RELEASE OF THE INHIBITION OF MESSENGER RNA TRANSLATION IN EXTRACTS OF INTERFERON-TREATED EHRLICH ASCITES TUMOR CELLS BY ADDED TRANSFER RNA

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SUMMARY. In an extract of Ehrlich ascites tumor (EAT) cells which had been "pre-incubated" for 45 min to lower endogenous protein synthesis ($S30_C$) the translation of exogenous encephalomyocarditis (EMC) viral mRNA proceeds at a constant rate for over 90 min. In a similarly treated extract of interferon-treated EAT cells ($S30_{INT}$) the translation proceeds at a lower rate than in the $S30_C$ for about 30 min and then stops. The impairment of the translation in the $S30_{INT}$ is mediated by one or more inhibitors. After the cessation of translation the viral mRNA in the $S30_{INT}$ is in large polysomes. The size of these changes little (if any) during a further 15 min incubation. The addition of mouse tRNA (but not ribosomal RNA or *E. coli* tRNA) to the $S30_{INT}$ after the cessation of viral mRNA translation results in the restart of translation at a rate close to that in the $S30_C$. This effect of tRNA is diminished by pactamycin, which inhibits peptide chain initiation but not elongation. These results indicate that addition of tRNA allows the elongation of incomplete peptide chains and the initiation of new chains. The need for added tRNA may be due to the fact that in $S30_{INT}$ the amino acid acceptance of some of the endogenous tRNA species (but not of added tRNAs) is impaired. This impairment is pronounced for leucine and very slight, if any, for five other amino acids tested (i.e. isoleucine, methionine, phenylalanine, threonine, and valine).

Interferons are macromolecules, probably glycoproteins, which are induced in a variety of vertebrate cells upon viral infection or some other stimuli. They are excreted from the producing cells, interact with other cells and make these inefficient in supporting the replication of a broad range of viruses (1).

The translation of added viral or cellular mRNA is impaired in $S30_{INT}$ (2-4). This impairment is caused by one or more inhibitors which are loosely bound to ribosomes (3,4). We reported earlier that the impairment can be partially overcome by tRNA added to the $S30_{INT}$ (4). In this communication we present further data on this effect of tRNA and on other characteristics of the interferon-mediated impairment of translation.

Materials and Methods

The sp. act. of the mouse interferon preparation used was 2×10^7 NIH mouse reference standard units/mg protein. This corresponds to 8×10^6 vesicular stomatitis virus (VSV) plaque reduction units/mg protein. The units throughout this paper are VSV plaque reduction units (5). All S30 extracts throughout this paper were prepared from either EAT cells treated with 60 units/ml of interferon for 18 hours ($S30_{INT}$) or untreated cells ($S30_C$) and were preincubated for 45 min to lower endogenous protein synthesis. For details of these and other preparations (including EMC RNA, mouse tRNA from L 929 fibroblasts, see (4)).

The reaction mixtures in which EMC RNA was translated included 25 mM Tris-Cl (pH 7.5), 120 mM KCl, 6 mM 2-mercaptoethanol, 5 mM phosphoenolpyruvate, 1 mM ATP, 0.6 mM

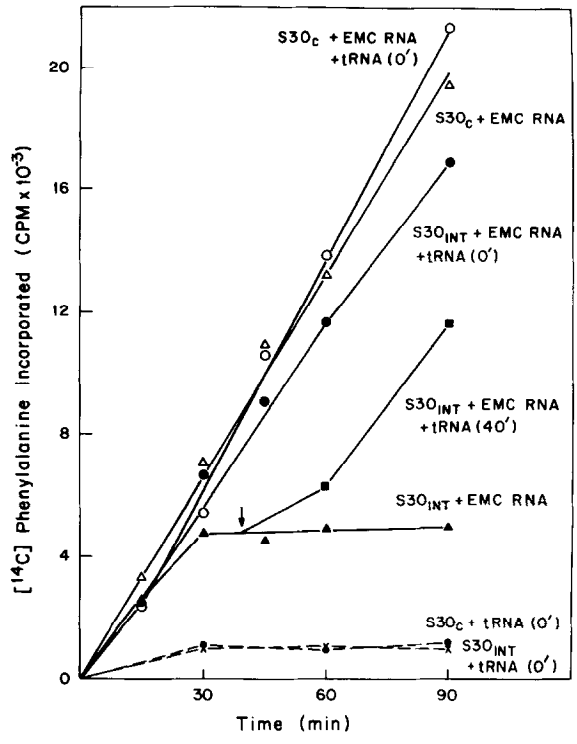

Fig. 1. Kinetics of EMC RNA translation in $S30_C$ and $S30_{INT}$. Release of the inhibition of translation in $S30_{INT}$ by added tRNA. The reaction mixtures (30 µl) included 5 mM $Mg(oAc)_2$ and either $S30_C$ (0.60 A_{260} units) or $S30_{INT}$(0.65 A_{260} units), 7.25 mM [^{14}C] phenylalanine (sp. act. 460 mCi/mmole), and if so indicated EMC RNA (1.4 µg) and mouse tRNA (7.5 µg, added at the times shown i.e. 0 or 40 min after the beginning of the incubation at 30°). The reactions were stopped at the times indicated and the amount of [^{14}C] phenylalanine incorporated into hot trichloroacetic acid-insoluble material was determined.

CTP, 0.2 mM GTP, 19 amino acids (except the one used radioactively labeled) 50 µM each, 2 mg/ml creatine phosphate, 0.3 mg/ml creatine phosphokinase. Further components of the reaction mixtures are listed in the figure legends.

Results and discussion

Characteristics of the translation of EMC RNA in $S30_C$ and $S30_{INT}$

In $S30_C$ the translation of added EMC RNA proceeds at a constant rate for 90 min or longer (curve $S30_C$ + EMC RNA in Fig. 1). In $S30_{INT}$ the same process takes place at a lower rate for about 30 min and then ceases (curve $S30_{INT}$ + EMC RNA in Fig. 1). To characterize the state of the EMC RNA translating machinery in the $S30_{INT}$ right after the cessation of protein synthesis we performed the following experiments: 1) We translated EMC RNA for 30 min in $S30_{INT}$ and as a control in $S30_C$. (No labeled amino acid was present during this time.) 2) Thereafter the polysomes containing EMC RNA from $S30_{INT}$ ($EMC\text{-}Rib_{INT}$) and from $S30_C$ ($EMC\text{-}Rib_C$) were isolated by centrifugation.

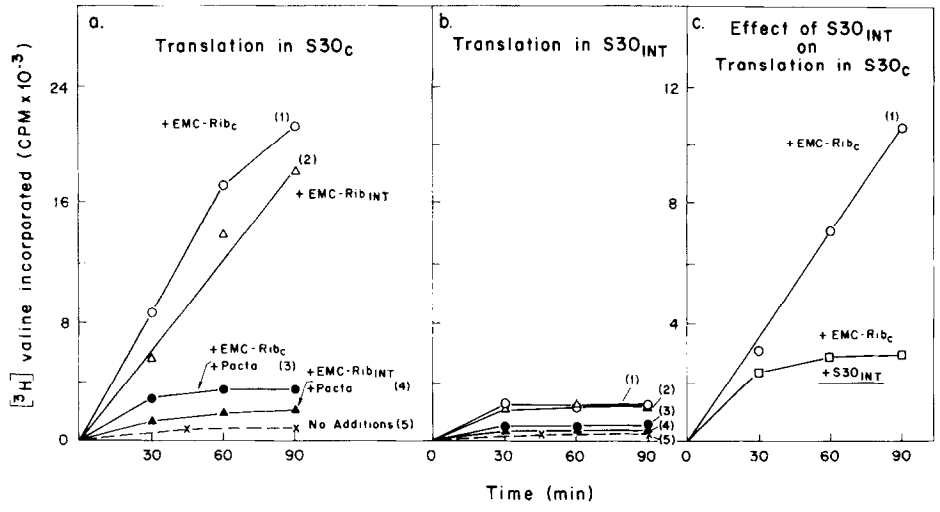

Fig. 2. EMC RNA-containing polysomes isolated from $S30_{INT}$ after their translation ceased can be translated in $S30_C$ as efficiently as EMC RNA-containing polysomes isolated from $S30_C$. The experiment was performed in two parts: i) preparation of $EMC\text{-}Rib_C$ and $EMC\text{-}Rib_{INT}$. The two reaction mixtures (180 µl each) included 4 mM $Mg(oAc)_2$, 3.2 A_{260} units of either $S30_C$ or $S30_{INT}$ and 7.5 µg EMC RNA. (No labeled amino acid was present.) Incubation was at 30° for 30 min. Thereafter 2.5 ml of ice cold buffer A (25 mM Tris-Cl pH 7.5, 80 mM KCl, 4 mM $Mg(oAc)_2$, 1 mM dithiothreitol) was added to each and the resulting solutions were centrifuged in the A321 rotor of the IEC B60 ultracentrifuge at 55,000 rpm (220,000 g) at 2° for 1 hour to sediment the ribosomes. The supernatant fractions were discarded. The sediments containing ribosomes with attached EMC RNA were rinsed each with 0.5 ml of ice cold buffer B (10 mM Tris-Cl pH 7.5, 10 mM KCl, 1 mM $Mg(oAc)_2$, 1 mM dithiothreitol) and each was suspended in 60 µl of buffer B. The resulting suspensions were designated as either $EMC\text{-}Rib_C$ (if containing ribosomes from $S30_C$) or $EMC\text{-}Rib_{INT}$ (if containing ribosomes from $S30_{INT}$). ii) Translation tests of $EMC\text{-}Rib_C$ and $EMC\text{-}Rib_{INT}$. Reaction mixtures (25 µl) including 4 mM $Mg(oAc)_2$ and either 0.55 A_{260} units of $S30_C$ (in a) or $S30_{INT}$ (in b) were supplemented with 5.4 mM [3H] valine (sp. act. 6.7 Ci/mmole) and if so indicated with 3 µl of either $EMC\text{-}Rib_C$ or $EMC\text{-}Rib_{INT}$ and 2 µM pactamycin; reaction mixtures (in c, 38 µl) contained 0.69 A_{260} units of $S30_C$, 3 µl of $EMC\text{-}Rib_C$, 4 µM [3H] valine and if indicated 0.69 A_{260} units of $S30_{INT}$. (The curves in c reveal the inhibitory effect of $S30_{INT}$ on the translation of $EMC\text{-}Rib_C$ in $S30_C$. It was shown in separate experiments that doubling of the concentration of $S30_C$ in the reaction mixture did not decrease the rate or extent of [3H] valine incorporation directed by EMC RNA.) The reaction mixtures were incubated at 30° for the times shown. Thereafter the amount of [3H] valine incorporated into hot trichloroacetic acid-insoluble material was determined. The composition of the reaction mixtures designated by the same number in parentheses in a, b and c was as indicated in a (with the exception of the S30 component).

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3) Aliquots of each of these polysome preparations were added together with [3H] valine to a reaction mixture containing $S30_{INT}$ and to another one containing $S30_C$. The kinetics of protein synthesis in each of the resulting four reaction mixtures was followed. The rates of translation of $EMC\text{-}Rib_C$ and $EMC\text{-}Rib_{INT}$ in $S30_C$ were similar and constant for about 90 min (Fig. 2a). Pactamycin, an inhibitor of chain initiation but not elongation, (6) impaired the translation revealing that chain initiation (and chain elongation)

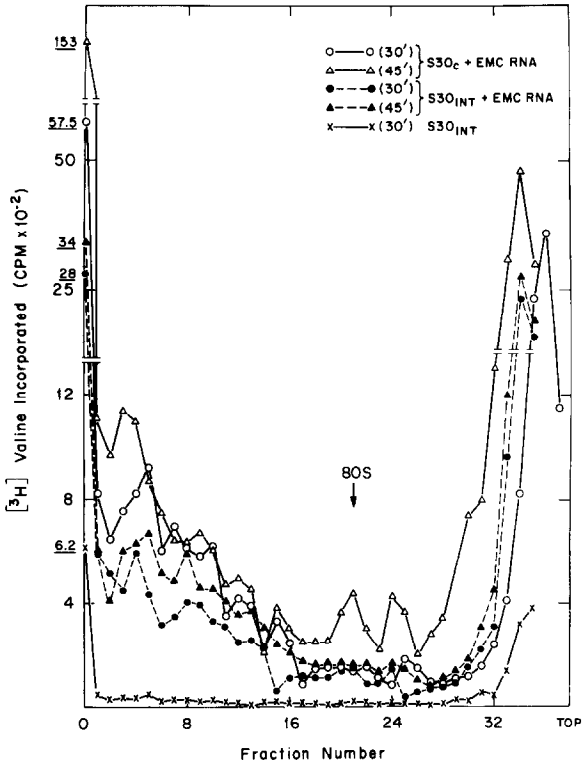

Fig. 3. Size distribution of EMC RNA-containing polysomes in $S30_C$ and $S30_{INT}$. The polysomes in $S30_C$ increase in size whereas those in $S30_{INT}$ are unchanged between 30 and 45 min of incubation. Reaction mixtures (60 µl) including 4 mM $Mg(OAc)_2$, 5.8 µM [^{3}H] valine (sp. act. 17.2 Ci/mmole), and if so indicated, 1.25 A_{260} unit of $S30_C$ or 1.3 A_{260} unit of $S30_{INT}$, and 3 µg EMC RNA, were incubated at 30° for either 30 or 45 min. Subsequently 0.15 ml of ice-cold buffer A was added to each and the resulting solutions were layered on exponential sucrose gradients (5-20.7% w/v) in buffer A supplemented with 0.2 mM dithiothreitol (11). Centrifugation was at 2° in the SB283 rotor of the IEC ultracentrifuge at 40,000 rpm (180,000g) for 1.8 hr. 20 drop fractions were collected. The amount of [^{3}H] valine incorporated into hot trichloroacetic acid-insoluble material was determined in each fraction. In the same experiment we also followed the kinetics of [^{3}H] valine incorporation in $S30_C$ and $S30_{INT}$ in conditions identical with those in which the samples applied to the sucrose gradients were prepared. In 30 µl reaction mixtures containing $S30_C$ 30,000 cpm of [^{3}H] valine was incorporated into hot trichloroacetic acid-insoluble material in a 30 min incubation and 58,900 cpm in a 60 min incubation. The corresponding values for incubations with $S30_{INT}$ were 15,500 cpm and 16,000 cpm.

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occurred in the reaction mixtures including $EMC\text{-}Rib_{INT}$ (as well as in those including $EMC\text{-}Rib_C$). The translation of both $EMC\text{-}Rib_C$ and $EMC\text{-}Rib_{INT}$ was much slower in $S30_{INT}$ than in $S30_C$ and ceased after 30 min (Fig. 2b). Addition of $S30_{INT}$ to $S30_C$ impaired the translation of $EMC\text{-}Rib_C$ (Fig. 2c).

These results indicate that a) the impairment of translation in the $S30_{INT}$ is reversible, at least the EMC RNA bound to ribosomes is not inactivated, and b) as reported earlier, the impairment is mediated by inhibitors in $S30_{INT}$.

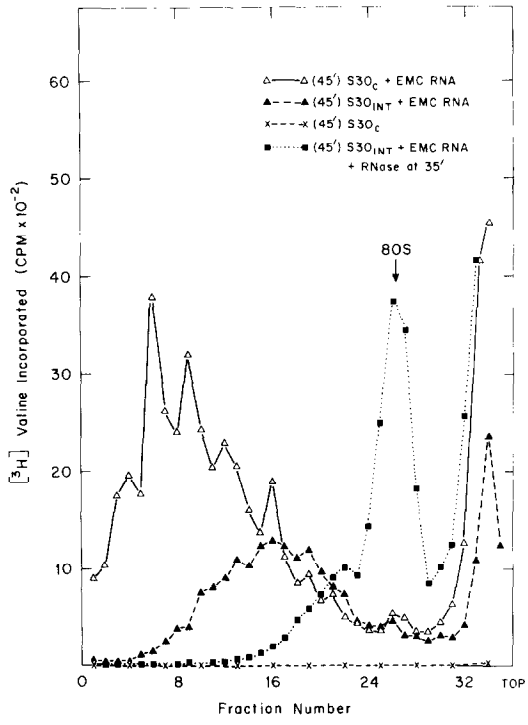

Fig. 4. Treatment with pancreatic ribonuclease cleaves the EMC RNA-containing polysomes in $S30_{INT}$ into monosomes. Reaction mixtures (60 µl) including [^{3}H] valine and if so indicated $S30_C$ or $S30_{INT}$ and EMC RNA were incubated at 30° for 45 min. (For further details see the legend to Fig. 3.) One reaction mixture containing $S30_{INT}$ and EMC RNA was supplemented after 35 min of incubation with 2 µg/ml of pancreatic ribonuclease and was incubated further until 45 min. Subsequently 100 µl of ice-cold buffer A was added to each reaction mixture and the mixtures were layered on exponential sucrose gradients and centrifuged in the conditions described in the legend to Fig. 3 except that the length of the centrifugation was 1 hr. 20 drop fractions were collected from each gradient and the amount of [^{3}H] valine incorporated into hot trichloroacetic acid-insoluble material was determined. In the same experiment we also followed the kinetics of [^{3}H] valine incorporation in $S30_C$ and $S30_{INT}$ in conditions identical with those in which the samples applied to the sucrose gradients were prepared. In 30 µl reaction mixtures containing $S30_C$ 65,000 cpm of [^{3}H] valine was incorporated into hot trichloroacetic acid-insoluble material in a 45 min incubation and 118,000 cpm in a 90 min incubation. The corresponding values for incubations with $S30_{INT}$ were 26,000 and 23,000.

The first of these conclusions is supported by the data in Figs. 3 and 4. These indicate that the EMC RNA is in large polysomes in $S30_{INT}$ after the cessation of translation (after 30 min). The size of these changes little (if any) during an incubation for further 15 min. The fact that treatment with pancreatic ribonuclease at low concentration degrades the EMC-RNA containing polysomes in $S30_{INT}$ to material sedimenting as monosomes (Fig. 4) indicates that we are not dealing with ribosomal aggregates. As a control we also checked the size of EMC RNA-containing polysomes formed in $S30_C$. As expected, the size of these increases greatly during the same time i.e. between 30 to 45 min of incubation (Figs. 3 and 4).

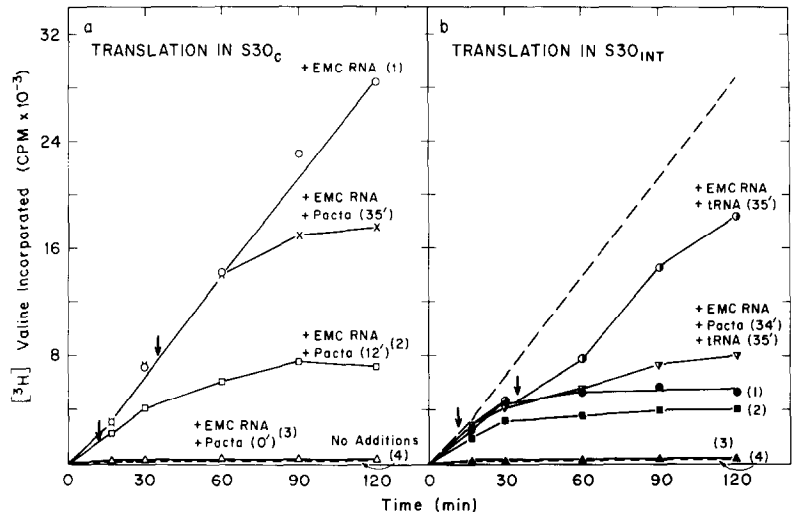

Fig. 5. Pactamycin impairs the effect of added tRNA in restoring the translation of EMC RNA in an $S30_{INT}$ extract. Reaction mixtures (60 µl) included 5 mM $Mg(oAc)_2$, 15 µM [^{3}H] valine (sp. act. 6.7 Ci/mmole) and as indicated 1.25 A_{260} unit of $S30_C$ (in a) or 1.3 A_{260} unit of $S30_{INT}$ (in b), 2.8 µg of EMC RNA, 1.6 µM pactamycin (added either at 0 time before adding EMC RNA, or at 12, 34, or 35 min after the beginning of the incubation, as shown by the arrows), and if so indicated 10 µg of mouse tRNA (added 35 min after the beginning of the incubation). The reaction mixtures were incubated at 30°. At the times indicated 10 µl aliquots were taken and the amounts of [^{3}H] valine incorporated into hot trichloroacetic acid-insoluble material were determined in each. The dashed line in b is identical with line 1 in a. The compositions of the reaction mixtures designated by the same number in parentheses (1 to 4) in a and b were as indicated in a (with the exception of the S30 component).

Added tRNA overcomes the impairment of translation of EMC RNA in $S30_{INT}$

We noted in a previous communication (4) that the inhibition of the translating capacity of $S30_{INT}$ could be partially overcome by added tRNA. In the experiments reported, however, adding of tRNA decreased the translating activity of $S30_C$. Recently we changed the conditions of these experiments by increasing the concentration of Mg^{++} ions from 4 to 5 mM and that of valine (the labeled amino acid) 3 fold. In the new conditions tRNA did not inhibit the translation by $S30_C$ (in fact it increased this very slightly) and at the same time it restored the translating capacity of $S30_{INT}$ to a level close to that of $S30_C$ (Fig. 1). Mouse ribosomal RNA or *E. coli* tRNA had no such effect. The addition of mouse tRNA to $S30_{INT}$ 10 min after the cessation of EMC translation resulted in the restart of translation after a short lag at a rate close to that in $S30_C$ (Fig. 1). This is in line with our other data indicating that the cessation of translation is not due to inactivation of the mRNA.

For further characterization of the effect of tRNA in restarting the translation in $S30_{INT}$ we tested the effect of pactamycin on this phenomenon. The curves in Fig. 5 reveal that, as expected, pactamycin added to the $S30_C$ prior to EMC RNA blocks translatior

completely. Adding the inhibitor at various times (12 or 35 min) after mRNA has been added i.e. at a time when translation is going on, allows translation to continue for about 20 to 30 min. This indicates that peptide chains are initiated in $S30_C$ during at least the first 35 min of the incubation. The curves in Fig. 5b reveal that pactamycin added to $S30_{INT}$ at 34 min (i.e. at the time when the translation of EMC RNA is leveling off) decreases greatly but not completely the effect of tRNA (added at 35 min) in restoring the translation. The fact that pactamycin decreases the effect of tRNA indicates that added tRNA allows the initiation of new peptide chains. The fact that the inhibition by pactamycin is not complete indicates that tRNA also allows the elongation of incomplete peptide chains. tRNA from interferon-treated cells could substitute for tRNA from untreated cells in releasing the impairment of translation in $S30_{INT}$ (data not shown).

tRNA deficiency in $S30_{INT}$ and interferon action

To learn about the basis of the apparent tRNA deficiency in $S30_{INT}$ we compared the capacity of $S30_{INT}$ with that of $S30_C$ to charge endogenous and added tRNAs with various amino acids. We found no significant difference between the two extracts in charging isoleucine, methionine, phenylalanine, threonine, and valine to endogenous tRNAs. However, the capacity of our $S30_{INT}$ to charge endogenous tRNA with leucine was about 50 per cent lower than that of $S30_C$. Moreoever, incubation of $S30_{INT}$ for a further 30 min (with or without sparsomycin, an inhibitor of protein synthesis (7)) resulted in an over 80 per cent decrease in this capacity. However, added tRNA was charged with leucine in the same $S30_{INT}$ to about the same extent as in $S30_C$ indicating that the activity of leucyl-tRNA synthetase was not impaired. These findings may account for both a) the cessation of mRNA translation in $S30_{INT}$ after 30 min incubation resulting in apparently stable, "stuck", paralyzed polysomes and b) the restoration of translation by added tRNA. It remains to be established if the amino acid acceptance of the various leucine-specific tRNA species is equally or unequally impaired in $S30_{INT}$.

It will have to be verified that the impairment of the aminoacylation of certain endogenous tRNA species is due to interferon and not to contaminants in the impure interferon preparations. Moreover, even if the impairment were due to interferon, its relationship to the antiviral effect of interferon would not be obvious. However, the following hypothesis could relate the two phenomena: It is conceivable that in the mRNAs of interferon-sensitive viruses certain codons occur more frequently than in cellular mRNAs (cf. ref. (8) and the proposed role of the tRNAs specified by bacteriophage T_4 (9,10)). If that was the case interferon could conceivably inhibit virus replication preferentially by causing a decrease in the amino acid acceptance by tRNAs translating such codons and thereby impairing viral protein synthesis more than host protein synthesis.

After these experiments were completed we learned that M. Revel and his associates

obtained results in agreement with many of those presented here (M. Revel, personal communication).

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Abbreviations: EAT, Ehrlich Ascites Tumor; EMC, encephalomyocarditis; $Mg(oAc)_2$, magnesium acetate; sp. act., specific activity; VSV, vesicular stomatitis virus.

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