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In Vitro Translation of Harvey Murine Sarcoma Virus RNA

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The viral RNA of the Harvey strain of murine sarcoma virus (Ha-SV), which does not encode for any known viral structural polypeptides, has been translated in a nuclease-digested, cell-free system. The major protein product of the in vitro translation reaction has a molecular weight of 21,000 and is initiated faithfully with [^{35}S]formylmethionine from formyl-[^{35}S]methionyl-tRNA$_F^{MET}$. This polypeptide is clearly distinct from the products of reactons translated in parallel experiments from the RNA of the Moloney strain of type C helper virus used to pseudotype the Ha-SV. The intensity of the 21,000-dalton polypeptide on gels correlates well to the concentration of Ha-SV RNA in different viral RNA preparations. These experiments indicate that a polypeptide marker for Ha-SV is now available for the first time. The possibility that this protein is the product of the rat portion of the Ha-SV genome is discussed.

Harvey sarcoma virus (Ha-SV) was the first isolate of a mammalian type C virus with the ability to produce the rapid formation of sarcomas in inoculated mice (6). Since the isolation of Ha-SV was accomplished by passaging Moloney leukemia virus (Mo-MuLV) in a Chester Beatty rat, the initial stocks of virus were composed of two viruses, namely, Mo-MuLV and Ha-SV. Subsequently, the Ha-SV was shown to transform fibroblasts (FT$^+$) in vitro and was cloned free from Mo-MuLV in transformed mouse and rat nonproducer cells (15, 29). Thus, as in the case of all mammalian FT$^+$ viruses, Ha-SV was shown to be a replication-defective virus and to be responsible for the focus-inducing capacity of the viral complex isolated from rats. In nucleic acid hybridization studies, the Ha-SV genome was shown to be smaller than that of Mo-MuLV and to be comprised of two distinct sets of nucleic acid sequences in a recombinant form (16, 17, 29). One set of sequences derived from a portion of the parental Mo-MuLV, and a second set consists of rat genetic sequences (1, 24, 29, 30, 34). Subsequent experiments have suggested that the rat genetic information represents a novel class of rat type C viral information (28) and plays an important role in the acquisition of the ability of Ha-SV and Kirsten sarcoma virus (Ki-SV) to transform cells (25).

Up to now, no proteins coded for by Ha-SV have been identified as markers for Ha-SV-transformed nonproducer cells, for basically two reasons. First, the recombinational events that formed Ha-SV were associated with an 80 to 90% deletion of the Mo-MuLV genone, and the residual Mo-MuLV sequences did not encode for any known structural proteins of Mo-MuLV, as judged by the inability to detect cross-reacting polypeptides by radioimmunoassays in Ha-SV-transformed cells (21). Second, no known protein marker exists for the rat genetic information that comprises the majority of the Ha-SV genome (31).

In an attempt to identify protein(s) coded for by the Ha-SV genome, we have developed an in vitro translation system in mouse cells that is stimulated by Ha-SV RNA. A protein has been identified, faithfully initiated on Ha-SV RNA, that has a molecular weight of 21,000.

MATERIALS AND METHODS

Viruses and cells. The Mo-MuLV pseudotype of the Ha-SV grown in NIH 3T3 cells was described previously (17). This culture produces approximately $10^{6.0}$ focus-forming units of Ha-SV and $10^{6.0}$ XC PFU of Mo-MuLV. Moloney helper virus, Mo-MuLV, was prepared from the parental NIH cells without the Ha-SV. Cells producing these viruses were maintained in plastic roller bottles (Corning) with 10% calf serum (Colorado Serum Co.) in Dulbecco modified Eagle medium and grown at 37°C.

Preparation of S10. Actively growing NIH 3T3 cells producing Mo-MuLV were rinsed three times with cold (4°C) HEPES (*N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid)-buffered saline (0.14 M NaCl 0.05 M HEPES [pH 7.8]) and then removed from the plastic rollers by scraping with a rubber policeman into the same buffer. After three washes in the HEPES-buffered saline, cells were collected by centrifugation at 500 × *g* and resuspended in buffer A (0.06 M KCl-0.003 M magnesium acetate-0.02 M HEPES [pH 7.8]-0.006 M β-mercaptoethanol).

After 10 strokes with loose- and tight-fitting glass Dounce homogenizers, respectively, the resulting extract was centrifuged at 10,000 × *g* for 15 min in a JA21 rotor (Beckman) at 4°C. The upper 80% of the resulting supernatant (S10) was dispensed in 0.3-ml amounts and immediately frozen at − 170°C. No dialysis step or Sephadex purification was used in preparation of the S10.

Protein synthesis conditions. Each reaction mixture was incubated at 30 to 32°C for 2 h and contained in 0.025 ml: 0.02 M HEPES (pH 7.8); 0.075 M KCl; 2×10^{-3} M magnesium acetate; 2×10^{-3} M dithiothreitol; 8×10^{-4} M ATP; 8×10^{-5} M GTP; 8 mM creatine phosphate; 4 μg of creatine phosphokinase (rabbit muscle; Sigma Chemical Co.); 1.2×10^{-5} M [^{35}S]methionine (680 Ci/mmol; Amersham/Searle); 1×10^{-4} M 19 other nonradioactive amino acids; 0.8 A_{260} (absorbance at 260 nm) units of S10; and 0.02 A_{260} units of viral RNA. After incubation at 30°C for specified times, a 0.005-ml portion was removed, treated with 0.1 N KOH for 10 min at 37°C, and then precipitated with 10% trichloroacetic acid. Pancreatic RNA (5 μg) was added to the remainder of the reaction mixture and incubated for 30 min at 37°C prior to preparation for gel electrophoresis.

Micrococcal nuclease digestion of S10 extracts was performed as described by Pelham and Jackson (23) with 10 μg of micrococcal nuclease (Boehringer-Mannheim) per ml, except that the nuclease digestion was performed at 25°C for 15 min at 4 mM $CaCl_2$; EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid] was added to a final concentration of 8 mM to terminate the nuclease reaction. The final molarity of EGTA added with the nuclease-treated S10 to the actual in vitro synthesis reactions was 3.2 mM.

Viral RNA purification. High-molecular-weight viral RNA was purified from virions disrupted with 1% sodium dodecyl sulfate (SDS) and digested with Pronase as described in the legend to Fig. 2. Viral subunits were isolated from various portions of the 55 to 70*S* peak (see below) after heating high-molecular-weight RNA at 80°C for 2 min. Centrifugation of heated RNA was in 15 to 30% sucrose gradients containing 0.01 M NaCl–0.01 M Tris-hydrochloride (pH 7.5) in an SW27 rotor (Beckman) at 24,000 rpm for 14 to 16 h. Gradient fractions were collected from below and pooled by comparison with 4, 18, and 28*S* [^{3}H]uridine cellular RNA markers in parallel gradients. Samples were precipitated with ethanol, collected by centrifugation at 104,000 × *g* for 30 min, resuspended in sterile distilled water, and stored at −170°C. Encephalomyocarditis virus RNA was kindly supplied by Robert Thach, Washington University, St. Louis, Mo. Rabbit hemoglobin mRNA was purchased from Searle Diagnostics, Amersham, England.

Preparation of ^{3}H-labeled cDNA's. The complementary DNA (cDNA) used to detect the RNA of Mo-MvLV was fraction 3 of Mo-MuLV as previously reported (21). This fraction represents the sequences in Mo-MuLV that are deleted from either S+L − Mo-SV or Ha-SV and thus could be used to detect specifically Mo-MuLV in the presence of Ha-SV. The cDNA used to detect Ha-SV represents the rat genetic sequences in common among Ki-SV, Ha-SV, and the endogenous rat genetic sequences (27, 30, 31). The Ha-SV-specific probe hybridized only 3.5% to Mo-MuLV RNA as opposed to 100% with Ki-SV, Ha-SV, or endogenous rat sarcoma virus RNA. The specific activity of each probe, labeled with [^{3}H]dCTP, was 2×10^{7} cpm/μg.

Hybridization. Each hybridization reaction mixture was incubated at 66°C and contained in 0.05 ml: 0.6 M sodium chloride, 0.02 M Tris-hydrochloride (pH 7.2), 5×10^{-4} M EDTA, 0.10% SDS, 1 μg of calf thymus DNA as carrier, and the indicated RNA. Analysis was performed with the use of S1 nuclease as previously described (2, 14). Hybridization kinetics were expressed as C_rt (moles · second per liter) as suggested by Birnstiel et al. (3).

Polyacrylamide slab gel electrophoresis. SDS slab gels were prepared by the method of Laemmli (13) and run at 4 mA. Samples were prepared for autoradiography by the method of Villa-Komaroff et al. (35). Gels were washed, dried, and subjected to autoradiography by using Kodak Royal Blue X-Omat film as described previously (4). Molecular weight markers were the *Escherichia coli* RNA polymerase B′ subunits (Boehringer Mannheim Corp.) (155,000), bovine serum albumin (68,000), ovalalbumin (43,000), Moloney viral p30 (30,000), and hemoglobin (15,500). Each marker was iodinated with ^{125}I by the procedure of Hunter and Greenwood (10) as described previously (22) and located on the gels in the autoradiograph procedure.

Preparation of F-[^{35}S]methionyl-tRNA. Rabbit liver tRNA was purchased from Grand Island Biological Co. Total tRNA was absorbed to benzoylated DEAE-cellulose, and $tRNA_F^{MET}$ was separated from $tRNA_M^{MET}$ by a sodium chloride gradient as described by Kerwar et al. (12). A preparation of aminoacyl synthetases and formylase from *E. coli* MRE600 was prepared by the procedure of Muench and Berg (20). Formylated [^{35}S]methionine-labeled tRNA was prepared as described (26). Each reaction was incubated at 30°C for 30 min and contained (per milliliter): Leucovorin (Lederle Laboratories), 0.24 mg; $tRNA_F^{MET}$, 0.80 A_{260} unit; enzyme protein, 1.0 mg; potassium cacodylate (pH 6.9), 0.1 M; ATP, 1×10^{-3} M; magnesium chloride, 0.01 M; 2-mercaptoethanol, 6×10^{-3} M; [^{35}S]methionine, 1.6×10^{-5} M; and the other 19 nonradioactive amino acids, 2×10^{-5} M. The specific activity of the formyl-[^{35}S]methionyl-$tRNA_F^{MET}$ was 4×10^{6} cpm/μg, and the $tRNA_F^{MET}$ was 7.5 to 10.0% pure, based on its methionine acceptor capacity. The formylation of the methionyl-$tRNA_F^{MET}$ was greater than 98% as judged by migration of the deacylated product on cellulose thin-layer plates at pH 3.5.

RESULTS

Analytical separation of Mo-MuLV and Ha-SV RNA. Since mammalian sarcoma viruses required a helper virus to productively infect cells, stocks of sarcoma virus and, therefore, sarcoma virus RNA are generally appreciably contaminated with helper viral RNA. To be able to prepare quantities of Ha-SV RNA suffi-

cient for translation, a one-step procedure was developed to enrich for Ha-SV RNA from a mixture of Ha-SV and Mo-MuLV RNAs. When 55 to 70S virion RNA from cultures releasing Mo-MuLV and Ha-SV had been examined previously heterogeneity of the high-molecular-weight RNA was noted as compared with Mo-MuLV high-molecular-weight RNA alone (17). Therefore, we prepared high-molecular-weight RNA from the Mo-MuLV/Ha-SV culture and analyzed the high-molecular-weight complex for Mo-MuLV-specific sequences and Ha-SV-specific sequences. As described in the legend to Fig. 1, cDNA's were used that were specific for Mo-MuLV or Ha-SV. Two distinct peaks of hybridization were noted; the faster-sedimenting peak hybridized to the Mo-MuLV-specific cDNA, and the slower-sedimenting peak hybridized to the Ha-SV-specific cDNA. Although overlap between the two peaks is apparent, appreciable separation can be achieved. Thus, by taking fractions from the slowest-sedimenting 20 to 30% of the high-molecular-weight RNA, a marked enrichment for Ha-SV is possible. These results are consistent with earlier work that indicated that heterodimers apparently do not form between helper virus RNA and the defective sarcoma virus RNA (5).

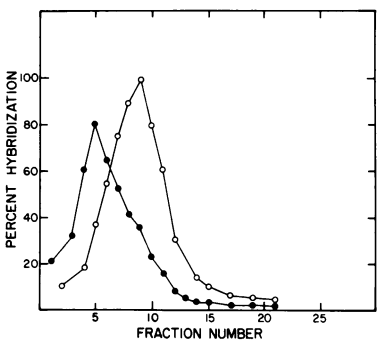

FIG. 1. *Separation of high-molecular-weight RNA of Mo-MuLV and Ha-SV. Six 75-cm² flasks of NIH 3T3 cells infected with Mo-MuLV and Ha-SV were fed with 10 ml of medium. After 14 h the medium was collected, clarified at 3,000 rpm for 10 min at 4°C, and pelleted for 3.0 h at 100,000 × g through a 5.0-ml cushion of 20% sucrose in a type 30 rotor (Beckman) at 4°C. The pelleted virus was suspended in 1.0 ml of buffer containing 0.1% diethyl-pyrocarbonate, 1.0% SDS, and TNE (0.1 M NaCl-10⁻³ M EDTA-0.02 M Tris-hydrochloride [pH 7.5]) and layered onto a 15 to 30% sucrose gradient in TNE. After centrifugation for 3.0 h at 39,000 rpm in an SW41 rotor (Beckman) at 16°C, fractions of approximately 0.5 ml were collected by puncturing from below. Each fraction was concentrated fivefold by ethanol precipitation and resuspended in 0.02 M Tris-hydrochloride (pH 7.5). A 0.005-ml portion of the indicated fractions was hybridized as described in the text for 24 h with approximately 3,000 cpm of each [³H]cDNA. The source of cDNA probes is given in the text. One hundred percent hybridization was approximately 2,500 cpm for each probe. Symbols: ○, [³H]cDNA from rat sequences of Ha-SV; ●, [³H]cDNA from S+L− minus fraction of Mo-MuLV.*

Preparative scale isolation of viral RNA. To prepare adequate quantities of viral RNA for cell-free translation studies, larger quantitities of freshly harvested, purified virus were then used. The 55 to 70S RNA from a typical preparation of approximately 7 liters of supernatant fluid from the culture releasing Mo-MuLV and Ha-SV was prepared as described in the legend to Fig. 2. The absorbancy profile of this RNA is shown in Fig. 2. A broad peak of absorbancy, with a peak at fractions 13 to 15 and a second peak at fractions 18 to 20, was observed. As will be shown subsequently, the higher-molecular-weight peak (fractions 6 to 12) represented helper Mo-MuLV, and the lower-molecular-weight peak (fractions 16 to 20) was predominantly the defective Ha-SV RNA component, which is consistent with the results of the analytic gradient (Fig. 1). Furthermore, an additional enrichment for the Ha-SV RNA could be achieved by starting with the slightly lighter fractions from the sucrose gradient banding of the viruses. It was noted that the Ha-SV RNA-containing particles banded at a slightly lower buoyant density in sucrose (1.155 g/cm) than did the Mo-MuLV (1.16 g/cm). Although the reasons for this buoyant density separation are not yet apparent to us, the combination of selecting slightly lower-buoyant-density virus fractions and the slowest-sedimenting portions of the high-molecular-weight RNA allowed a significant enrichment for Ha-SV RNA.

In vitro conditions for viral RNA translation. RNA from 55 to 70S preparative gradients was concentrated by ethanol precipitation and employed as RNA in a mammalian cell-free, protein-synthesizing system. The addition of this RNA to a nuclease-digested S10 extract from NIH 3T3 cells producing Mo-MuLV virus markedly stimulated incorporation of [³⁵S]-methionine into protein. Optimal conditions for radioactive incorporation were determined, and representative optima are shown in Fig. 3. Figure 3A shows a broad potassium chloride optimum between 60 and 90 mM; however, at lower or higher KCl concentration, incorporation diminished sharply. In contrast to the rather broad KCl curve, the optimal concentration of magnesium acetate has a narrow range, with an optimum of 2 mM (Fig. 3B). Added calf liver tRNA stimulated incorporation 50 to 100% in the presence of added mRNA (Fig. 3C); apparently some loss of tRNA may occur

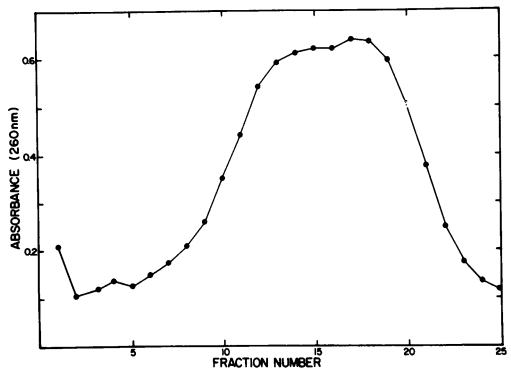

FIG. 2. *High-molecular-weight RNA profile from virions released from Mo-MuLV/Ha-SV cultures. Seven liters of supernatant fluid from confluent NIH 3T3 cell cultures releasing the Moloney pseudotype of Ha-SV were collected 16 h after the addition of 100 ml/plastic roller bottle (Corning). Virus-containing fluid was rapidly chilled at 4°C and clarified in a J-CF rotor (Beckman, Fullerton, Calif.) by centrifugation at 12,000 rpm at a flow rate of 10 to 12 liters/h in a J21 centrifuge. Clarified supernatant fluid was then centrifuged in a CF-32 (Beckman) continuous-flow rotor by using a linear 15 to 60% sucrose density gradient in 0.05 M Tris-hydrochloride (pH 7.6). Fractions were monitored by absorbancy at 280 nm, and six individual fractions (density range, 1.10 to 1.19 g/cm³) were concentrated about 1,000-fold after a 2- to 3-fold dilution with 0.05 M Tris-hydrochloride (pH 7.8) and centrifugation in a type 35 rotor (Beckman) at 142,800 × g for 60 min. Without freezing, fractions with the highest virus concentration (density, 1.15 to 1.18 g/cm³) were made 1% in SDS and incubated with self-digested Pronase (Sigma Chemical Co., St. Louis, Mo.) (750 μg/ml) for 30 min at 37°C. This mixture was then applied to a 15 to 30% linear sucrose gradient in TNE in an SW27 rotor and centrifuged for 16 h at 14,000 rpm and 24°C. Tubes were punctured from below, 35-drop fractions were collected for fractions 1 to 30 and 70-drop fractions were collected thereafter, and absorbancy at 260 nm was determined. All materials were autoclaved or rinsed with 0.1% diethyl-pyrocarbonate before use.*

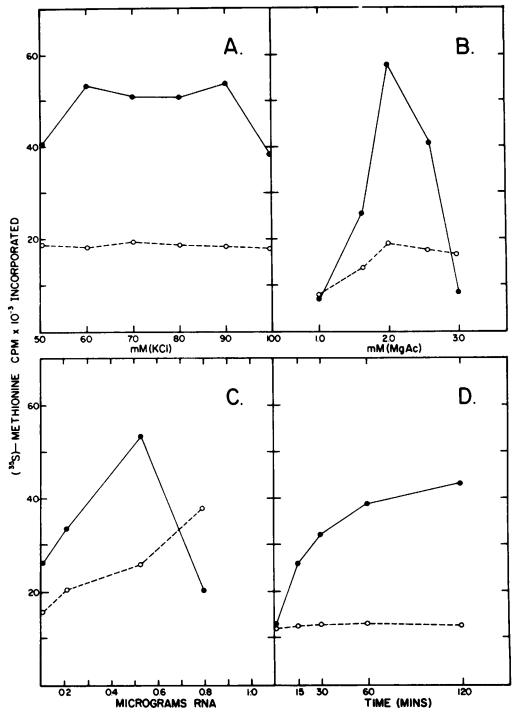

FIG. 3. *Conditions for protein synthesis. Reactions were performed as indicated in the text. Symbols: ●, plus mRNA; ○, no added mRNA. (A) Potassium chloride dependence; (B) magnesium acetate dependence; (C) tRNA dependence: (●) plus tRNA plus mRNA, (○) no added tRNA plus mRNA; (D) kinetics of incorporation.*

with our nuclease digestion conditions. As shown in Fig. 3D, the digested extract without added message shows a very low background that does not increase with time; in the presence of added Mo-MuLV and Ha-SV RNA, incorporation increases with time for at least 2 h. Thus, when a nuclease-digested S10, programmed with Mo-MuLV and Ha-SV RNA, was used, clearcut stimulation of [^{35}S]methionine incorporation over background was noted.

Autoradiographic analysis of in vitro products. After treating the extracts with RNase to degrade acylated tRNA, radioactivity incorporated in the in vitro reactions described above was precipitated in acetone, dissolved in SDS, and analyzed by polyacrylamide slab gel electrophoresis. The results of a representative 7.5% acrylamide slab are shown in Fig. 4. The nuclease digestion procedure of Pelham and Jackson (23) largely eliminates background incorporation, and only very faint bands with approximate molecular weights of 43,000 and 98,000 were noted in in vitro reactions containing no added RNA (Fig. 4, column 1). When the in vitro synthesis system was programmed with Mo-MuLV RNA sedimenting at either 60 to 70S or 55S (Fig. 4, columns 2 and 3), a predominant polypeptide with a molecular weight of 65,000 was noted. However, additional polypeptides at much lower concentrations were also noted in Mo-MuLV RNA primed reactions. Notably high-molecular-weight polypeptides occasionally existed as doublets at 140,000 and 82,000 and a polypeptide at 70,000. Low-molecular-weight polypeptides also were noted with the Mo-MuLV by itself and were approximately 57,000, 38,000, and 25,000. The predominant band at 65,000 to 70,000 and fainter bands of higher molecular weight have been noted previously by Kerr et al. with Mo-MuLV

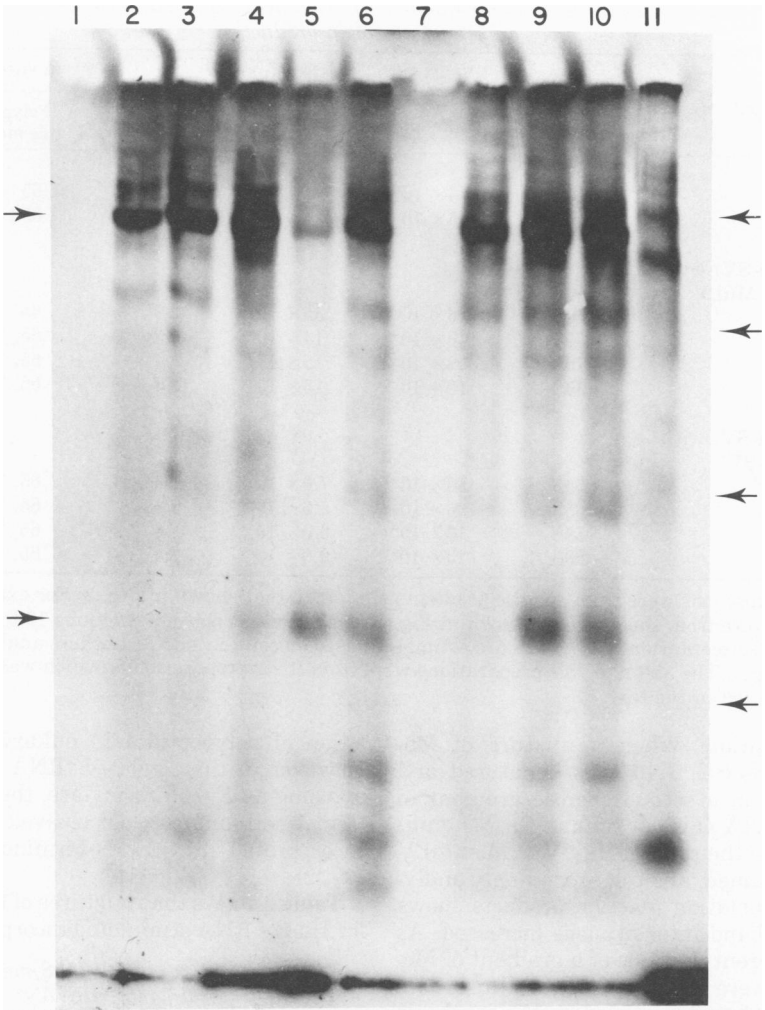

FIG. 4. *Autoradiographic analysis of slab gel electrophoresis of products of cell-free translation stimulated with different RNAs. Conditions for reactions are described in the text. Migration is from top to bottom. (1) No added RNA to nuclease-digested S10; (2) Mo-MuLV 70S RNA (0.65 μg)-programmed reaction products (Table 1 RNA preparation no. 1); (3) Mo-MuLV 55S RNA (0.8 μg) reaction products (Table 1 RNA preparation no. 2); (4) Mo-MuLV pseudotype of Ha-SV 70S peak (Table 1 RNA preparation no. 4); (5) same as 4, 55S peak (Table 1 RNA preparation no. 7 [0.5 μg]); (6) Table 1 RNA preparation no. 8 (1.0 μg); (7) blank; (8) Table 1 RNA preparation no. 6 (0.5 μg); (9) Table 1 RNA preparation no. 10 (0.5 μg) 28S RNA; (10) 20S (0.5 μg) from same gradient yielding 28 and 35S RNA peaks; (11) EMC RNA (0.43 μg)-programmed reaction products. Molecular weight markers indicated on right-hand margin are bovine serum albumin (68,000), ovalalbumin (43,000), Moloney p30, (30,000), and hemoglobin (15,500). The arrows on the left-hand margin indicate the p65 product of Mo-MuLV and the p21 of Ha-SV.*

RNA in an in vitro system and identified as precursors to various structural proteins of Mo-MuLV by immunoprecipitation and tryptic digest analysis (11). We have confirmed their identification in results to be published elsewhere.

Using RNA enriched for Ha-SV sequences, polypeptide bands noted in response to pure Mo-MuLV RNA (columns 4 to 6) diminished in intensity, and an additional polypeptide band with a molecular weight of 21,000 (p21) was noted (Fig. 4, columns 5 and 6). Several preparations of RNA were tested for their ability to direct the synthesis of this 21,000-molecular-weight (Table 1). Each preparation containing Ha-SV RNA that represented slower-sedimenting viral RNA, as previously discussed, directed the synthesis of the p21. Furthermore, the intensity of the p21 was in direct proportion to the concentration of the Ha-SV RNA in a

TABLE 1. *Correlation of ratios of Ha-SV and Mo-MuLV nucleic acid sequences in various RNA preparations and in vitro protein product*

RNA prepn[a]	Size[a]	½ C_rt with [³H]cDNA from:		Fold excess	In vitro protein product	
		S+L− minus	Sarc		Polypeptide mol wt	Relative amt of p21
Mo-MuLV						
1	60–70S	5×10^{-2}			65	...
2	55S	5×10^{-2}			65	...
Mo-MuLV (Ha-SV) with excess Mo-MuLV						
3	60–70S	7.6×10^{-2}	35×10^{-2}	4.8	65	...
4	60–70S	9.0×10^{-2}	14×10^{-2}	1.5	65, 21	+
5	60–70S	7.2×10^{-2}	15×10^{-2}	2.0	65, 21	+
6	35S	10×10^{-2}	15×10^{-2}	1.5	65, 21	+
Mo-MuLV (Ha-SV) with excess Ha-SV						
7	55S	50×10^{-2}	7.0×10^{-2}	7.0	65, 21	+++
8	55S	36×10^{-2}	5.6×10^{-2}	6.4	65, 21	+++
9	55S	10×10^{-2}	6.0×10^{-2}	1.6	65, 21	++
10	28S	13×10^{-2}	9.0×10^{-2}	1.4	65, 21	++

[a] RNA preparations were from sucrose gradients similar to that shown in Fig. 2. For example, preparations 8 and 4 were from the gradient shown in Fig. 2 and represent pools of fractions 16 to 20 and 6 to 12, respectively. Sedimentation coefficients are estimates based on cellular rRNA markers analyzed in parallel sucrose gradients. The 35S and 28S preparations were from RNA preparation 5, which was denatured and respun as detailed in the text.

given preparation. When a mixture of Mo-MuLV and Ha-SV RNA was denatured and resedimented in a second sucrose gradient to obtain 28S RNA (Fig. 4, column 9), a significant change in the ratio of Ha-SV to Mo-MuLV RNA was obtained, and correspondingly analysis of the translation reaction products shows that the p21 band intensity was increased. As controls, different portions of a gradient of Mo-MuLV RNA were tested in in vitro translation, and none was found to stimulate the synthesis of the p21 band (Fig. 4, columns 2 and 3). Furthermore, in studies to be presented elsewhere (T. Shih and E. Scolnick, manuscript in preparation), oligonucleotide fingerprints of ^{32}P-labeled Ha-SV RNA from sucrose gradients similar to those shown in Fig. 1 were performed. Complexity analysis indicated that the Ha-SV RNA was not detectably contaminated with any other species of RNA besides the Mo-MuLV RNA indicated in Fig. 1. The results thus indicate that the p21 protein is a translational product of Ha-SV RNA.

Labeling of Ha-SV product with formylmethionine. As a means for establishing that the RNA-dependent in vitro synthesis was faithfully initiating the p21 protein on the added Ha-SV RNA, in vitro incubation was performed with formyl-[^{35}S]methionyl-$tRNA_F^{MET}$. Normally, methionine is incorporated from $tRNA_F^{MET}$, but cleaved from the N-terminal position of polypeptides in eukaryotic systems. However, if this acylated tRNA is formylated by using an *E. coli* formylase, the incorporated formylmethionine is not cleaved and is stably incorporated into the N-terminal position (7, 19, 32).

Table 2 shows that mixtures of RNA enriched for Ha-SV RNA stimulated incorporation of for-

TABLE 2. *Incorporation of [^{35}S]methionine with different mRNA's*[a]

RNA added	Substrate Used (trichloroacetic acid cpm)	
	formyl-[^{35}S]methionyl-$tRNA_F^{MET}$	[^{35}S]methionine
None	1,355	5,885
Hemoglobin	32,220	50,495
EMC virus	17,645	131,935
Mo-MuLV (Ha-SV)	4,418	24,840

[a] Reaction mixtures (0.025 ml) were essentially as described in the text and the legend to Fig. 3, except 1×10^6 cpm of formyl-[^{35}S]methionyl-$tRNA_F^{MET}$ and 5×10^{-6} M nonradioactive methionine replaced the [^{35}S]methionine where indicated. The amounts of added RNA were 0.5, 0.53, and 0.48 μg for hemoglobin, EMC, and Mo-MuLV (Ha-SV) (preparation no. 10), respectively, and reactions were incubated for 2 h at 30°C. [^{35}S]methionine incorporation represents a 0.005-ml portion, and formylmethionine incorporation represents the entire 0.025 ml.

myl-methionine to significant levels. Hemoglobin and encephalomyocarditis (EMC) RNA stimulated more incorporation of formylmethionine than comparable amounts of Ha-SV RNA. The levels of formylmethionine incorporation correlated with the levels of free [S^{35}]methionine incorporation and presumably reflected more rounds of initiation with the hemoglobin and EMC RNAs. The products of the reactions with the Ha-SV and EMC RNAs were analyzed on gels (Fig. 5). The EMC RNA-primed reaction product demonstrates a predominant band at a molecular weight of 7,800 (9). Globin mRNA directs the synthesis of a band at a molecular weight of 15,500 (not shown). The enriched Ha-SV RNA stimulated incorporation of formyl-[^{35}S]methionine into two main polypeptide bands, a p65 and p21 corresponding to the two major bands from the Mo-MuLV RNA and the Ha-SV RNA. Too little label was incorporated to comment on the higher-molecular-weight bands for Mo-MuLV RNA. Thus, the in vitro protein synthesis system initiates properly with the various RNAs tested and indicates that the Ha-SV encodes for at least a 21,000-dalton protein that is faithfully initiated.

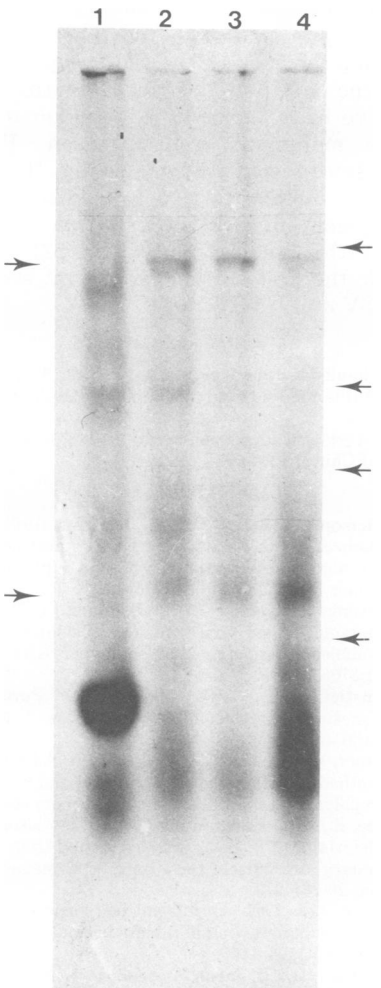

FIG. 5. *Autoradiographic analysis of formylmethionine-labeled reaction mixture products. Gels were electrophoresed as detailed in the text using ^{35}S products labeled with formylmethionyl-tRNA$_F^{MET}$. Reaction mixtures added to different lanes were: (1) EMC RNA (0.43 μg); (2 to 4) Table 1 RNA preparations 4 (0.5 μg), 7 (1.0 μg), and 10 (0.5 μg), respectively. All Ha-SV preparations incorporated formyl-[^{35}S]methionine to levels comparable to that shown in Table 2. Films were exposed for 96 h. Molecular weight markers and arrows are as described in the legend to Fig. 4.*

DISCUSSION

From avian and mammalian hosts, several type C viruses have been isolated that have the ability to induce malignant transformation of fibroblasts in cell culture. Without exception, such viruses have been shown to be recombinants between type C viral information present in various strains of replicating helper viruses and additional sarcoma virus-specific information designated "sarc." Genetic and molecular data (18, 27, 33, 36, 37) have suggested that these sarc sequences play an important role in the ability of the various FT$^+$ viruses to transform cells and probably code for a protein responsible for the viral-mediated cellular transformation.

Two strains of such FT$^+$ viruses, isolated from rodent species Ki-SV and Ha-SV, have been of particular interest to us because the genomes of these particular viruses contain a relatively small proportion of helper sequences as compared with the sarcoma virus-specific sequences as compared with other strains of avian or mammalian sarcoma viruses that have been reported (27). Second, consistent with hybridization data, immunological studies have failed to detect any murine type C structural viral proteins in cells transformed by Ki-SV or Ha-SV (21). Thus, in an attempt to identify protein(s) coded for by these viruses, a method has been developed for isolating in a one-step manner with high yield, large quantities of the RNA of one of these particular strains of sarcoma viruses free from helper virus RNA.

The purified Ha-SV RNA has been used to program an in vitro protein-synthesizing system rendered highly dependent on added mRNA by micrococcal nuclease treatment as

suggested by Pelham and Jackson (23). The use of the nuclease-digested S10 was essential since no protein markers or sera exist that would have allowed the detection of proteins translated from Ha-SV RNA in the absence of the very low endogenous protein synthesis background. Thus, by utilizing this low background system, the Ha-SV RNA has been used to direct the synthesis of a protein that has a molecular weight of 21,000 and is initiated properly from Ha-SV RNA since it incorporates formylmethionine into stable linkage from the substrate formyl-methionyl-$tRNA_F^{MET}$. Thus, the protein represents a faithful translational product of at least the N-terminal portion of a protein coded for by the Ha-SV genome and thus should be a useful marker for the first time for Ha-SV-transformed nonproducer cells.

In the absence of any bona fide protein markers for the Ha-SV genome, it is not possible to determine whether this protein is the completed size or only the N-terminal portion of a bona fide Ha-SV coded for protein. In this regard, our in vitro system directed the synthesis of several high-molecular-weight proteins from both Mo-MuLV RNA and EMC viral RNA. The p70 and p140 proteins synthesized from Mo-MuLV RNA have also been noted by others (11) using in vitro translation systems and shown to be authentic precursors of viral structural proteins. Furthermore, in studies not shown, only p21 was detected in Ha-SV RNA-directed reactions, even when KCl shifts in an attempt to prevent premature termination were performed (9) or when shorter incubation times were used in an attempt to reduce cleavage events. Thus, it seems unlikely that the p21 from Ha-SV RNA represents a prematurely terminated product of a putatively larger polypeptide, although these results do not exclude this possibility. In other studies (Scolnick and Parks, unpublished data), the RNA of the Kirsten sarcoma virus propagated in mink cells with a feline type C helper virus (28, 31) has also directed the synthesis of a p21 protein in this in vitro system. Since the Ki-SV RNA used for translation has a 20-fold excess of Ki-SV over FeLV RNA, it appears highly likely that the p21 is translated from the Ki-SV and Ha-SV genomes rather than from the helper virus used to pseudotype either sarcoma virus.

The relatively small size of the product of the in vitro translation reaction also must be reconciled with the molecular weight of Ha-SV, which is approximately 1.8×10^6 (16, 17). The genome is composed of approximately 65% rat sequences and 35% Mo-MuLV sequences (17), and the potential coding capacity of either sequence is substantially greater than that necessary to code for the p21 protein. Presumably, the translation starts from the 5′ end of the Ha-SV RNA, and it is possible that additional proteins are coded for by Ha-SV from other internal initiation sites. Alternatively, a large part of the sarcoma virus genome could be composed of redundant sequences, and the actual coding capacity might be significantly less. Nevertheless, despite the many unanswered questions, since no protein marker has been heretofore available for Ha-SV, the p21 polypeptide provides the first protein marker for this type of defective RNA tumor virus with the ability to induce malignant transformation. Furthermore, since recent studies (Young, Howk, and Scolnick, unpublished data) indicate that the rat sequences of Ki-SV occur within 100 nucleotides of the 5′-end of Ki-SV, the p21 seems to provide the first marker for the rat sequences of Ki-SV and Ha-SV.

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