PRODUCTION OF DEFECTIVE VIRUS BY TERMINALLY DIFFERENTIATED MYOTUBES INFECTED WITH ROUS SARCOMA VIRUS

H.T. PARK[1], H. HARA[1], M. FUJIHARA[1#], K.J. HSIA[1#], A. TANAKA[1#], T. FURUSE[2], Y. IWASAKI[2], A. KAJI[1*]

[1]Department of Microbiology, University of Pennsylvania School of Medicine, 573 Maloney Building, 36th and Spruce St., Philadelphia, Pennsylvania, 19104-4283, USA; [2]National Center for Nervous, Mental and Muscular Disorders, Tokyo, Japan

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Summary. – The generally accepted concept that the replication of Rous sarcoma virus (RSV) is dependent on host cell DNA synthesis was reexamined. As the host we used terminally differentiated myotubes (MT), in which no cellular DNA synthesis is observed. As an extension of our previous study which indicated that RSV-infected MT produce various virus components, we examined viral particles produced by infected MT. Electron microscopy showed presence of viral particles released from infected MT. Immunoprecipitation analysis revealed that these particles contained an equal amount of the gag but a decreased amount of the env proteins as compared with the particles from infected chicken embryo fibroblasts (CEF). Consequently, viral particles from infected MT had an infectivity only 6% of that of particles from infected CEF cells. In a parallel experiment, we microinjected molecularly cloned RSV DNA into MT. In contrast to the infection mediated by viral particles, both MT and CEF cells produced the same amount of infectious particles when microinjected with viral DNA. We conclude that RSV replicates in the complete absence of host DNA synthesis, though infectivity of the progeny virus depends on the initial condition of the infection.

Key words: Rous sarcoma virus; retrovirus replication; chicken fibroblast; myotube; microinjection; defective virus

Introduction

Integration of viral DNA into the host chromosomal DNA has been believed to be an obligatory step for the replicative life cycle of RSV (Varmus and Swanstrom, 1984). Earlier studies suggested that integration is dependent on the host DNA synthesis (Varmus *et al.*, 1977; Varmus *et al.*, 1979; Varmus and Swanstrom, 1984), leading to the concept that viral production does not occur in the absence of host DNA synthesis. Recent development of the *in vitro* retrovirus integration system still does not solve the exact molecular mechanism as to the requirement for host DNA synthesis (Goff, 1990).

In order to re-examine the role of host DNA synthesis in the viral DNA integration and replication, we used as the host terminally differentiated multinucleated MT, in which chromosomal DNA synthesis is irreversibly and completely stopped by the natural differentiation process (Stockdale and Holtzer, 1961; Pullman and Yeoh, 1978; Kobayashi and Kaji, 1978; Lim and Hauschka, 1984; Kaufman and Robert-Nicoud, 1985). In our previous studies, we found that the viral DNA remained unintegrated in infected MT, indicating that host DNA synthesis is required for integration *in vivo*. Contrary to the general belief, all three species of viral

*Corresponding author

#Present address: M. Fujihara, Research Section, Hokkaido Red Cross Blood Center, 2-2 Yamanote, Nishi-ku, Sapporo, Hokkaido 063, Japan; K.J. Hsia, University of California, San Diego, Medical Center, 225 Dickinson Street, H-814-H, San Diego, California, 92103-1990, USA; A. Tanaka, Department of Oral Bacteriology, Tokyo Medical and Dental University Dental School, 5–45 Yushima 1-Chome, Bunkyo-ku, Tokyo 113, Japan.

Abbreviations: AMV = avian myeloblastosis virus; ara-C = cytosine arabinoside; CEF = chicken embryo fibroblast; FFU = focus forming unit; MF = myogenic fibroblast; MEM = minimum essential medium; MOI = multiplicity of infection; MT = myotube; PAGE = polyacrylamide gel electrophoresis; p.i. = post infection; RSV = Rous sarcoma virus; RT = reverse transcriptase, reverse transcription; SDS = sodium dodecyl sulphate; SRA = Schmidt-Ruppin subgroup A strain of RSV; TBR = tumor bearing rabbit

mRNAs (38 S, 28 S and 21 S) were detected at their normal level in infected MT (Tanaka *et al.*, 1992). However, synthesis of the env and v-src proteins (translated from the 28 S and 21 S mRNA, respectively) was significantly reduced in infected MT, while the gag and pol proteins (both translated from the 38 S mRNA) were synthesized at the normal level (Hsia *et al.*, 1992).

In this paper, we provide electron microscopic evidence that RSV-infected terminally differentiated MT release viral particles into media. However, the low env/gag and focus forming unit (FFU)/reverse transcriptase (RT) ratios of the viral particles produced by infected MT suggested that these particles mostly consisted of env-deficient, infection-defective virions. In contrast, microinjected viral DNA produced infectious virus in MT.

Materials and Methods

Cells and virus. Preparation of MT and CEF cells from 10-day-old C/O chicken embryos was described previously (Kobayashi and Kaji, 1978). For the preparation of infected MT, the latter were cultured without cytosine arabinoside (ara-C) for at least 15 hrs after ara-C treatment. Cultures were then infected at multiplicity of approximately 1 – 5 FFU per nucleus with a Schmidt-Rupin subgroup A strain of RSV (SRA), which was a gift from Dr. H. Hanafusa of the Rockefeller University. Cultures at 4 – 5 days post infection (p.i.) were used for the analysis of progeny viral particles. Mononucleated cells from MT culture (myogenic fibroblast, MF) were prepared by trypsinization of 5-day-old MT cultures. These represented the small number (about 1%) of mononucleated cells present in the MT culture. Focus assays were carried out essentially as previously described (Vogt, 1969).

RT assay. The virion RT activity was assayed using poly(A)-oligo$(dT)_{15}$ as the template as described previously (Grandgenett *et al.*, 1973). The RT activity was expressed as units per ml of the original medium containing the virus. One unit incorporates 1 nmole of deoxynucleotide into trichloroacetic acid-precipitable form in 10 mins at 37 °C (Houts *et al.*, 1979).

Purification of radiolabelled virus by sucrose gradient. Preparation and purification of radiolabelled virus were done essentially according to Hunt *et al.* (1979) with some modifications. Four plates of infected secondary CEF cultures were labelled on days 1 to 2 and 4 to 5 p.i. using 8 ml of methionine-free Eagle's minimum essential medium (MEM) containing ^{35}S-methionine (50 μCi/ml; 1200 Ci/mmol), 2% foetal calf serum and 1% dimethylsulfoxide. The labelled viruses were pelleted by ultracentrifugation and purified by gradient centrifugation first over the 10 – 50% linear sucrose gradient and then over the 20 – 50% sucrose gradient. The purified particles were lysed in RIPA buffer (150 mmol/l NaCl, 10 mmol/l Tris-HCl pH 7.2, 1% (w/v) Na-deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS), immunoprecipitated and examined by polyacrylamide gel electrophoresis (PAGE) as described previously (Tanaka and Kaji, 1983).

Pulse labelling and analysis of the virion proteins. The infected MT culture (10^7 nuclei) and the infected MF culture (10^7 cells) on day 4 p.i. were pulse-labelled with ^{35}S-methionine at 100 μCi per ml of Earle's balanced salt solution per 10 cm dish at 36 °C for 1 hr. Immediately after removal of the labelling medium, each culture was chased by an incubation with 7 ml of muscle medium at 36 °C for 3.5 hrs. The labelled viral particles released into medium during the chase were clarified at 1,500 x g for 20 mins at 2 °C. The viral particles were pelleted in the Beckman SW27 rotor at 25,000 rpm at 2 °C for 1 hr. The pellet was resuspended in NTE buffer and pelleted again as above. The viruses were then disrupted and immunoprecipitated according to the procedure of Oppermann *et al.* (1977) and analyzed as above.

Electron microscopy was performed as described in our previous work (Yoshimura *et al.*, 1981).

Microinjection was performed according to the published method (Graessmann *et al.*, 1980). The molecularly cloned SRA, pSRA2 (DeLorbe *et al.*, 1980), was purchased from the American Type Culture Collection (catalog No. 45000). The purified pSRA2 DNA (1.15 mg in 17 μl) was digested with *Sal*I to completion, and the released SRA2 DNA was purified by agarose gel electrophoresis. The RSV expression vector pRSV$^{v\text{-}src}$ (Nemeth *et al.*, 1989) was a gift from Dr. J.S. Brugge of Ariad Pharmaceuticals Inc., Cambridge, MA. The SRA2 DNA and pRSV$^{v\text{-}src}$ DNA were suspended in sterile, 1% KCl at a final concentration of 6 ng/μl for SRA2 DNA or 8.6 ng/μl for pRSV$^{v\text{-}src}$ DNA (Kopchick *et al.*, 1981). Following the microinjection, a greased cloning ring (7 mm inner diameter) was placed around the microinjected cells (600 nuclei), and 0.3 ml of medium was placed in the ring. The culture fluid was harvested every day for 5 days and the focus assay was done as described previously (Vogt, 1969).

Results

Electron microscopic observation of the infected MT

In order to determine whether or not infected MT produce viral particles, electron microscopy was carried out. Fig. 1 shows a section of MT releasing progeny viral particles. The areas where the particles were present in Fig. 1 were *not* intracellular vacuoles but indented extracellular areas as evidenced by the double membrane layer surrounding them. A control electron microscopy picture of uninfected MT did not show any particles resembling those shown in Fig. 1.

Analysis of the virus particles released from infected MT

In the experiment shown in Fig.2, viral proteins in the released viral particles were examined. Infected MT culture was labelled with ^{35}S-methionine and the radioactivity was chased with regular medium. The labelled viral materials in the medium were purified by centrifugation through sucrose density gradient (10 – 50%). Fractions corresponding to the peak of radioactivity were collected and subjected to immunoprecipitation analysis. The sedimentation pattern

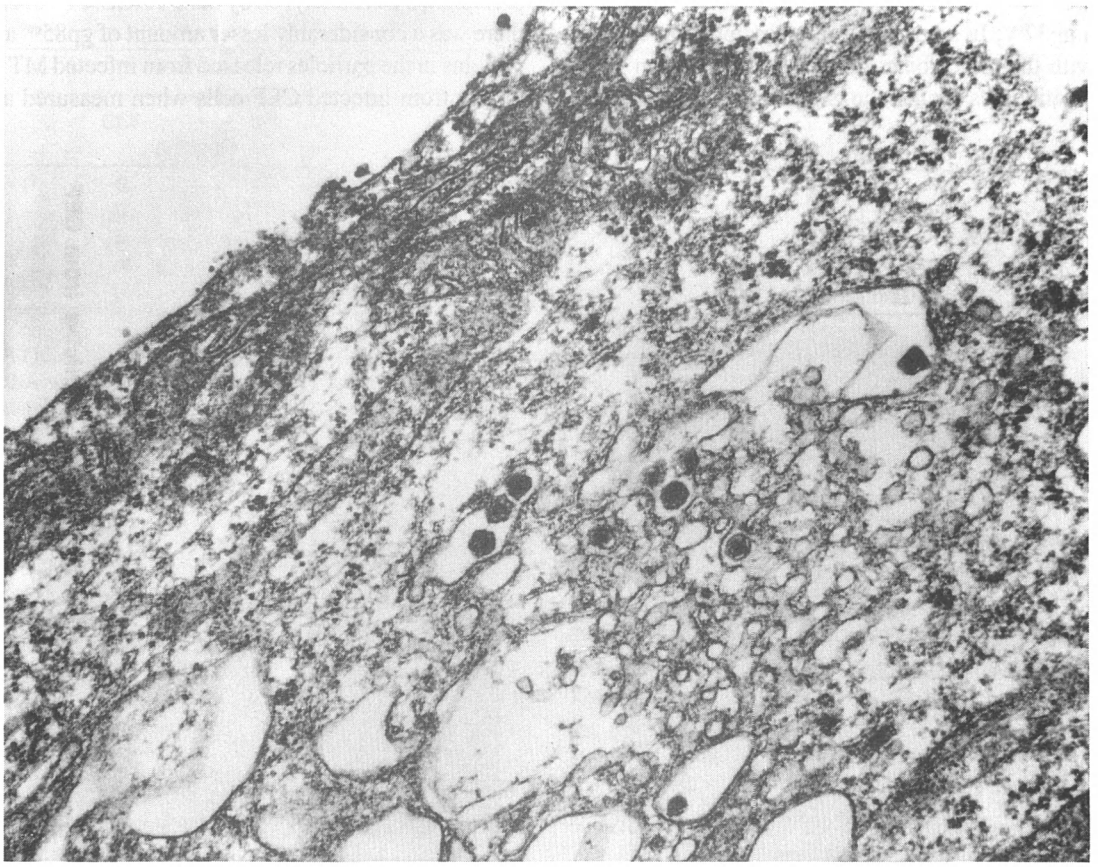

Fig. 1
Electron micrograph of RSV-infected MT showing released progeny virus particles
Magnification 60,000 x.

of the viral particles from infected MT was identical to that of the viral particles from infected CEF cells (buoyand density = 1.17 g/ml) (Bader, 1975). Solubilized particles were immunoprecipitated with either a goat anti-avian myeloblastosis virus (AMV) serum or a goat anti-AMV RT serum, and were analyzed by SDS-PAGE. As a control, the mononucleated fibroblasts present (1%) in the MF culture were used. As shown in Fig. 2, the virion gag proteins ($p27^{gag}$, $p19^{gag}$ and $p15^{gag}/p12^{gag}$) from infected MF (lane a) and infected MT (lane c) were immunoprecipitated by the goat anti-AMV serum. Virion RT proteins $p95^{pol}$ (ß) and $p65^{pol}$ (α) were precipitated from MF (lane b) and MT (lane d) by the goat anti-AMV RT serum. Although the latter bands were not as distinct as the gag proteins, the fact that they appeared at the expected positions, and the fact that the RT activity was detected in the culture media as shown in Table 1 indicated that these bands represented polymerase α and ß. The goat anti-AMV RT serum also precipitated $p19^{gag}$, which is visible in all four lanes of Fig. 2, and to a lesser extent $p27^{gag}$; The non-immune serum control precipitated no viral proteins.

Table 1. Analysis of viral particles released from RSV-infected MT

Cells infected with RSV	(I) FFU/host DNA (unit/μg)	(II) RT/host DNA (unit/μg)[a]	(III) FFU/RT I/II
MT	$6.6 \pm 1.9 \times 10^4$	1.1 ± 0.5	6.1×10^4
CEF	$8.9 \pm 3.1 \times 10^6$	8.4 ± 1.4	1.1×10^6

The secondary CEF culture (3×10^6 cells/10 cm plate) and MT cultures (5×10^6 cells/10 cm plate) were infected with RSV at MOI of 5, and the medium was changed every day. The virus was harvested on day 5 p.i. (6 hrs after the media change) and analyzed for FFU titer and RT activity.
[a] One unit incorporates 1 nmole of deoxynucleotide into acid precipitable form in 10 mins at 37 °C, using poly(A)-oligo$(dT)_{15}$ as template primer as defined by Houts *et al.* (1971). Under our experimental condition, one unit corresponds to 6×10^5 cpm/hr.

In the experiment shown in Fig. 3, ^{35}S-methionine-labelled particles released into the culture media were purified by sucrose density gradient. Solubilized particles were precipitated

with an anti-gp85 serum which contained antibodies against gp85^{env} an gp37^{env}; In some cases, the precipitation was carried out with the tumor bearing rabbit (TBR) serum which contained antibodies against the gag proteins. The immunoprecipitates were analyzed by SDS-PAGE. Fig. 3A shows that there was a considerably lesser amount of gp85^{env} and gp37^{env} proteins in the particles released from infected MT compared to that from infected CEF cells when measured against t

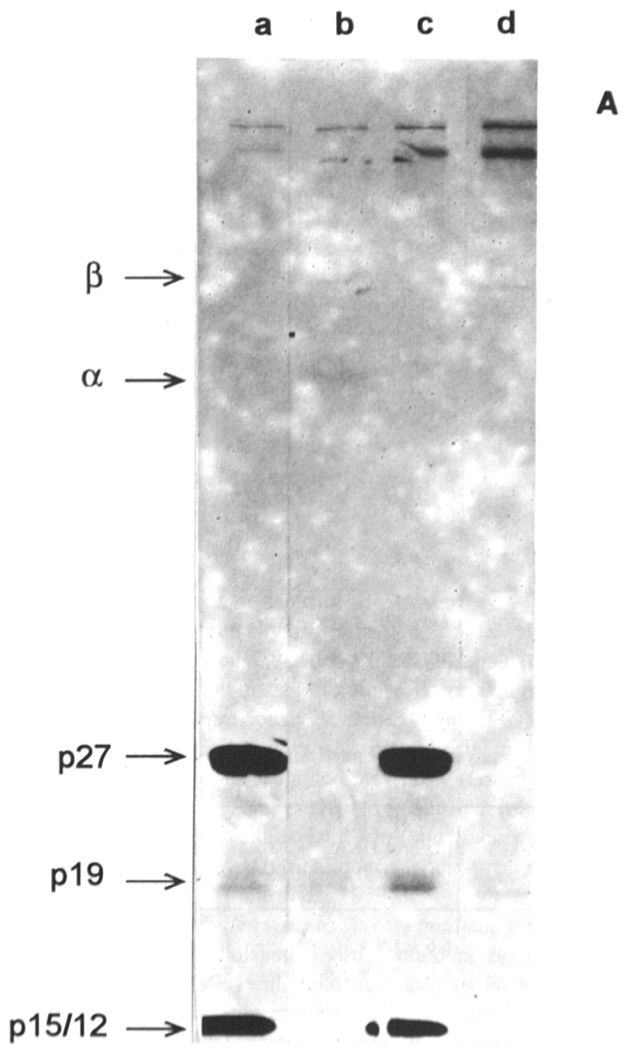

Fig. 2
SDS-PAGE of radiolabelled proteins released from RSV-infected MT and MF cultures

The RSV-infected MF culture (SR-MF, lanes a and b) and RSV-infected MT culture (SR-MT, lanes c and d) at 4 days p.i. were pulse-labelled with ^{35}S-methionine. Two equal portions of disrupted virus suspensions were immunoprecipitated with immune serum (goat anti-AMV serum, lanes a and c) or goat anti-AMV RT serum (lanes b and d). The amount of radioactivity in each immunoprecipitated sample was as follows: (a) 4,500 cpm; (b) 960 cpm; (c) 3,450 cpm; (d) 1,200 cpm. Immunoprecipitated radioactive antigens were analyzed by SDS-PAGE (12% acrylamide/0.108% bisacrylamide). The gel was prepared for fluorography and exposed to a Kodak X-Omat R film at -70 °C for 8 days.

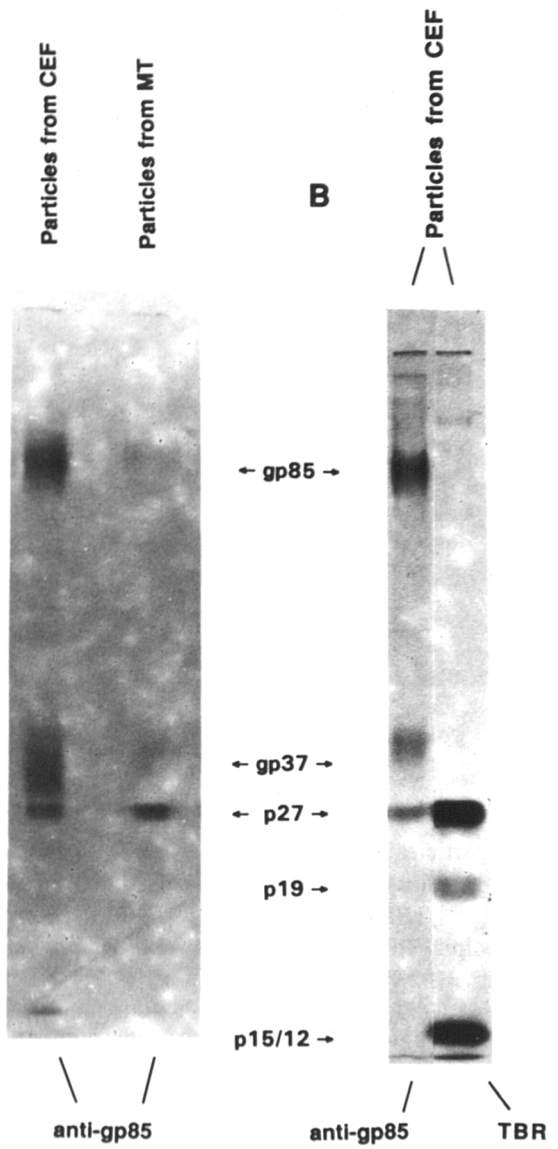

Fig. 3
Analysis of envelope proteins of radiolabelled RSV particles released by RSV-infected MT and CEF cells

The viral particles were labelled with ^{35}S-methionine (50 μCi/ml; 1,200 Ci/mmol in panel A, 100 μCi/ml; 1,369 Ci/mmol in panel B) in 8 ml methionine-free MEM. The viral particles were purified by sucrose gradient. Virion proteins (2 – 4 x 10^4 cpm in panel A and 2.7 x 10^6 cpm in panel B) were immunoprecipitated with anti-gp85 serum or TBR serum under the antibody excess condition and analyzed by SDS-PAGE. Cells were infected with tsNY68 at MOI of 1, and the viral particles were harvested on day 4 (panel A) and day 6 (panel B) p.i.

Table 2. FFU titer of the culture fluid of microinjected MT and CEF cells from day 1 to day 5 post microinjection

Days post microinjection	Exp. 1[a]		Exp. 2[b]		
	CEF	MT	CEF[c]	MT[c]	MT[d]
	(FFU/ml)		(FFU/ml ± standard error)		
1	0	0	4.3 ± 4.3	9.0 ± 9.0	0 ± 0
2	0	0	$2.0 \pm 1.5 \times 10^2$	3.6 ± 1.8x10	0 ± 0
3	5	0	$1.0 \pm 5.5 \times 10^2$	$3.1 \pm 2.3 \times 10^2$	2.7 ± 2.8x10
4	40	10	$1.6 \pm 1.1 \times 10^4$	$8.8 \pm 8.1 \times 10^3$	$1.8 \pm 0.6 \times 10^2$
5	166	55	$3.6 \pm 2.2 \times 10^4$	$1.2 \pm 1.0 \times 10^5$	$4.5 \pm 0.8 \times 10^3$

[a] SRA2 DNA (12 molecules/cell) was used. Samples were collected from a single ring.
[b] $pRSV^{v\text{-}src}$ DNA was used. Each value is an average of three rings.
[c] Approximately 120 molecules/cell were microinjected.
[d] Approximately 12 molecules/cell were microinjected.

same amount of radioactive proteins. The anti-gp85 serum is known to react with $p27^{gag}$ in addition to env proteins (Hsia *et al.*, 1992). This notion was further supported by the experiment in Fig. 3B, showing that the 27 K protein precipitated by anti-gp85 serum electrophoresed to exactly the same position as the $p27^{gag}$ precipitated by TBR serum. Relative mobility of $p27^{gag}$ against $gp37^{env}$ or $gp85^{env}$ in Fig. 3B was identical within an experimental error to that in Fig. 3A. We therefore used the $p27^{gag}$ protein band as an internal marker to determine the relative amounts of the env proteins to that of the gag proteins in Fig.3A. In order to quantitate the amounts of radiolabelled proteins, we subjected the autoradiogram in Fig. 3A to densitometric analysis. The results indicated that the ratio env/gag of the particles harvested from infected MT was 4-fold less than that of the particles from the infected CEF cells. In a separate experiment, the released viral particles were examined every day from day 1 through day 4 p.i. The reduction of the env proteins was observed throught this period (data not shown).

Infectivity of the virus particles released by RSV-infected MT

The FFU titer and RT activity of the particles from infected MT and CEF cells were determined. FFU per unit amount of cellular DNA as shown in Table 1 indicated that infectivity released by infected MT was less than 1% of that released by infected CEF cells (column I). On the other hand, infected MT culture released RT to the extent of 13% of those released by CEF cells (column II). This indicates that particles released from infected MT culture had a low infectivity relative to the amount of RT. Together with the notion that env protein was much less in these particles (Fig. 3), the data suggest that the particles produced by infected MT culture were deficient in env protein, and that due to this deficiency the particles had only 6% of specific infectivity (FFU/RT) compared with the normal virus released from CEF cells (column III). Because the infected MT cultures contain some (approximately 1%) mononucleated cells, the FFU titer observed in this experiment may partly or totally have come from these mononucleated cells. Therefore, 6% infectivity calculated in these experiments is the upper limit of the infectivity of these defective particles, and the real value could be lower.

Microinjection of RSV DNA into MT and CEF cells

Since microinjected viral DNA is known to behave differently from naturally occuring viral DNA in the infected cells (Luciw *et al.*, 1984), we then studied MT microinjected with molecularly cloned RSV DNA in order to determine if these MT could produce infectious particles. Table 2 shows the results of two independent microinjection experiments. In contrast to the virus-infected MT mentioned above, microinjected MT produced infectious particles. Infectivity released by microinjected MT was approximately equal to that released by microinjected CEF cells, the positive control. Time course of the release of infectivity by microinjecte4d MT and by microinjected CEF cells appears similar. Production of a significant amount of infectious virus from microinjected CEF cells is in accordance with the observation by Kopchick *et al.* (1981). Cells microinjected with $pRSV^{v\text{-}src}$ plasmid DNA (Nemeth *et al.*, 1989) showed similar results as those microinjected with SRA2 DNA except that higher efficiency of virus production was observed in the former. This is probably due to the fact that the $pRSV^{v\text{-}src}$ plasmid already contains the complete RSV sequence in the provirus form. On the other hand, the RSV sequence in SRA2 is interrupted within the *env* gene and has to be ligated in the correct order *in vivo* to form a complete virus genome. In a separate control experiment, SRA2 DNA was injected into the media surrounding the desig-

nated area of MT. No infectivity was produced under such a condition (data not shown). This observation ruled out the possibility that the DNA that might have leaked out from the microinjection needle contributed to the production of viral particles.

Discussion

Our previous work (Tanaka *et al.*, 1992) was consistent with the earlier finding that RSV DNA integration is dependent on host DNA synthesis (Varmus *et al.*, 1977; Varmus *et al.*, 1979; Humphries *et al.*, 1981; Varmus and Swanstrom, 1984). In MT, in which chromosomal DNA synthesis is irreversibly stopped by the terminal differentiation process (Stockdale and Holtzer, 1961; Pullman and Yeoh, 1978; Kobayashi and Kaji, 1978; Lim and Hauschka, 1984), no viral DNA integration was observed and the viral DNA remained unintegrated. In this paper we presented evidence indicating that viral particles are produced by RSV-infected MT, establishing that RSV replication is not dependent on host cell DNA synthesis. However, the particles produced by RSV-infected MT were mostly defective. We conclude that infected MT produced env-deficient particles with a greatly reduced infectivity. In contrast, viral DNA microinjected into MT produced particles with a normal infectivity. The exact reason why RSV DNA injected into MT resulted in infectious particles, while infected MT produced defective particles, remains obscure.

Here we postulate a mechanism based on the fact that RT often falls from the RNA template, producing incomplete, single-stranded, antisense DNA fragments (Rothenberg and Baltimore, 1977). In contrast, microinjected molecularly-cloned viral DNA does not produce antisense DNA. It is possible that these antisense DNA may interfere with the translation process by means of heteroduplex formation. Since RT starts at the position very close to the 5'-end of the viral genome and jumps to the 3'-end, it is likely that more incomplete fragments homologous to the *src* and *env* sequences are produced than those homologous to *gag* and *pol*. In fact, we have observed that infected MT synthesize a significantly reduced amount of src and env proteins (Hsia *et al.*, 1992). It is known that the production of env protein in the infected cells results in superinfection interference (Keshet and Temin, 1979; Weller *et al.*, 1980; Weller and Temin, 1981; Chen and Temin, 1982). The reduction in env protein may therefore prevent establishment of superinfection interference. This would allow continuous infection of the already infected MT by the newly produced particles, resulting in persistent RT and hence production of antisense fragments.

Is the amount of antisense DNA in infected MT enough to cause translational inhibition by hybridizing to viral mRNA? Schincariol and Joklik (1973) reported that CEF cells infected with a Prague C strain of RSV contains about 300 copies of virus-specific RNA per cell 4 days p.i. Survey of literature indicates that the amount of viral DNA in infected cells varies from 0.5 to 100 copies per cell depending on the virus and experimental conditions used (Schincariol and Joklik, 1973; Ali and Baluda, 1974; Varmus *et al.*, 1974; Guntaka *et al.*, 1976; Varmus and Shank, 1976; Khoury and Hanafusa, 1976; Temin *et al.*, 1979; Estis and Temin, 1979). It is not known how much viral DNA accumulates if infected cells are not allowed to establish superinfection interference. Our previous results show that RSV-infected MT persistently harbor at least 2.5 unintegrated viral DNA molecules per cell over a period of 7 days p.i. (Tanaka *et al.*, 1992). This number does not include small fragments of viral DNA which appeared as a smear in the blotting experiments (data not shown). In fact, the detailed analysis of *in vitro* RT reported by Boone and Skalka (1980, 1981a, 1981b) indicates that a great majority of the first strand synthesis reactions are terminated well before the completion of full length cDNA. The failed reactions may results in a large number of incomplete fragments, many of them too short to be detected by the conventional blotting method. Therefore, it is plausible to assume that MT continuously superinfected by progeny particles contain enough amount of antisense DNA fragments to preferentially inhibit translation of src and env proteins.

The production of defective virus in our system took place under the condition where integration of viral DNA is prohibited. This situation is analogous to infection with integration-defective mutants of retroviruses. Indeed such mutants are often associated with a very low titer of progeny virus. Panganiban and Temin (1983) reported that alterations in the integration-specific sequence in LTR create an integration-defective spleen necrosis virus which produces progeny particles with a titer 10^{-6} of that of the wild type. Cobrinik *et al.* (1983) reported that deletions in the integrase recognition site within the LTR sequence of RSV result in a significantly reduced RT activity released in the media. Introduction of mutations or deletions in the integrase gene (IN) of the viral genome also results in a greatly reduced progeny titer (Donehower and Varmus, 1984; Panganiban and Temin, 1984; Schwartzberg *et al.*, 1984; Stevenson *et al.*, 1990a, 1990b). Because unintegrated DNA is abundant in the host cells in these cases, it is possible that a similar translational inhibition of the 3'-end genes as observed in our system may be responsible for the lower titer of progeny virus.

If the above hypothesis holds, a similar translational control is expected in an environment where integration is inhibited and incomplete RT products are expected to be abundant. Indeed Sharpe *et al.* (1990) reported that the murine neurotropic retrovirus, Cas-Br-E, does not express env pro-

tein in infected neuron despite the fact that the spliced *env* mRNA is present. Also RSV-infected CEF cells in the early period after infection may have a preferential inhibition of env and src proteins production due to the possible presence of abundant antisense DNA in these cells. We are currently investigating this possibility.

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