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Biochemical Characterization of Endogenous Type C Virus Information in Differentiated and Undifferentiated Murine Teratocarcinoma-Derived Cell Lines

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Undifferentiated teratocarcinoma cells express sixfold-higher levels of endogenous xenotropic type C virus-related RNA than differentiated cells. Three species of polyadenylated viral RNA (35S, 24S, and 14S) have been identified in the undifferentiated teratocarcinoma cells. Paradoxically, neither viral particles nor viral proteins have been detected in these cells.

The stable genetic transmission of endogenous retroviral genes through the germ line of most avian and mammalian species has suggested that these genes play a role in biological processes other than neoplasia. Electron microscopic studies of early embryos from a variety of laboratory mouse strains and feral mice (2, 18) have demonstrated the presence of endogenous type C retroviral particles which were not in evidence at later stages of development. These observations have led to the speculation that the expression of endogenous type C viral genes may be involved in embryogenesis and differentiation (10). However, attempts to experimentally examine this possibility have been impeded by the scarcity of embryonic material as well as by the rapid transition of cells through successive states of differentiation. To avoid this problem, we have used lines of undifferentiated stem embryonic carcinoma cells (ECC) and differentiated tissue culture cells derived from the 129/J st murine teratocarcinoma (11, 12). In this communication, we have correlated the level of expression of type C viral RNA with the state of differentiation, and we present a preliminary characterization of this RNA.

Previous attempts by our laboratory as well as others to isolate an infectious endogenous type C virus from 129/st tissue culture cells have not been successful. Moreover, Chattopadhyay et al. (5) have shown that cellular DNA from mouse strain 129 hybridizes only partially with a complementary DNA (cDNA) probe prepared with the AKR ecotropic type C virus, suggesting that this class of endogenous murine type C viral genes is missing in the mouse strain 129 cellular genome. To determine whether the xenotropic class of endogenous type C viral genome is represented in the cellular DNA of mouse strain 129, we have prepared a cDNA probe with *Mus musculus molossinus* xenotropic type C virus (MOL-MuLV-x). We have previously shown that the genome of this virus is highly related to that of other xenotropic viral isolates from a variety of inbred strains of mice (4). The extent of the sequence homology between the MOL-MuLV-x [^{3}H]cDNA and mouse strain 129 cellular DNA as well as the reiteration frequency of these sequences was assessed by C_0t analysis (Fig. 1). The MOL-MuLV-x [^{3}H]cDNA hybridized to a similar final extent as the mouse strain 129 cellular DNA and the DNA from dog thymus cell line FCf2th chronically infected with MOL-MuLV-x virus (designated MOL-8155). Cellular DNA annealing kinetics show that, compared with unique sequences, the virus-related sequences are reiterated 8 to 10 times per haploid genome in both undifferentiated and differentiated cells. These results show that the cellular DNA of mouse strain 129 contains multiple copies of sequences related to the genome of the xenotropic class of endogenous type C viruses and that there is no apparent modulation of the number of virogenes between undifferentiated and differentiated cells.

The extent of transcription of endogenous xenotropic virogenes in ECC (PCC4) and differentiated (PCD1) cells was compared by kinetic analysis of nucleic acid hybridization of cytoplasmic RNA from the respective cell lines and MOL-MuLV-x [^{3}H]cDNA (Fig. 2). A plateau value of hybridization was not reached at a C_rt of 2.5×10^4 for PCC4 or PCD1. This result is not due to partial sequence homology between the MOL-MuLV-x [^{3}H]cDNA and the viral RNA sequences detected in these cell lines, since the T_m (thermal stability) of these hybrids is the same as that found with xenotropic 70S viral

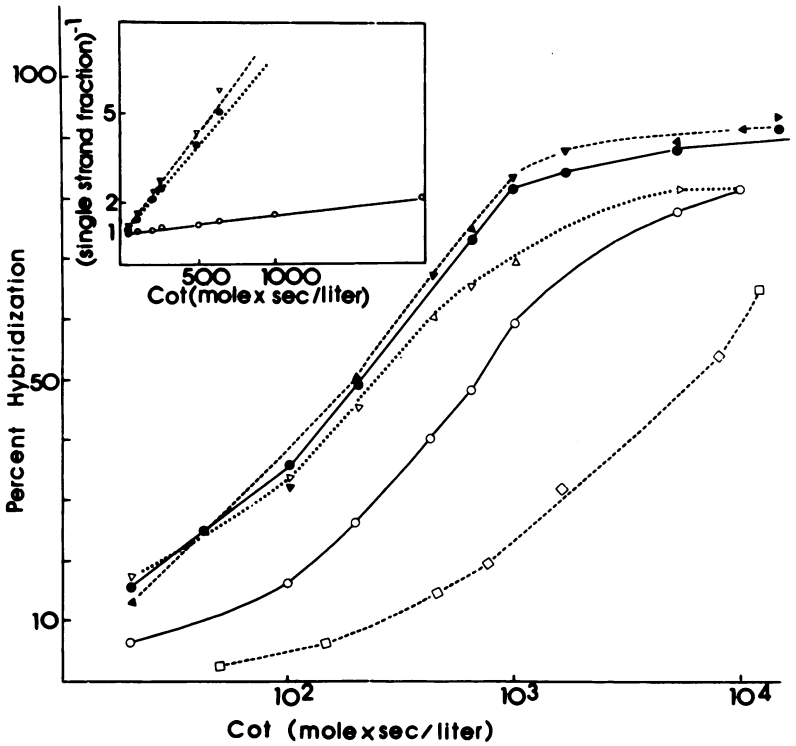

FIG. 1. *Kinetics of hybridization of the MOL-MuLV-x [^{3}H]cDNA to DNA extracted from various cell lines. Cellular DNA was extracted from nuclei in NTE buffer (pH 8.4) by treatment with 0.5% sodium dodecyl sulfate (SDS) and proteinase K. The mixture was then deproteinized three times with phenol-chloroform-isoamyl alcohol, and DNA was precipitated by ethanol. Viral cDNA was synthesized by a modification of the method previously described by Benveniste and Scolnick (1), allowing the synthesis of longer and more representative transcripts. MOL-MuLV-x [^{3}H]cDNA was prepared by the incubation for 4 h of harvested viruses in vitro in the endogenous RNA-dependent DNA polymerase reaction. Purified viruses were added to an incubation mixture containing the following: 0.04 M Tris (pH 7.8); 0.06 M KCl; 0.02% Triton; 2.8 mg of $MgCl_2$; 0.3 mg of dithiothreitol per ml; 1 mM each dATP, dCTP, and dGTP; 4×10^{-5} M [^{3}H]dTTP (50 Ci/mM); and 30 μg of actinomycin D per ml. The [^{3}H]cDNA prepared in this way had a size between 16 and 20S in an alkaline sucrose gradient and a specific activity of 2×10^7 cpm/μg, and it contained 90% of the respective 70S viral RNA at a molar ratio of [^{3}H]cDNA to [^{32}P]RNA of 1.5. Hybridization reactions of 1,000 cpm of [^{3}H]cDNA and sonicated DNAs (3 to 4 μg/ml) were performed in a total mixture volume of 0.020 ml containing 0.01 M Tris (pH 7.4), 0.75 M NaCl, 0.002 M EDTA, and 0.05% SDS. Hybridizations were initiated by heating the reaction mixtures to 98°C for 10 min, cooling on ice, and incubating at 68°C. The [^{3}H]cDNA probe was annealed to nuclear DNA from the following cellular lines: PCC4 (mouse strain 129 teratocarcinoma derived, totipotential, undifferentiated cell) (◀); PCD1 (mouse strain 129 teratocarcinoma derived, myeloblastic, differentiated cell) (●); D152 (BALB/c mouse, fibroblasts) (△); MOL-8155 (chronically infected Fcf 2th dog cell line) (○). The self-annealing of unique sequences of DNA extracted from mouse strain 129 fibroblasts is shown (□). The insert shows the Wetmur and Davidson plot (19) of the same data.*

RNA (87°C) (Fig. 2, insert). The amount of virus-specific RNA detected in these cell lines was estimated by comparing the C_rt values obtained at 30% hybridization, as described by Varmus et al. (17). As shown in Fig. 2, these values are 1.8×10^{-2} for the viral RNA, 6.6×10^1 for MOL-8155, 4.5×10^3 for PCC4 RNA, and 2.3×10^4 for PCD1 RNA. By comparing these values for cellular RNAs with those of purified viral RNA, one can estimate the amount of virus-specific RNA at 2.7×10^{-2}% in MOL-8155 RNA, 4×10^{-4}% in PCC4 RNA, and only 7.8×10^{-5}% in PCD1 RNA. Assuming that the viral RNA genome has a molecular weight of 3.0×10^6 and that about 10 pg of RNA is present per cell, one finds that there are approximately 540 molecules of viral RNA per MOL-8155 cell, 8 molecules per PCC4 cell, and only 1.5 molecules per PCD1 cell. Thus, the undifferentiated PCC4 cells express five- to sixfold-higher levels of viral RNA than the differentiated PCD1 cells. The level of expression in the PCC4 cells represents

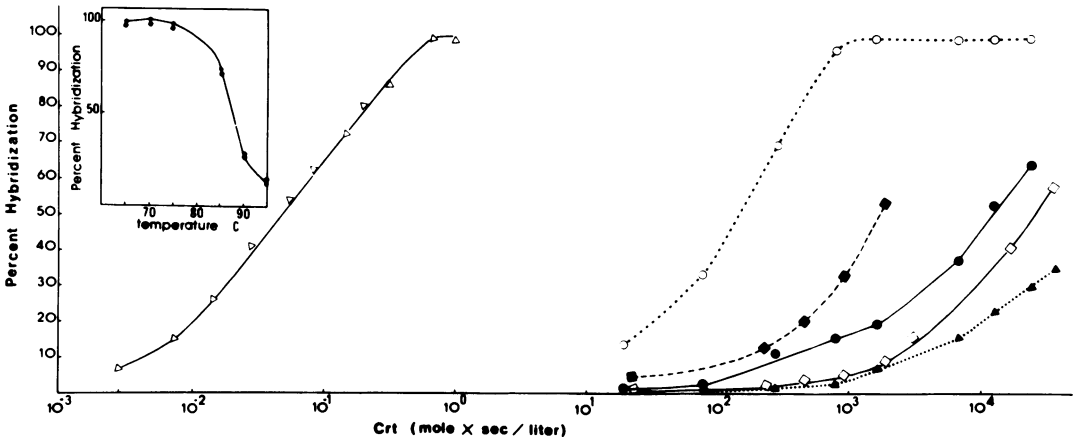

FIG. 2. *Kinetics of hybridization of MOL-MuLV-x [^{3}H]DNA probe to homologous viral 70S RNA (△) and to cytoplasmic RNA extracted from the following cell lines: PCD1 (▲), PCC4—total (●), poly(A$^+$) (■), poly-(A$^-$) (□); and MOL-8155 (○). 70S RNA was extracted from purified MOL-MuLV-x by disruption with 1% sodium dodecyl sulfate (SDS) followed by phenol deproteinization (6). Cellular cytoplasmic RNA was extracted in NTE buffer (pH 7.3) after treatment with 1% SDS. For deproteinization, we performed three extractions with chloroform, neutralized phenol, and isoamyl alcohol (24:24:1, vol/vol/vol), followed by ether treatment, lyophilization, and exhaustive dialysis four times against 0.01 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 10^{-4} M EDTA. Cytoplasmic RNA was fractionated into poly(A$^+$) RNA and poly(A$^-$) RNA by affinity chromatography on oligo(dT)-cellulose. The material bound to the column after two passages was eluted in water and precipitated with 2 volumes of ethanol after adjusting to 0.1 M NaCl. Hybridization conditions have been described in Fig. 1. Insert, Melting profiles of hybrids formed between viral [^{3}H]cDNA and RNA from PCC4 (●) and from homologous viral 70S RNA (◆) at the C$_r$t of 1 × 10^4 at 65°C. Samples were diluted 10-fold in 0.75 M NaCl, heated at the indicated temperature for 5 min, and then digested with S1 nuclease.*

only 1 to 2% of that found in chronically infected cells.

To determine whether the higher level of type C viral RNA expression detected in PCC4 cells, relative to PCD1 cells, is peculiar to them or related to their undifferentiated state, we compared the extent of hybridization of the MOL-MuLV-x [^{3}H]cDNA with cytoplasmic RNAs extracted from several other undifferentiated and differentiated cell lines. The data presented in Table 1 show that the [^{3}H]cDNA hybridized to a greater extent when RNA was extracted from undifferentiated teratocarcinoma cells and was independent of the cells' potential for further differentiation. Moreover, when PCC3d$^-$ cells were permitted to differentiate in vitro (becoming PCC3d$^+$), we observed a decrease in viral RNA expression. In this case, the extent of hybridization was comparable to that observed with RNA from all differentiated cell lines tested. These results suggest that differentiation alters either the level of stable viral RNA synthesized in the undifferentiated cells or the size of the subpopulation of cells expressing viral RNA.

Although the undifferentiated teratocarcinoma cells express a significant level of xenotropic viral RNA, these cells do not release infectious viral particles. Moreover, in other experiments (not shown) we have been unable to detect the expression of type C virus-related p30 protein or, using a group-specific competitive radioimmunoassay, murine type C-related gp70 protein. We therefore have focused on a characterization of the viral RNA expressed in undifferentiated teratocarcinoma cells. Cytoplasmic RNA from the undifferentiated cell line PCC4 was fractionated into polyadenylated [poly(A$^+$)] and non-polyadenylated [poly(A$^-$)]

TABLE 1. *Expression of endogenous type C oncornavirus RNA in various cell lines from mouse strain 129 teratocarcinoma*

Source of RNA	Strain	State of differentiation	% Hybridization to cell RNA[a]
PCC4	Mouse 129	−	52 ± 2
PCC3d$^-$	Mouse 129	−	52 ± 2
PCC6	Mouse A/He	−	50 ± 2
PCC3d$^+$	Mouse 129	+	23 ± 1
PCD1	Mouse 129	+	24 ± 1
PCD3	Mouse 129	+	23 ± 1
FES 129	Mouse 129	+	24 ± 1
FES A/He	Mouse A/He	+	4 ± 1
Fcf 2th	Dog	+	2 ± 1
DBS FRhL$_1$	Rhesus monkey	+	2 ± 1

[a] Quantitative values (C$_r$t 1.3 × 10^4).

RNA by two passages over an oligodeoxythymidylate [oligo(dT)]-cellulose column. The fraction of virus-related RNA in each preparation was determined by comparing the C_rt values at 30% hybridization of the MOL-MuLV-x [^{3}H]-cDNA with those obtained with 70S viral RNA (Fig. 2). The results show that the virus-specific RNA is found predominantly in the poly(A^+) preparation (2×10^{-3}%) as compared with the poly(A^-) preparation (2×10^{-4}%). We conclude that a significant portion of the viral RNA expressed in these cells is poly(A^+).

In cells chronically infected with murine type C virus, both genomic-length viral RNA and the subgenomic, 24S, size class have been observed (7). In the latter species, a region at the extreme 5′ end of the genome has been transposed to the sequences from the 3′ half of the viral RNA genome (8). It has been shown that genomic-length viral RNA can be translated into the *gag*

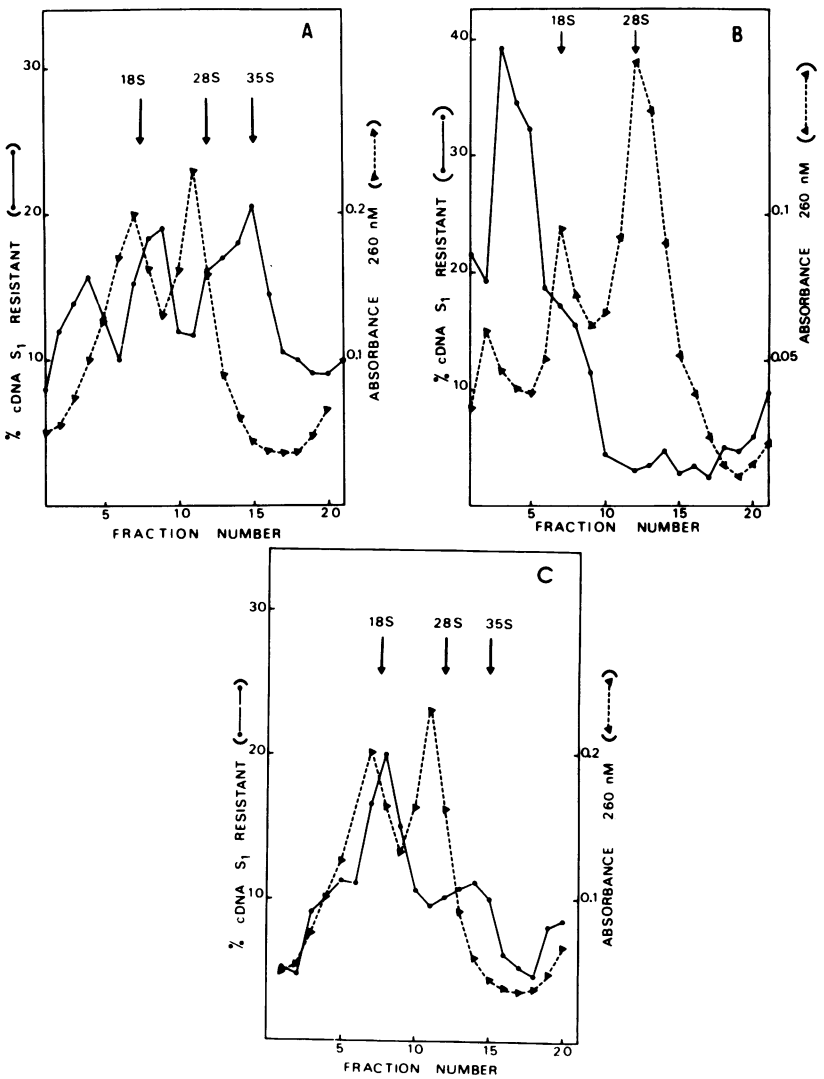

FIG. 3. *Sedimentation analysis of virus-specific RNA from poly(A^+) (A) and poly(A^-) (B) PCC4 RNA and poly(A^+) (C) MOL-8155 RNA. RNA samples were dissolved in Tris buffer containing 0.4% sodium dodecyl sulfate (SDS) and layered onto a 5 to 20% sucrose gradient also containing 0.4% SDS. Centrifugation was conducted in an SW50 rotor (Dupont de Nemours) at 45,000 rpm and 22°C. Fractions were collected from the top and precipitated with ethanol after the salt concentration was adjusted to 0.1 M NaCl and hybridized with [^{3}H]DNA in 20 μl of hybridization mixture in sealed capillaries. Symbols: (●) Radioactivity; (▲) absorbance.*

pol viral precursor proteins and that the 24S viral RNA species is the envelope mRNA (16). We have analyzed the poly(A^+) and poly(A^-) fractions of cytoplasmic RNA from PCC4 cells to determine whether virus-related RNA is composed of similar sized species. As shown in Fig. 3A, sucrose gradient analysis of the poly(A^+) RNA fraction reveals three major size classes of virus-related RNA: 35S, 22S, and 14S RNA. Similar RNA species were detected in the poly(A^+) RNA fraction from the chronically infected MOL-8155 cells (Fig. 3C). The proportionately lower levels of 35S RNA probably reflect the packaging of this species of RNA into viral particles which are released from the cell. In contrast, poly(A^-) RNA from PCC4 cells contains mostly 14S and some 22S viral RNA species. The significance of the large peak of 14S RNA is unclear at this time. It may represent broken or processed pieces of RNA. The presence of such a population has also been observed by others (3). Thus, within the limitations of the techniques employed, the PCC4 tissue culture cells express poly(A^+) RNA which is processed into genomic and subgenomic classes of sizes similar to those observed in chronically infected cells.

The lack of expression of viral proteins by PCC4 cells may reflect a regulatory control of the cellular components involved in the translation of mRNA or simply a low level of viral proteins not detectable by the assay employed. Alternatively, the viral RNA itself may be expressed in a form which requires further modification or lacks appropriate sequences required for efficient initiation of translation. It is relevant to note the recent observation that the "capping" modification of mRNA, at the 5′ end of the molecule, is required for its efficient translation in eucaryotic systems (13).

The apparent higher level of expression of viral RNA in undifferentiated stem embryonic carcinoma cells compared with that in differentiated cells suggests that this system will be useful for studying at a molecular level the mechanisms involved in the regulation of gene expression during embryonic development. The function of the viral RNA expressed in the embryonic carcinoma cells is unknown. However, recently we and others (9, 14, 15) have observed that mouse embryonic carcinoma cells, in contrast to a variety of differentiated cell lines, are resistant to exogenous infection by murine type C viruses. It is possible that the expression of the endogenous type C viral genes plays a role in this resistance phenomenon.

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