INCREASED ENDONUCLEASE ACTIVITY IN AN EXTRACT FROM MOUSE EHRLICH ASCITES TUMOR CELLS WHICH HAD BEEN TREATED WITH A PARTIALLY PURIFIED INTERFERON PREPARATION: DEPENDENCE ON DOUBLE-STRANDED RNA

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SUMMARY. Reovirus messenger RNAs are degraded faster in crude extracts (S30) from mouse Ehrlich ascites tumor cells which have been treated with either a partially purified interferon preparation or the interferon inducer poly(I)·poly(C) than in corresponding extracts from untreated cells. The faster degradation appears to be a consequence of endonuclease action. The endonuclease activity *in vitro* depends on the presence of double-stranded reovirus RNA in the reaction mixture.

INTRODUCTION

INTs are glycoproteins whose synthesis is induced in a variety of vertebrate cells upon viral infection. They are released from the producing cells, interact with other cells and make these inefficient in supporting the growth of a large variety of viruses (1). We have been investigating the way in which the replication of reovirus is inhibited in INT-treated mouse L cells and EAT cells as well as in their extracts.

Studies with intact cells revealed that the early steps in reovirus replication, i.e., the adsorption of virus to cells, its penetration and partial uncoating (resulting in the formation of subviral particles) are not affected in INT-treated cells (2,3). The accumulation of virus-specific mRNA, ds RNA and protein is however impaired (4-6). The studies with intact cells did not allow us to establish whether the block in viral protein accumulation is a consequence of an effect on viral RNA metabolism (e.g., transcription, processing, or turnover), or on the translation of viral mRNA, or on both (5).

Earlier studies revealed that the treatment of EAT cells with INT is manifested in their extract in various ways. Thus, a crude extract of INT-treated cells ($S30_{INT}$), if preincubated and passed through Sephadex G25, has

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Abbreviations: INT, interferon; EAT, Ehrlich ascites tumor; ds, double-stranded; $S30_{INT}$ and $S30_C$, extracts from cells treated with interferon and from control cells; reo $mRNA_U$ and reo $mRNA_M$, unmethylated and methylated reovirus mRNA; m^7G, 7-methylguanosine; Gm, 2'-o-methylguanosine; PBS, phosphate buffered saline; VSV, vesicular stomatitis virus.

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an impaired capacity to translate added mRNA (7-10). Furthermore, it inactivates some of its endogenous tRNA species faster than an extract of control cells ($S30_C$) does (11) and the addition of tRNAs overcomes, at least partially, the impairment of translation (12,13). $S30_{INT}$ which has been neither preincubated, nor passed through Sephadex G25 is also impaired in its capacity to translate added mRNA (11,14). However, tRNAs are more stable in the latter type of extract, and the addition of tRNA does not overcome the impairment of translation (11). The methylation of the 5'-terminal and the penultimate guanylate residues of unmethylated reo mRNA is also inhibited in $S30_{INT}$ (15). The impairment of methylation in $S30_{INT}$ is clearly not due to the degradation of the reo mRNAs (manuscript in preparation).

In this communication, we present data on a further effect of INT-treatment of EAT cells as manifested in their extracts. We find that added reo mRNAs are degraded faster in $S30_{INT}$ than in $S30_C$. The increased rate of degradation appears to be due to endonuclease action, and this action requires the addition of ds reo RNA.

An impairment of viral RNA accumulation in INT-treated cells was described in the cases of SV40 virus (16), VSV (17,18), and vaccinia virus (19). Moreover, it was reported that nuclease activity is increased in a membrane fraction of chick embryo fibroblasts which had been treated with a crude INT preparation (20). It has been shown that ds RNA blocks peptide chain initiation in eucaryotic cells (21), apparently by inactivating one of the initiation factors (22). INT-treated mouse L cells were found to be more sensitive to the cytopathic effect of ds RNA than control cells (23). Moreover, extracts of INT-treated L cells were shown to be more prone to the inhibition of translation of exogenous mRNA by ds RNA than corresponding extracts of control cells (24).

RESULTS

Increased endonuclease activity in $S30_{INT}$

Added reo mRNAs are degraded faster in $S30_{INT}$ than in $S30_C$. The agent(s) responsible for the faster degradation is/are present both in not preincubated and not Sephadex-treated $S30_{INT}$ (Fig. 1A) and in $S30_{INT}$ which has been preincubated and from which small molecules have been removed by gel filtration through Sephadex G-25 (Fig. 1B).

In the experiments in Fig. 1A, methylated reo mRNA (reo $mRNA_M$, with 5' terminal $m^7GpppGm$ or ppG) was used; in the experiments in Fig. 1B, unmethylated reo mRNA (reo $mRNA_U$, with 5' terminal ppG or GpppG) was used (25). These and other experiments failed to reveal a clearcut difference in the degradation pattern in $S30_{INT}$ between reo $mRNA_M$ and reo $mRNA_U$.

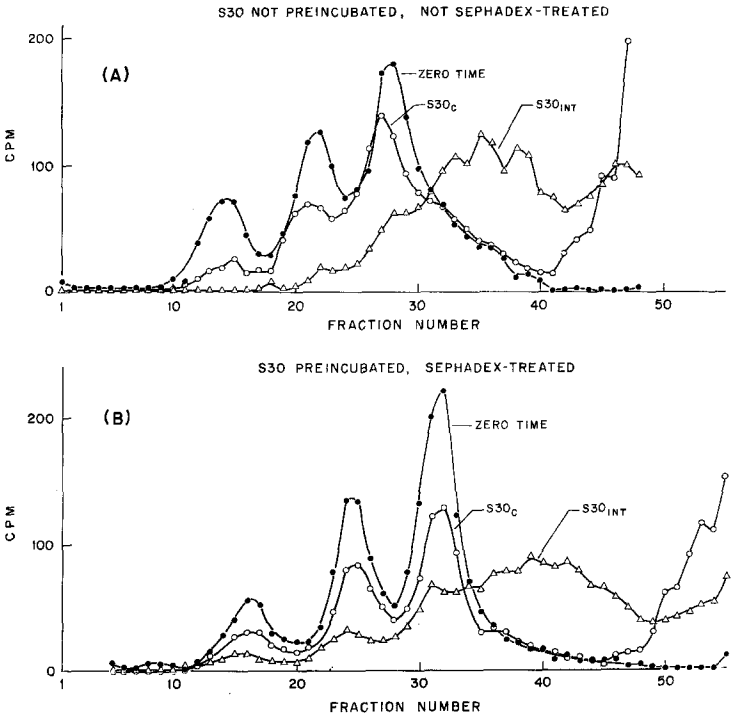

Fig. 1. Reo mRNAs are degraded faster in $S30_{INT}$ than in $S30_C$: A) not preincubated, not Sephadex-treated S30; B) preincubated, Sephadex-treated S30. Mouse INT. The specific activity of the partially purified preparation was 1.1×10^6 VSV plaque reduction units (4) (this corresponds to 1.1×10^7 NIH mouse reference standard units/mg protein). S30 extracts were prepared from EAT cells grown in suspension culture which had been treated with 200 units/ml of INT for 18 hours ($S30_{INT}$) or from cells grown under identical conditions but not treated with INT ($S30_C$) as described in ref. 11. The yield of VSV in a single growth cycle was reduced by over 95%. Not preincubated and not Sephadex-treated S30s were used throughout the study unless otherwise indicated. Reo mRNA: ^{32}P-labeled reo $mRNA_M$ and reo $mRNA_U$ were generous gifts from Dr. A. Shatkin (26). Reaction mixture for assaying reo mRNA degradation: The composition of the reaction mixture (30 µl) was identical to that developed for reo mRNA translation (27) except that all amino acids were unlabeled, no tRNA was added, 5 mM magnesium acetate, 120 mM KCl, and 18 A_{260} units/ml of S30 were used and in A) 100 µg/ml of reo $mRNA_M$ and not preincubated, not Sephadex-treated S30s were used and the reaction mixtures were incubated at 30° for 15 mins whereas in B) 100 µg/ml of reo $mRNA_U$ and preincubated, Sephadex-treated S30s were used and the reaction mixtures were incubated at 30° for 30 mins. Processing of the reaction mixture: The reactions were terminated by adding 0.4 ml of buffer A (0.1 M NaCl, 10 mM Tris Cl (pH 7.5), 1 mM EDTA) containing 0.5% (w/v) SDS and 0.4 ml water-saturated phenol. The resulting samples were agitated at room temperature for 10 mins. The aqueous layers were separated from the phenol layers by centrifugation and were washed with 3 ml ether. The residual ether was removed by a stream of N_2. 0.2 ml samples were applied onto 12.5 ml linear sucrose gradients (7 to 25%, w/v) in buffer A. The gradients were centrifuged at 2° and 39,000 rpm for 12 hours in the SB283 rotor in an IEC B60 ultracentrifuge. Radioactivity in the fractions was determined by counting in a xylene-based scintillator. Zero time samples were processed from not incubated reaction mixtures containing $S30_C$.

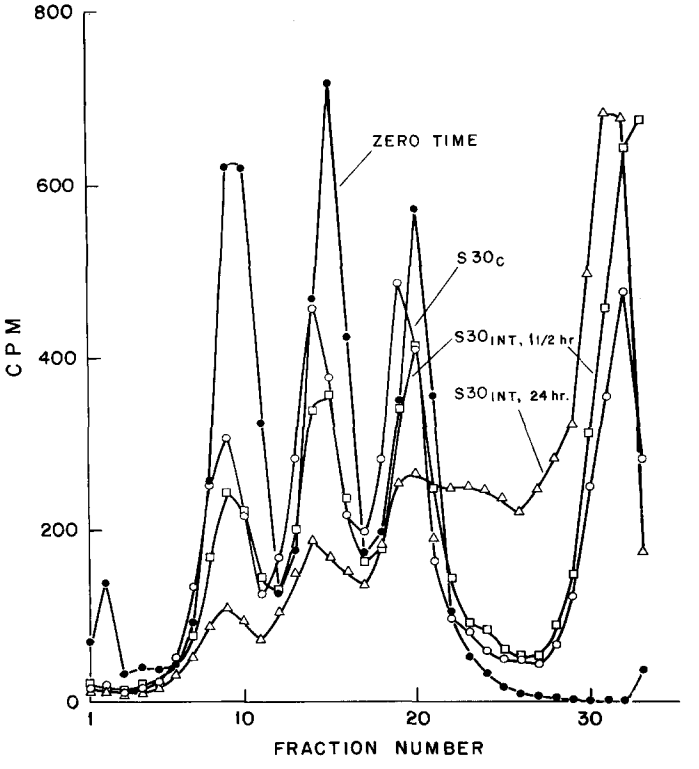

Fig. 2 Reo mRNAs are degraded faster in $S30_{INT}$ than in $S30_C$: effect of the duration of the exposure of cells to INT. S30s were prepared from cells not exposed to INT ($S30_C$) and cells exposed to 60 units/ml of INT for either 1-1/2 hr ($S30_{INT}$ 1-1/2 hr)or 24 hrs ($S30_{INT}$ 24 hrs). Reo $mRNA_M$ labeled in [^{3}H] guanylate residues was prepared according to the procedures in ref. (29) except that the concentration of ATP was 2 mM and that of magnesium acetate 15 mM, 10 µM S-adenosyl-methionine was added (to allow for the methylation of the RNA) and the incubation was at 37° for 2 hrs. The reaction mixtures (15 µl) contained 30 A_{260} units of S30 extracts/ml. For further details see the legend to Fig. 1. The yield of VSV in a single growth cycle was reduced by 75% in cells exposed to INT for 1-1/2 hr, and by over 99% in those exposed for 24 hrs.

The following observation indicates that the faster degradation of reo mRNA in $S30_{INT}$ may be due to endonuclease action: the rate of formation of RNA cleavage products that are soluble in cold trichloroacetic acid (i.e., mono- and short oligonucleotides) is not faster in $S30_{INT}$ than in $S30_C$ (data not shown). We will designate the agent(s) responsible for the faster degradation of reo mRNA in $S30_{INT}$ than in $S30_C$ as $endonuclease_{INT}$.

Possible relationship between INT action and $endonuclease_{INT}$ activity

Not having homogeneous INT available, we cannot prove that the $endonuclease_{INT}$ has been induced by INT and not by other components in the INT preparation. The following results are consistent with the possibility that $endonuclease_{INT}$ is induced by INT:

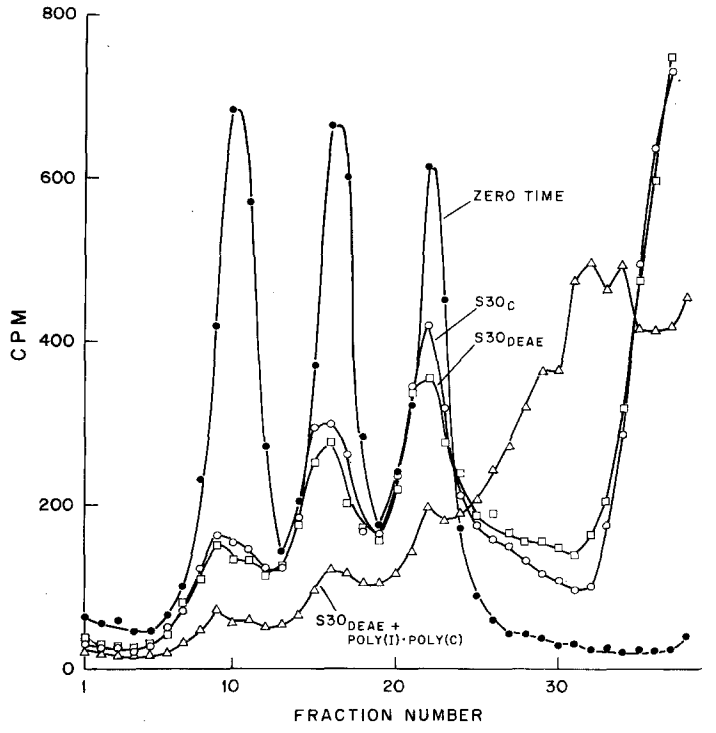

Fig. 3. Reo mRNAs are degraded faster in S30 from cells treated with the INT inducer poly(I)·poly(C) than in S30 from control cells. Confluent monolayers of cells grown in Eagle's minimal essential medium (F15, Gibco) supplemented with 7% fetal calf serum were treated with either poly(I)·poly(C) (10 µg/ml ; Miles) and DEAE-dextran (20 µg/ml; Pharmacia)(S30 DEAE + poly(I)·poly(C)) or with DEAE-dextran (20 µg/ml) (S30 DEAE). Both poly(I)·poly(C) and DEAE-dextran were dissolved in PBS. Control cells were treated with PBS only ($S30_C$). After a 90 min incubation at 37° the media were replaced by Eagle's minimal essential medium (F15, Gibco) and the cells were incubated for 20 hrs. Thereafter, the cells were detached by incubation in PBS containing 1 mM EDTA at 37° for 10 min and S30s were prepared as described in the legend to Fig. 1. Reo $mRNA_M$ was prepared as described in the legend to Fig. 2. The reaction mixtures (15 µl) contained 30 A_{260} units of S30/ml. For further details see the legend to Fig. 1. The yield of VSV in a single growth cycle was reduced by the exposure of cells to DEAE dextran + poly(I)·poly(C) by over 99%, it was not reduced in cells exposed to DEAE dextran.

(a) $Endonuclease_{INT}$ activity is much less pronounced in cells exposed to INT for 1-1/2 hours than in those exposed for 24 hours (Fig. 2). At the same time, it has been established that the induction of the maximal antiviral state (i.e., the state in which the virus replication is inhibited the most) requires an exposure of cells to interferon for several hours (28).

(b) Reo mRNA is also degraded faster in an extract of cells which were not exposed to INT, but to the INT-inducer poly(I)·poly(C) (30) (in Fig. 3 $S30_{DEAE}$ + poly(I)·poly(C)). DEAE-dextran added together with poly(I)·poly(C) potentiates the effect of the latter in inducing INT, but in itself DEAE-

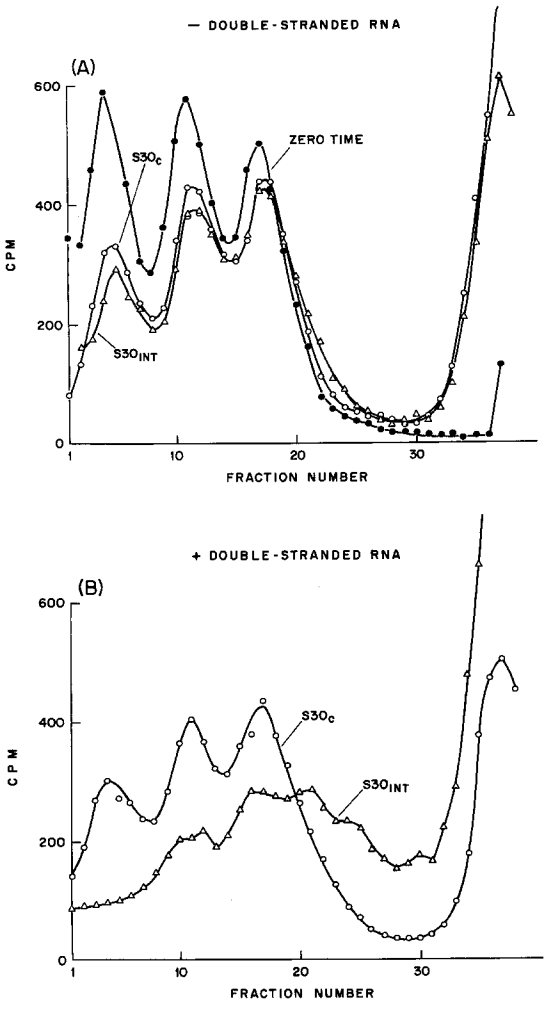

Fig. 4. Reo mRNAs are degraded faster in $S30_{INT}$ than in $S30_C$: requirement for ds reo RNA. ds reo RNA was extracted from virions according to ref. 33 except that the adenylate-rich oligonucleotides were removed by gel filtration through a Sephadex G-100 column equilibrated with 10 mM Tris Cl (pH 7.5). Treatment for removal of ds RNA from reo mRNA (see refs. 32 and 33). [^{3}H]-guanylate labeled reo $mRNA_M$ (see the legend to Fig. 2) was precipitated 5 times from aqueous solution by the addition of an equal volume of 4 M LiCl at 0° and thereafter once by the addition of 2 volumes of ethanol. (LiCl-precipitated reo mRNA.) In the course of this procedure, 12% of the UV-absorbing material, presumably, mostly ds reo RNA, but less than 1% of the [^{3}H]-labeled material, presumably, mostly reo mRNA, was lost in the fractions not precipitated in 2 M LiCl. Reaction mixture for assaying reo $mRNA_M$ degradation: in A), the reaction mixtures (15 µl) included 30 A_{260} units of S30/ml and 100 µg of [^{3}H]-labeled LiCl-precipitated reo mRNA/ml. In B), the reaction mixtures were supplemented in addition with 5 µg of ds reo RNA/ml. The centrifugation through sucrose gradients was as described in the legend to Fig. 1, except that the volume of the gradient was 12 ml and it was centrifuged at 2° for 10 hrs, and 41,000 rpm in the SW 41 rotor in a Beckman L2-50 centrifuge. For further details see the legend to Fig. 1. The yield of VSV in a single growth cycle was reduced by over 99% in cells treated with INT.

dextran is not known to induce INT (31). Treatment with DEAE-dextran (in Fig. 3 $S30_{DEAE}$) enhanced the rate of reo mRNA degradation only slightly above that observed in extracts of control cells (in Fig. 3 $S30_C$).

Dependence of $endonuclease_{INT}$ action on ds RNA

Contaminating ds RNA had not been removed from the reo mRNA preparation used in the experiments shown in Figs. 1, 2, and 3. However, the reo mRNA preparation used for the experiment in Fig. 4 was precipitated five times with 2 M LiCl to remove as much of the contaminating ds RNA as possible (32).

Remarkably, the degradation of the LiCl-precipitated reo mRNA was only slightly, if at all, faster in $S30_{INT}$ than in $S30_C$ (Fig. 4A). To test if the lack of a faster degradation of the LiCl-precipitated reo mRNA in $S30_{INT}$ was due to the absence of ds RNA, we added ds reo RNA (5 µg/ml) to the reaction mixtures. This addition greatly enhanced the rate of degradation of the LiCl-precipitated reo mRNA in $S30_{INT}$, whereas it affected the rate of degradation in $S30_C$ barely, if at all (Fig. 4B).

DISCUSSION

The data presented reveal that : (a) reo mRNAs are degraded faster in $S30_{INT}$ than in $S30_C$; (b) the faster degradation is due to endonuclease action; and (c) the endonuclease action is dependent on ds reo RNA.

Preliminary results (to be presented in detail elsewhere) indicate that the increased rate of degradation is elicited by ds reo RNA at concentrations as low as 0.3 µg/ml and as high as 15 µg/ml (the highest concentration tested), whereas at 0.06 µg/ml no effect was noted. Poly(I)·poly(C) (5 µg/ml) could substitute for reo ds RNA in enhancing the rate of reo mRNA degradation. Thus, it is conceivable that any ds RNA may enhance endonuclease activity in $S30_{INT}$. The cleavage of ds reo RNAs was not observed in either $S30_C$ or $S30_{INT}$ under the conditions of reo mRNA degradation.

The $endonuclease_{INT}$ mediating the faster degradation is probably not a lysozomal enzyme. At least, the specific activity of N-acetyl beta glucosaminidase (an enzyme known to be located in lysozomes (34)) was not higher in $S30_{INT}$ than in $S30_C$. Protein synthesis may not be required for $endonuclease_{INT}$ action, at least the addition of sparsomycin (an inhibitor of protein synthesis)(35) to the reaction mixture did not affect the rate of reo mRNA cleavage.

It might be relevant to the above considerations that we find that reovirus subviral particles isolated by CsCl density gradient centrifugation from an extract of cells which had been treated with the partially purified INT preparation manifest an endonuclease activity and synthesize short RNA products whereas subviral particles isolated from untreated cells have little

or no endonuclease activity and synthesize full size reo mRNAs (3).

Further characteristics of endonuclease$_{INT}$ are under investigation. It is conceivable that the endonuclease$_{INT}$-ds RNA system will turn out to be a major mediator of INT action. Whether this is the case or not will have to be established, however, in experiments with intact cells.

In conclusion, the results presented reveal a novel way in which ds RNA might be involved in mediating the functioning of the INT system.

Acknowledgments

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