MUTANTS OF NONPRODUCER CELL LINES TRANSFORMED BY MURINE SARCOMA VIRUS

III. Detection and Characterization of RNA Specific for Helper and Sarcoma Viruses

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A nonvirus producer (NP)[1] BALB/3T3 cell line transformed by the Kirsten sarcoma virus (Ki-SV) can be induced to produce both sarcoma and helper Type C (nontransforming) viruses by treatment with 5′-bromodeoxyuridine (BrdU) (1–3). Viral genome RNA produced by such a cell is composed of two RNA subunits having distinct nucleotide sequences: one subunit has a sedimentation coefficient of 35S, is derived from the helper virus, and is indistinguishable in nucleotide sequences from Rauscher leukemia virus (RLV); the other has a sedimentation coefficient of 30S and is derived from Ki-SV. The in vitro DNA transcripts of the induced virus have nucleotide sequences complementary to both RNA species. This was shown by use of a Ki-SV specific transcript, purified by eliminating sequences homologous to RLV.[2]

We previously described four mutant cell types derived by BrdU treatment of the BALB/3T3 NP cell line (2, 3). These include both producing and nonproducing cells which could be divided further into tumorigenic and nontumorigenic categories. The expression of viral RNA in these cell lines has now been studied making use of DNA transcripts capable of detecting helper virus and sarcoma virus-specific sequences in separate assays.

Materials and Methods

Cells.—The BALB/3T3(A-31), and a derivative, K-234, transformed by Ki-SV (NP cells), were originally obtained from Doctors G. Todaro and S. Aaronson (4, 5). Mutant cell lines, M-57-1, M-58-4, M-58-2, M-43-2, and M-50, were derived from the NP cell after short-term treatment with 5′-BrdU (2, 3). The original selection of these cell lines was based on "flat" morphologic appearance. 58-2T is a cell line established from a slow growing tumor in a BALB/c mouse which was inoculated with M-58-2 cells. The 58-2T cells appeared to produce more virus than the original M-58-2 cells based on RNA-dependent DNA polymerase activi-

[1] *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; Cr·t, product of initial concentration of RNA(mol) and time (sec); Ki-SV, Kirsten strain of murine sarcoma; Nd^{25}, refractive index at 25°C; NP, nonvirus producer BALB/3T3 transformed by Ki-SV; NRK, normal rat kidney; RLV, Rauscher leukemia virus; SSC, standard saline-citrate, 0.15 M NaCl and 0.015 M sodium citrate buffer, pH 7.2.

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ties in culture media. JLS-V9 infected with RLV (6); TG-8 (RLV), a random bred Swiss-mouse embryo cell infected with RLV; and normal rat kidney (NRK) cells were used in various aspects of this work. Mouse embryo fibroblasts from BALB/c mice were obtained from Flow Laboratories, Inc. (Rockville, Md.). All cells were grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum and antibiotics.

Preparation of RNA.—Cell RNA was prepared as described previously (7). For size analysis RNA was treated with dimethyl sulfoxide as described previously (8). For reassociation kinetic analysis, cellular RNA was further purified by centrifugation in Cs_2SO_4 (Nd^{25} = 1.3556, ρ = 1.256) for 60 h at 20°C in a Spinco SW41 rotor at 33,000 rpm (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Under these conditions RNA sedimented to the bottom of the tube, and DNA around ¼ from the bottom. The supernate was carefully removed and the RNA at the bottom of the tube was dissolved in water and dialyzed against 1 × SSC (1 × SSC–0.15 M NaCl and 0.015 M Na_3 citrate), precipitated by 2 vol of ethanol, collected by centrifugation, and redissolved in 0.1 × SSC. Viral high molecular weight RNA was isolated as described previously (8). RNA concentrations were determined by the orcinol reaction (9) giving values which agreed with those calculated from absorbancy at 260 nm (23 A_{260} U = 1 mg RNA/ml).

Preparation of [^{3}H]Viral DNA.—[^{3}H]viral DNA was prepared by the endogenous RNA-directed DNA polymerase reaction using RLV and virus produced by 58-2T cell (58-2T virus) as described previously.[2,3]

Preparation of Ki-SV Specific [^{3}H]DNA (58-2TS DNA).—[^{3}H]DNA made from 58-2TS virus was incubated with RLV 70S RNA (2 μg/ml) in 2 × SSC and 40% formamide at 45°C for 48 h. After hybridization, single-stranded [^{3}H]DNA (about 50% of input) was separated from hybridized DNA by differential batch elution on hydroxyapatite (8). More than 80% of the [^{3}H]DNA thus obtained hybridized with 58-2T cell RNA and NP cell RNA, while no significant hybridization was observed with RLV RNA and BALB/3T3 cell RNA.[2]

Hybridization.—For reassociation kinetic analysis, sample RNA and yeast RNA (Sigma Chemical Co., St. Louis, Mo.) were mixed to give a final concentration of 10 mg/ml. Mixtures were incubated with 500 cpm of [^{3}H]DNA in 2 × SSC and 40% formamide (10). 10-μl reaction mixtures in capillary tubes were incubated at 45°C for various times and the extent of hybridization then determined using the single-strand specific, S-1 nuclease (11). All other hybridizations were made in 100 μl volumes containing 250 or 500 cpm [^{3}H]DNA, RNA and 2 × SSC at 66°C for 20 h. The hybridization mixture was digested at 45°C for 2 h with S-1 nuclease and the radioactivity of trichloroacetic acid insoluble material after digestion was determined as described previously (12).

The percent of hybridization was plotted against the product of the initial concentration of sample RNA and time (Cr·t) as defined by Birnsteil et al. (13). Since the level of self-annealing of [^{3}H]DNA was less than a few percent above background even after long periods of incubation in the absence of sample RNA, data have been corrected only for the background levels obtained in samples without incubation. Maximum hybridization between [^{3}H]DNA and sample RNA containing homologous RNA was 70–80% of the corrected counts (input counts minus background counts), and comparative data were normalized taking this value as 100%.

RESULTS

Kinetics of Reassociation of Cellular RNA from Mutant Clones.—RLV DNA: Three mutant clones activated to produce virus by BrdU and two which re-

[3] Tsuchida, N., R. V. Gilden, M. Hatanaka, A. E. Freeman, and R. J. Huebner. Type C virus-specific nucleic acid sequences in cultured rat cells: evidence for transcriptional variation. *J. Natl. Cancer Inst.* Manuscript submitted for publication.

mained virus-negative (2, 3) were tested for virus-specific RNA. The Rauscher virus DNA transcript (RLV DNA) was found to be adequate for this purpose based on comparisons of cells actively producing RLV and the activated mutant cells (Fig. 1 A). RNA from the JLS-V9 (RLV) and the 58-2T cell lines

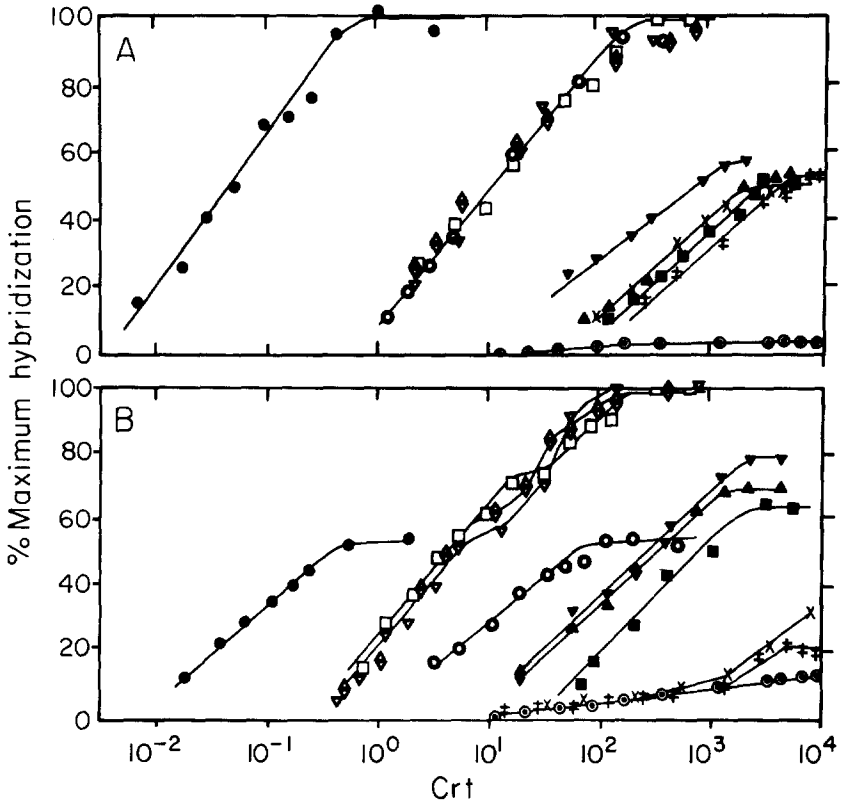

FIG. 1. (A) Hybridization of RLV[^{3}H]DNA product with various RNA's. [^{3}H]DNA was hybridized with the following RNA: RLV HMW RNA (●—●), 0.004 mg/ml; RLV producing JLS-V9 cell RNA (○—○), 0.7 mg/ml; 58-2T cell RNA (□—□), 1.26 mg/ml; M-57-1 cell RNA (▽—▽), 1.26 mg/ml; M-58-4 cell RNA (◇—◇), 1.26 mg/ml; NP cell RNA (■—■), 4.7 mg/ml; BALB/3T3 cell RNA (X—X), 6.4 mg/ml; M-50 cell RNA (▲—▲), 3.2 mg/ml; M-43-2 cell RNA (▼—▼), 3.2 mg/ml; MEF cell RNA (‡—‡), 5.6 mg/ml; and NRK cell RNA (⊙—⊙), 4.2 mg/ml. (B) Hybridization of 58-2T[^{3}H]DNA product with various RNA's. All symbols are as in Fig. 1 A.

hybridized with similar kinetics and protected the [^{3}H]DNA transcript to the same maximal extent. In both cases, the final percent hybridization of the viral DNA was the same as observed with homologous RLV 70S RNA. In addition to 58-2T, two other producing cell lines, M-58-4 and M-57-1, gave similar results.

The virus-negative cell lines all showed partial protection of the RLV transcript and required much higher concentrations of RNA to achieve hybridization. All showed similar kinetics with the exception of M-43-2, which contained about three times the viral-specific RNA of the other cells based on relative Cr·t values (13). This cell line was previously noted to have an unusually high modal chromosome number (135) compared to the parental NP cell and other mutants (3). Thus, while the RLV probe readily detected viral RNA in all mutant cells, there were no obvious differences between virus-negative mutant cells and normal BALB/c cells.

58-2T DNA: Kinetic analyses were also made with the DNA transcript (58-2T DNA) of the virus induced in 58-2T cells by BrdU. 58-2T DNA contains both helper-specific and Ki-SV-specific sequences.[2] Virus specific RNA was readily detected in the virus-negative mutant cells and NP cells (Fig. 1 B). The extent of hybridization and amount of virus-specific RNA detected were clearly distinguishable from BALB/c cells. In virus-producing M-58-4, M-57-1, and 58-2T cells the kinetic analysis seems to give evidence of at least a two-component system. This transcript hybridized with RLV RNA only to 50% of the maximum obtained with 58-2T cell RNA, consistent with previous results.[2]

Size of virus-specific RNA in mutant cells: The size of Ki-SV and helper RLV specific RNA sequences in mutant cells was characterized by sucrose gradient analyses. 58-2TS DNA and RLV transcripts were used to detect these RNA sequences. M-57-1, a virus-producing tumorigenic clone, contained 35S RNA reactive with the RLV transcript and 30S (also a large peak with a sedimentation coefficient of approximately 16S) RNA reactive with the 58-2TS transcript (Fig. 2 A). M50, a virus-negative tumorigenic clone contained both 30 and 16S RNA size classes capable of hybridizing to 58-2TS DNA, but no significant viral RNA was detected with RLV DNA in 35-10S region (Fig. 2 B). Thus, no obvious qualitative differences were noted between the producing and nonproducing tumorigenic clones as far as hybridization with 58-2TS DNA was concerned. M-43-2, a virus-negative, nontumorigenic clone also showed 30S RNA specific for 58-2TS transcript (no 16S peak); however, in contrast to other cell lines producing infectious virus, the RLV-reactive subunit sedimented at 26-27S.

DISCUSSION

A summary of virus-specific RNA synthesis in mutant and control cells (Table I) indicates a clear expected correlation between copy number of RLV-specific sequences and presence of overt virus production. Of interest is the observation that helper virus RNA can be detected at similar levels in both control and transformed NP cells. The presence of viral RNA in normal cells is now known for a variety of species (10, 14–16). The smaller than normal subunit found in M-43-2 cells might have resulted from a genuine viral mutation

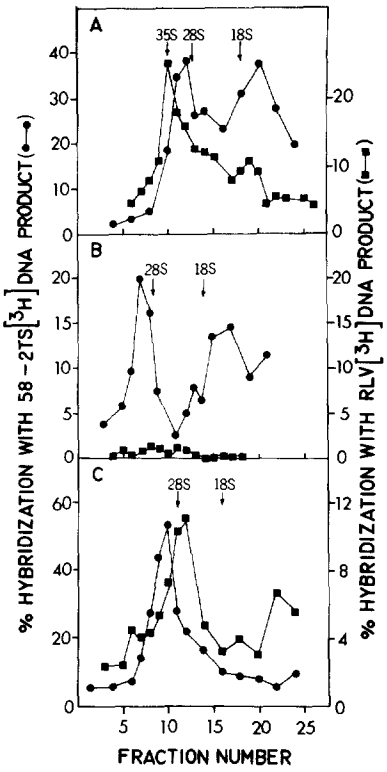

FIG. 2. Rate zonal sedimentation patterns of sarcoma and leukemia specific RNA in sucrose gradients. The cell RNA was isolated from: (A) M-57-1; (B) M-50, or (C) M-43-2, and layered on a 15–30% sucrose gradient in NTE buffer (0.01 M Tris HCl, pH 7.0, 0.1 M NaCl, 0.001 M EDTA) containing 0.5% SDS, and centrifuged at 20°C in a Spinco SW41 rotor for 6 h at 36,000 rpm. Fractions (0.5 ml) were collected from the bottom. Absorbancy was measured at 260 nm, and RNA was precipitated with 50 μg of carrier yeast tRNA and dissolved in 100 μl of 0.1 × SSC. 10 μl of M-57-1 cell RNA, 30 μl of M-50 cell RNA, and 40 μl of M-43-2 cell RNA in each fraction was hybridized with 250 cpm of 58-2TS[^{3}H]DNA product. 10 μl of M-57-1 cell RNA, 30 μl of M-50 cell RNA, and 40 μl of M-43-2 cell RNA in each fraction was hybridized with 500 cpm of RLV[^{3}H]DNA product. After hybridization, the extent of hybridization was measured using S-1 nuclease as described previously (12).

(deletion) induced by BrdU treatment. It is noteworthy that 30S Ki-SV-specific sequences were detected in all varieties of mutant cells, regardless of in vivo transplantability. Thus, transcription of these sequences is not sufficient to predict malignant behavior in vivo.

In previous studies we noted that the tumorigenic mutant M50 cell line did not yield sarcoma virus in repeated rescue attempts by direct superinfection (3). With the more sensitive fusion rescue technique, the M50 cell line did yield sarcoma virus, but at levels $\frac{1}{25}$ that of parental NP cells. However, sarcoma-specific RNA (30S size class) was readily detected, confirming the origin of M50 from the parental NP cell.

TABLE I

Summary of Virus-Specific RNA Synthesis in Mutant Cells

Cell line	Copy no./cell* RLV 70S RNA equivalent	Virus production‡	Size of major peak of:	
			Leukemia-specific RNA	Sarcoma-specific RNA
BALB/3T3	9×10^1	−	—§‖	—
NP	7×10^1	−	—§‖	30S‖
58-2T	2.5×10^3	+	35S‖	30S‖
M-57-1	2.5×10^3	+	35S	30S
M-58-4	2.5×10^3	+	ND¶	ND
M-50	7×10^1	−	—	30S
M-43-2	2.5×10^2	−	26–27S	30S
JLS-V9(RLV)	2.5×10^3	+	35S‖	—

* Calculated from $Cr \cdot t_{\frac{1}{2}}$ assuming: (*a*) that one molecule of viral 70S RNA contains 1.7×10^{-11} μg (mol wt 1×10^7) and that the cellular RNA content is 8×10^{-6} μg/cell, and (*b*) that the kinetics of hybrid formation are not affected by the change in viral RNA concentration as the reaction progresses (10).

‡ Reference 3.

§ Not detected at 10-35S region.

‖ See footnote 2.

¶ Not done.

SUMMARY

BALB/3T3 cells transformed by the Kirsten sarcoma virus (nonvirus producer BALB/3T3 cells) and mutant cell lines derived therefrom by treatment with bromodeoxyuridine (BrdU) were analyzed for expression of virus-specific RNA using single-stranded DNA transcripts of Rauscher leukemia virus (RLV), a virus activated in one of the cell lines (58-2T), and Ki-SV-specific DNA transcript; the latter transcript after removal of all sequences cross-reactive with RLV RNA. The Rauscher virus DNA detected multiple copies of viral RNA in virus-producing cells ($\sim 2.5 \times 10^3$/cell) whether infected with RLV or activated to produce virus with BrdU. Nonproducer (NP) cells and normal BALB cells showed small numbers of RNA genomes (70-250/cell) and only partial saturation of the transcript. The intracellular RNA sedimented at 35S (main peak) with a variable minor peak at 20S with the exception of one mutant cell, M-43-2 (main peak at 26-27S). The 58-2T transcript reacted preferentially in NP cells and their derivatives with biphasic kinetics suggesting the possibility of sequences specific for the original transforming virus. The size of Ki-SV specific sequences were 30S in mutant cells whether or not complete virus was being produced and independent of in vivo transplantability.

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