bound to rat DNAs at time 0 or to E coli DNA at high Cot values.

The modified method was utilized to compare the hybridization of 70 S ^{32}P RNA from both AMV and P-RSV and that of ^{32}P rRNA with the same preparation of XC-cell DNA and rat liver DNA respectively (see table II). For low Cot values the quantity of viral ^{32}P RNA bound to XC-cell DNA or liver DNA was decreased by a factor of 10 or more in the presence of non labelled In-RNA, that confirmed previous results (5,7). For high Cot values this competition effect of In-RNA did not prevent the hybridization

DNA from	Non labelled In-RNA	P cent of input ^{32}P RNA Acid-precipitable				P cent of ^{32}P RNA Hybridized	
		Time 0		Cot 1.3×10^4			
		No RNAase	+ RNAase	No RNAase	+ RNAase	A	B
XC cells 2 mg	None	100	16.0	90	33.0	20.6	ND
	300 µg	100	22.2	92	34.9	15.7	13.1
Rat liver 2 mg	None	100	16.0	93	17.2	2.5	ND
	300 µg	100	17.5	96	15.9	0	1.2

TABLE I. Hybridization of ^{32}P AMV-RNA with XC cell DNA as compared with liver DNA, using the method of Melli et al (A) or the modified method (B). 70 S ^{32}P AMV RNA (about 0.05 µg, 9600 cts/min) was incubated with DNA in the presence or absence of In-RNA from rat liver. A : the quantity of RNAase resistant acid-precipitable (AP) ^{32}P RNA found at time 0 was substracted from that found after incubating at Cot 1.3×10^4. The difference was expressed as p cent of AP ^{32}P RNA in non RNAase treated samples incubated at Cot 1.3×10^4. B : Aliquote samples incubated at Cot 1.3×10^4 were filtered through nitrocellulose membranes. The quantity of ^{32}P RNA bound to DNA after treating the membranes with RNAase was expressed as p cent of AP input ^{32}P RNA as in A. Means of 2-3 determinations. ND : not done.

Input ^{32}P RNA			Rat liver In-RNA µg	DNA/ ^{32}P RNA ratio	Cot	^{32}P RNA hybridized with DNA from			
	µg	cpm				XC-cells cpm	XC-cells P cent	Rat liver cpm	Rat liver P cent
AMV	0.05	5,800	200	10^3	1.8	29	0.5	18	0.3
	0.05	5,800	200	4×10^4	1.5×10^4	760	18.1	83	1.9
P-RSV	0.02	2,650	200	10^5	2×10^4	1140	52.2	66	3.3
	0.04	5,300	200	5×10^4	2×10^4	1230	28.0	111	2.7
r-RNA	0.04	5,300	200	10^4	4×10^3	5	NS	8	NS
	1.20	159,000	none	50	1.8	795	0.50	910	0.57

TABLE II. Hybridization of 70 S RNA from AMV and P-RSV as compared with rRNA, using the modified method. Cpm : cts/min. DNA concentration : 10 mg/ml at high Cot, 0.1 mg/ml at low Cot. P cent hybridized calculated as indicated in table I (B). Means of 2, 3 or 4 determinations. NS : non significant. The amount of input P-RSV RNA (in µg) was possibly overestimated (see text).

of a lerge proportion of the input viral RNA from both P-RSV and AMV with XC-cell DNA, whereas at most 2-3 p cent were bound to liver DNA. Moreover when decreasing by half the P-RSV RNA concentration, the quantity annealed with XC-cell DNA was not significantly diminished and exceeded 50 p cent of the acid-precipitable input ^{32}P RNA. It is to note that if the specific radioactivity of 70 S AMV RNA could be measured that of 70 S P-RSV RNA could not, because in our experimental conditions the extraction yield of the later (about 2.4×10^4 cts/min) was much smaller than that of the former ($1-1.6 \times 10^6$ cts/min). It was assumed that the SR* of 70 S P-RSV RNA was the same as that of rRNA from the virus producing cells, despite the fact that 70 S AMV RNA is generally 2-3 times more radioactive than rRNA from the AMV producing cells. So an underestimation of the SR of P-RSV RNA might account for its superior hybridization rate as compared with AMV RNA.

For low Cot values roughly similar amounts of chick ^{32}P rRNA were hybridized with both XC-cell DNA and rat liver DNA. We had obtained comparable hybridization rates when annealing chick rRNA with chick DNA (unpublished). This may be attributed to extensive cross hybridizations between rRNAs and DNAs from fowls and mammals (8). Finally non labelled In-RNA almost completely inhibited the hybridization of ^{32}P rRNA with XC-cell as well as liver DNA. These controls indicate that hybridization of viral RNA with XC-cell DNA may be considered as specific.

DISCUSSION. The discovery of viral reverse transcriptases (9,10) gave a new impulse to the search for provirus DNA. We had previously shown that, using conventionnal methods, only a small fraction (3-4 p cent) of the 70 S RNA from oncornaviruses, fraction much enriched in adenine, can be readily hybridized with redundant sites of DNA (11,12,13). This was confirmed by different methods : zonal and isopycnic centrifugations, selection of fragments of the viral RNA widely differing in their capacity to anneal with DNA or studies of the change in the hybridization rate of viral RNA after stepwise hydrolysis with an exonuclease (5,7). It was also found that In-RNA from normal cells, a cytoplasmic RNA fraction much enriched in messenger RNAs, is a seemingly specific competitor for partial hybridization of the viral RNAs(5,7), suggesting some homology between certain sequences of viral RNAs and cellular messenger RNAs (in preparation). Finally no meaningful difference was found in the hybridization rates of viral RNAs with DNAs from either transformed or normal cells.

* SR : Specific Radioactivity

This conclusion was confirmed by others using radioactive viral RNA (14, 15) or DNA copies transcribed by the viral polymerases (16,17). Some reports were conflicting. Baluda and Nayak (18,19) found that the DNA of transformed cells can hybridize more viral RNA than that of normal cells. Great variations in the hybridization yield were interpreted as evidence of variations in the quantity of integrated provirus DNA but nothing more than 0.1 p cent of the input RNA was hybridized, which leaves some doubt on the interpretation.

The discovery of "infectious DNA" in rat cells transformed by RSV (3,4) or in chick cells transformed by AMV (20) or RSV (21) seems to demonstrate, quite unequivocally, the validity of the provirus theory. Although very promising this biological approach is not easily adaptable to quantitative studies at the molecular level. The present results demonstrate that adequate annealing methods can reveal a large proportion of the provirus DNA molecule in the genome of transformed cells. Moreover the alkaline treatment of DNA rules out any possibility that some RNAase resistant RNA copy of the viral genome is involved. Finally it seems that the provirus sites were saturated by small quantities of viral RNA. If this is true, at most 0.01 µg of viral RNA can be hybridized with 2 mg of XC-cell DNA. The DNA content of rat cells (data in ref 22) is roughly equivalent to 4×10^{12} daltons per cell, as compared with 10^7 for viral RNA. So it may be tentatively assumed that XC-cells contain an average of 1 or 2 provirus DNA copies per cell genome. More elaborate work is needed to obtain safer quantitative data that may clarify some current concepts.

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