Viral RNA Subunits in Cells Transformed by RNA Tumor Viruses

Abstract. *Single-stranded 35S and 20S viral RNA species are synthesized in virus-producing mouse and rat cells transformed by the murine sarcoma virus. A transformed hamster cell line that does not produce virus synthesizes 35S, but not 20S viral RNA.*

RNA extracted from RNA tumor viruses sediments at 70*S*, but little or no 70*S* RNA can be extracted from infected or transformed cells (*1*). Nevertheless, hybridization tests show large amounts of virus-specific RNA in the nucleus and cytoplasm of virus-producing rat and mouse cells transformed by murine sarcoma virus (MSV) (*2*). Much smaller quantities of viral RNA were found in "cryptic," nonvirus-producing hamster cells (HT-1) transformed by MSV (*2, 3*). To determine the relation between the intracellular viral RNA and the 70*S* genome, we have studied the size of the virus-specific RNA species in cells transformed by MSV. We report here that virus-producing transformed cells synthesize at least two species of single-stranded viral RNA molecules with sedimentation coefficients of 35*S* and 20*S*. The nonvirus-producing HT-1 cells synthesize only 35*S* viral RNA. These findings have implications for the transformed state of the cell and offer an explanation for the lack of viral 70*S* RNA in virus-producing cells.

We determined the amount of virus-specific RNA as follows. Increasing amounts of 70*S* viral RNA isolated from virus particles were annealed with a constant amount of the [^{3}H]DNA product prepared with the RNA-directed DNA polymerase (reverse transcriptase) of purified MSV(M) (that is, Moloney strain) grown in 78A1 cells. The extent of DNA-RNA hybrid formation was measured by differential elution from hydroxyapatite (*2*). The relation between the concentration of viral RNA and the extent of hybrid formation is given in Fig. 1A. Saturating amounts of 70*S* viral RNA (0.05 to 0.1 μg) converted 70 to 80 percent of [^{3}H]DNA to a hybrid form. The reaction is highly specific for viral RNA, for the addition of as much as 200 μg of RNA from normal rat or adenovirus-transformed rat cells did not affect the hybridization of 0.01 μg of 70*S* viral RNA to MSV [^{3}H]DNA (inserted in Fig. 1A).

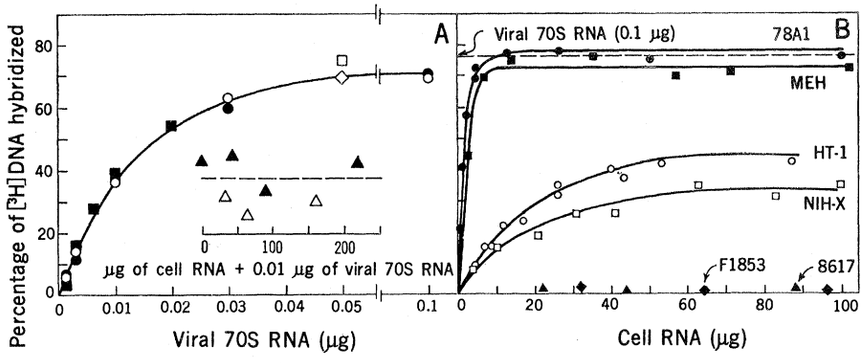

Fig. 1. (A) Quantitation of virus-specific RNA. Viral 70*S* RNA was prepared from purified MSV(M) lysed in NTE buffer (0.1*M* NaCl, 0.01*M* tris, *p*H 7.6, 0.001*M* EDTA) with 1.0 percent sodium dodecyl sulfate (SDS), extracted with phenol, and centrifuged in sucrose gradients (15 to 30 percent) in NTE buffer containing 0.1 percent SDS for 2.5 hours at 36,000 rev/min at 20°C in the Spinco SW41 rotor. The MSV(M) [^{3}H]DNA was prepared by incorporation of [^{3}H]thymidine triphosphate (11 to 12 c/mmole) into purified MSV(M) by the endogenous RNA-directed DNA polymerase reaction (*2*). This [^{3}H]DNA (500 count/min) was annealed with increasing amounts of 70*S* viral RNA, and the extent of RNA-DNA hybrid formation was measured by batch elution from hydroxyapatite crystals; centrifugation was used instead of the chromatographic procedure (*2*). The annealed sample was diluted and incubated at 60°C for 20 minutes in 5.0 ml of 0.12*M* sodium phosphate buffer (*p*H 6.7) and 0.4 percent SDS containing 0.28 g of hydroxyapatite. The hydroxyapatite removed by centrifuged was washed twice by incubation with 5-ml portions of the same buffer at 60°C for 10 minutes. The absorbed DNA-RNA hybrid was eluted by two portions (5 ml each) of 0.4*M* phosphate buffer (10 minutes at 60°C). The radioactivity in the fraction precipitable by trichloroacetic acid was determined as described (*1*). Hybridization of various amounts of 70*S* RNA with [^{3}H]DNA in different experiments are indicated by the symbols ○, ●, ■, □, ◇. Normal rat cells (F1853) and adenovirus type 2–transformed rat cells (8617) (*13*) were grown and RNA was extracted as described (*8*). The 70*S* RNA (0.01 μg) was hybridized with [^{3}H]DNA product in the presence of various amounts of 8617 cell RNA (▲) or F1853 cell RNA (△). (B) Hybridization-saturation of MSV(M) [^{3}H]DNA with RNA from virus-producing and nonvirus-producing transformed cells. The 78A1, MEH, and HT-1 cells were grown as described (*2, 3*). The NIH-X cells (*5*) were grown in suspension culture in Eagle's minimal essential medium with nonessential amino acids, sodium pyruvate, 2 m*M* L-glutamine, and 7 percent calf serum. Various amounts of RNA extracted from whole cells (*8*) were annealed with MSV(M) [^{3}H]DNA (500 count/min) and RNA-DNA hybrid formation was measured as described.

The MSV(M) [^{3}H]DNA was annealed with increasing amounts of RNA from (i) MSV(M) virus, (ii) virus-producing MSV(M)-transformed rat cells (78A1), (iii) virus-producing MSV(H)- (that is, Harvey strain) transformed mouse cells (MEH), (iv) "cryptic" nonvirus-producing MSV(M)-transformed hamster cells (HT-1)—which fail to synthesize the group specific (gs) antigen of the mouse leukemia-sarcoma virus group (*4*), (v) nonvirus-producing mouse cells (NIH-X) derived from the leukemic spleen of an x-irradiated NIH mouse by Dr. Hartley (*5*), (vi) human adenovirus type 2–transformed rat cells (8617), and (vii) normal rat cells (F1853). As shown in Fig. 1B, no viral RNA sequences were detected in control 8617 and F1853 cell lines. Virus-specific RNA was detected readily in virus-producing 78A1 and MEH cells (70 to 80 percent hybridization) and in nonvirus-producing HT-1 and NIH-X cells (30 to 40 percent hybridization); this is the first demonstration that NIH-X cells contain virus-specific RNA.

From the half-saturation values (Fig. 1B), we estimate that nonvirus-producing cells (HT-1 and NIH-X) contain 1/15 as much virus-specific RNA as do virus-producing cells, although the DNA product used may not be an equimolar mixture of the DNA sequences in the 70*S* RNA genome. The finding that "cryptic" cells contain much smaller amounts of virus-specific RNA is consistent with the previous report (*2*). The HT-1 cell RNA formed a hybrid with only 40 percent of MSV(M) DNA (Fig. 1B), suggesting that some viral RNA sequences transcribed in virus-producing cells are absent from nonvirus-producing HT-1 cells; conceivably this could be due

to differential expression of sarcoma and leukemia virus specific information. However the MSV(M) strain which transformed HT-1 cells may differ in base sequence from the MSV(M) strain used to prepare the [^{3}H]DNA product. To exclude this possibility, we obtained a sample of the original MSV(M) virus which induced the HT-1 cell (*6*). The virus was then grown in mouse BALB/3T3 cells (*7*). Viral 70*S* RNA molecules isolated from purified virus of this strain of MSV(M) and from the strain of MSV(M) growing in 78A1 cells gave identical hybridization-saturation curves. Furthermore they both hybridized with 70 to 80 percent of the MSV(M) (78A1 cell) [^{3}H]DNA product. Thus the MSV(M) strains appear to be identical.

Only 30 percent of the MSV(M) [^{3}H]DNA hybridized with saturating amounts of RNA from NIH-X cells. Since the nature of the viral genome in the NIH-X cell line is not known, we cannot, in this case, conclude with certainty that there is incomplete transcription of viral RNA.

The size of the virus-specific species in 78A1, MEH, and HT-1 cells was determined. Total cellular RNA was extracted by the hot phenol method (*8*), treated with dimethylsulfoxide to dissociate RNA aggregates (*9*), and fractionated by zone sedimentation in sucrose gradients (15 to 30 percent). A constant volume of each fraction was then annealed with [^{3}H]DNA to detect virus-specific RNA. The 78A1 RNA (Fig. 2A) and MEH RNA (not shown) displayed two distinct peaks of virus-specific RNA with sedimentation coefficient of 35*S* and 20*S*. If cellular RNA was not treated with dimethylsulfoxide we observed that, in addition to 35*S* and 20*S* viral RNA species, variable amounts of viral RNA sedimented between 35*S* and 60*S*, but that there was no distinct peak of 70*S* RNA. The RNA from cryptic HT-1 cells revealed a 35*S* virus-specific RNA peak but no 20*S* viral RNA (Fig. 2B). These species of RNA were reproducibly detected in numerous experiments. The peaks of 35*S* and 20*S* virus-specific RNA in 78A1 cells were isolated from the gradients and treated with ribonuclease or deoxyribonuclease or both. We found that both 35*S* and 20*S* viral RNA species behave as single-stranded RNA, since they are digested by ribonuclease but not by deoxyribonuclease.

Our data indicate that the intracellular viral RNA consists of at least two size classes of single-stranded RNA molecules sedimenting at 35*S* and 20*S*. These viral RNA species are mostly, if not entirely, plus (+) strands since they hybridize to a large extent with the MSV [^{3}H]DNA product, which itself hybridizes 70 to 80 percent with viral RNA (+). The 35*S* and 20*S* viral RNA species may be transcribed on viral DNA (−) templates formed by the virion RNA-directed DNA polymerase. The possibility that viral RNA (+) species are made not on viral DNA but on viral RNA (−) strands is consistent with one report (*10*): but most workers have failed to detect viral RNA (−) sequences by hybridization of cellular RNA with 70*S* viral RNA (*1*).

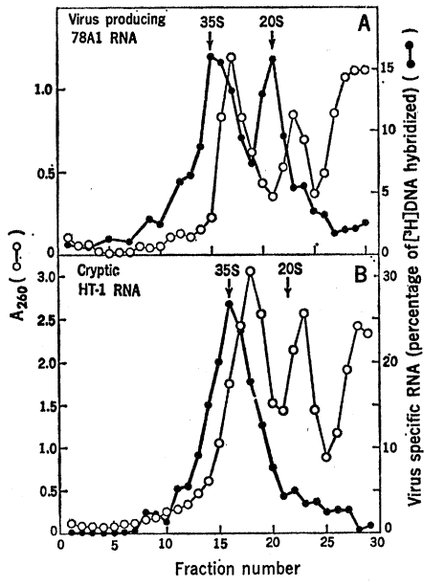

Fig. 2. Zone sedimentation of virus-specific RNA from 78A1 and HT-1 cells in sucrose density gradients. The RNA was isolated from 78A1 and HT-1 cells (*8*), dissolved in NTE buffer, treated with dimethyl sulfoxide (*9*) and dialyzed at 25°C for several hours against 500 volumes of NTE buffer containing 0.5 percent SDS. The RNA was precipitated with two volumes of ethanol and dissolved in NTE buffer; this solution was layered on sucrose gradients (15 to 30 percent) in NTE buffer containing 0.5 percent SDS, and centrifuged at 25°C in a Spinco SW25.1 rotor for 16 hours at 17,000 rev/min. Fractions (1 ml) were collected, and the ultraviolet absorbance was measured at 260 nm. RNA was precipitated from each fraction with two volumes of ethanol after the addition of yeast RNA (50 μg). The precipitate was dissolved in 0.3 ml of 0.1 × SSC (SSC is 0.15*M* NaCl and 0.015*M* sodium citrate), and portions were annealed with the MSV(M) [^{3}H]DNA (500 count/min). The amount of RNA-DNA hybrid formed was measured as described in the legend of Fig. 1. (A) 78A1 RNA: 3 μl from each fraction was annealed with [^{3}H]DNA product. (B) HT-1 RNA: 50 μl was annealed with [^{3}H]DNA product.

The 35*S* and 20*S* viral RNA species that we detected in MSV-transformed mouse and rat cells may serve two functions: (i) precursors to the 70*S* viral RNA genome and (ii) mRNA's in viral protein synthesis. In support of a precursor role, we note that (i) the 70*S* viral RNA genome is irreversibly dissociated under denaturing conditions to 35*S* and heterogeneous smaller RNA's (*9*), and that (ii) we have data showing that some intracellular virus-specific RNA is aggregated unless RNA is treated with dimethylsulfoxide. Possibly viral RNA aggregates are intermediate in viral RNA assembly at the cellular membrane. The second function of viral 35*S* and 20*S* RNA as mRNA's for protein synthesis is consistent with recent findings that viral RNA sequences are present in polyribosomes of 78A1 cells (*11*).

The cryptic state of HT-1 cells is characterized by two defects; a level of RNA only 2 to 6 percent of that in the virus-producing cells, and a specific deficiency of viral 20*S* RNA. To determine whether these two characteristics are related to the cryptic state of MSV-transformed cells, we have studied the virus-specific RNA of three other nonproducing mouse cell lines transformed by MSV(M). We found in each case (i) small amounts of virus-specific RNA, (ii) a deficiency in some viral gene sequences, and (iii) no 20*S* RNA (*12*).

The absence of viral 20*S* RNA in MSV-transformed cells that do not produce virus suggests that 20*S* RNA may not be essential to maintain the transformed state of the cell. The mechanism of cell transformation by RNA tumor viruses appears to be similar in many respects to that of DNA tumor viruses such as the adenoviruses where it has been shown that only a portion of the viral genome is transcribed (*1*). The proteins coded for by the viral RNA found in nonvirus-producing cryptic cells are likely candidates for "transforming proteins."

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5. NIH-X cells were established by Dr. Janet Hartley, who provided us with a seed culture.
6. We thank Dr. R. J. Huebner for providing us with a seed culture of the virus.
7. We thank A. Rankin for growing the virus.
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Spontaneous Transformation of Human Brain Cells Grown in vitro and Description of Associated Virus Particles

Abstract. *A human brain cell culture grown in vitro has spontaneously transformed, as determined by morphology, growth characteristics, and karyotype analysis. Virus particles morphologically akin to oncogenic RNA viruses are present in the transformed cells, which are now in subculture 60.*

In our studies of the subacute spongiform viral encephalopathies, numerous human and primate brain tissues have been grown in vitro (*1*, *2*). In this report we present data on the spontaneous transformation of one of the human brain cultures and a description of the morphological characteristics of the virus particles observed in this cell culture.

A specimen of human brain was obtained at biopsy from a patient (S.G.) with Creutzfeldt-Jakob disease; the cell line that was derived from this tissue is termed SG. The tissue was placed in Eagle's minimal essential medium supplemented with penicillin, 100 unit/ml; streptomycin, 100 μg/ml; and 20 percent fetal bovine serum inactivated at 56°C for 60 minutes. The tissue was cut into small pieces and divided into two samples. The first sample was explanted into Falcon flasks according to the technique described by Rogers *et al.* (*2*). The second sample was washed with medium not containing bovine serum, treated with 0.025 percent trypsin, and seeded into Falcon flasks. Both kinds of cultures were incubated at 35°C in atmosphere containing 5 percent CO_2 and observed and fed at approximately 4-day intervals. When the cell sheets were about three-fourths confluent, the cells were treated with a mixture of 0.05 percent trypsin and 0.02 percent ethylenediaminetetraacetic acid and subcultured into new flasks.

The primary cultures prepared by both methods initially grew out as monolayers of fibroblast-like cells. After 60 days, foci of cells with altered morphology (Fig. 1a) were noted in subculture 3 of the cultures that had been treated originally with trypsin. During the next 30 days foci of similarly changed cells appeared in subcultures from all flasks of the culture treated originally with trypsin, and in subcultures from two of the three flasks of primary explants. By subculture 5 the transformed cells were the only cell types observed in all cultures (Fig. 1b). (Subcultures derived from the one untransformed flask of primary explant and from transformed cultures at various subculture levels had earlier been frozen and stored in liquid nitrogen.) The transformed cells appeared initially in small focal areas of altered morphology. These cells were larger than the original fibroblast-like cells and were epithelial in appearance; they rapidly overgrew the untransformed cell sheet. They displayed the characteristics of transformed cells; loss of contact inhibition was shown by continued growth of confluent cultures, which caused overlapping and piling. Subcultures derived from the lines prepared originally with or without trypsin were maintained as two cell lines until subculture 10, then the subcultures from both lines were pooled.

The transformed SG cell line has now been maintained for 14 months and is in subculture 60. The culture was diluted 1 : 3 for subcultures 1 to 5, 1 : 4 for subcultures 6 to 35, and 1 : 5 for subcultures 36 to 60. The cells now show a rapid rate of growth with enormously increased capacity to persist in serial subcultures; subculture 60 is a 10^{38} dilution of the primary cultures. The cells have a 60 percent plating efficiency, and as few as ten cells are needed to initiate cell growth on a surface area of 25 cm^2.

Chromosome studies on subcultures 47 and 54 (*3*) indicate that the cells are of human origin and contain a chromosome number ranging from 70 to 80. The majority of cells contain 75 chromosomes; one is a large accrocentric marker with a long arm longer than that in the No. 13 to 15 group, and one or two chromosomes are minutes. The incidence of chromosomal aberrations is less than 1 percent.

Virus particles morphologically resembling the known oncogenic RNA viruses were detected in all cultures examined since the transformation occurred. At various subculture levels, SG cells were removed from the flasks with rubber policemen and sedimented at 600*g* for 10 minutes. The cell pellets were fixed in 2 percent glutaraldehyde, post-fixed in Dalton's chrome osmium, dehydrated in alcohol, and embedded in Epon. The cells were stained with lead citrate and examined in a Siemens electron microscope. Virus particles were not observed when the brain culture prepared with trypsin was harvested after 40 days of incubation and before morphological changes had occurred. Virus particles were found in all of the brain cultures examined after morphological changes—that is, in subculture 4 of the explant culture, subcultures 4 and 6 of the culture prepared with trypsin, subcultures 14, 26, and 36 of the pooled culture. Three morphological forms of virus particles were observed in the transformed SG brain cultures (Fig. 2). Intracellular particles about 60 to 90 nm in diameter were observed only within the cytoplasm and contained an electron-transparent center, giving a doughnut-shaped appearance (Fig. 3a). Particles that were budding from the plasma membrane (Fig. 3b) were also seen; the particles appeared to acquire an outer envelope in this process. Finally, extracellular virus particles were observed; these were oval or spherical, about 100 to 120 nm in diameter (Fig. 3c). Most of the extracellular virus particles contained a partially dense nucleoid. The virus forms observed appear to represent one type of particle that shows dif-